THE EFFECT OF LIPIDS AND SERUM ALBUMIN ON BACTERIAL GROWTH

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We have previously reported that certain water-dispersible lipids promote diffuse growth of tubercle bacilli in liquid media (6–9). These same compounds have also been found to enhance the growth of other microbial species, in particular of a certain micrococcus (strain C) recently isolated in our laboratory (7). Although the addition of crystalline serum albumin to the medium often permits initiation of growth of minute inocula (one or a very few cells) which would not grow in the absence of the protein, the latter substance does not increase appreciably the final density of the culture (5–9, 15). The purpose of the present paper is to describe and analyze the interrelationships between lipids, albumin, and bacterial growth.

EXPERIMENTAL

Cultures.—The origin of the different strains of mycobacteria used in the experiments which are to be described has been indicated elsewhere (8). Since all strains gave essentially similar results only those obtained with avian strain TA_2 and human strain H37S will be reported in the present paper.

The micrococcus strain C was recently isolated as a contaminant on albumin oleate agar in our laboratory. This organism gives only scant growth on ordinary bacteriological media but grows abundantly in media to which 2 per cent defibrinated rabbit blood has been added. The cultures of micrococcus C and of tubercle bacilli were maintained in the "Tween 80"-albumin medium described in earlier communications (8, 9).

Media.—The synthetic lipids used in the experiments to be reported have been described elsewhere (8). They consist of esters of long chain fatty acids which are dispersible in water due to the multiplicity of hydrophilic oxygen containing groups of the polyhydric alcohols and ethylene oxide chains present in their molecular structure. These soluble lipids are prepared for industrial purposes and, therefore, do not constitute pure chemical entities. In particular, the fatty acids used in their manufacture are commercial products obtained from natural sources and are contaminated with related and unrelated substances.[1]

In order to test the effect of the free unesterified long chain fatty acids on bacterial growth, preparations of a high degree of purity were obtained from a number of different sources. A sample of pure palmitic acid was prepared by Dr. W. E. Doering of Columbia University, New York, by treating a commercial product with excess bromine in order to remove unsaturated impurities, and recovering the palmitic acid by fractional crystallization from methanol. Capric acid, and the methyl esters of lauric, myristic, palmitic, stearic, and oleic acid were kindly supplied by Dr. L. Shedlovsky of the Colgate-Palmolive Peet Research

[1] The different preparations of water-soluble esters of long chain fatty acids were prepared and generously supplied by the Atlas Powder Company of Wilmington, Delaware.

Laboratories. These methyl esters had been purified by fractional distillation under reduced pressure; the fatty acids were prepared by treatment of these esters with 10 per cent aqueous sodium hydroxide, followed by acidification with 10 per cent hydrochloric acid and washing with cold distilled water. Linoleic acid, the ethyl ester of linolenic acid, and arachidonic acid were prepared and supplied by Dr. G. O. Burr and Dr. R. T. Holman of the University of Minnesota; these products were derived from the polybromides and had iodine values very near the theoretical. I wish to extend my most sincere thanks to Dr. Doering, Dr. Shedlovsky, Dr. Burr, and Dr. Holman for their generous cooperation. It is hardly necessary to emphasize that experiments carried out with commercial preparations of fatty acids give at best equivocal results and in particular do not permit a satisfactory differentiation between the effects of saturated and unsaturated acids on bacterial growth.

The serum albumin was a crystalline preparation obtained from bovine plasma and supplied by the Armour Laboratory, Chicago. It was sterilized by filtration of a 5 per cent solution through Selas porcelain candles and was added aseptically to the culture medium.

Measurement of Growth.—Growth could be detected within 24 hours in the case of the micrococcus culture, and within 3 to 4 days in the case of the tubercle bacilli. The data reported in the present paper correspond to the amount of growth observed after 7 days' incubation at 37 C.

The density of growth in liquid media was recorded in terms of optical density or by determining the amount of bacterial sediment deposited under standardized conditions of centrifugation (20 minutes at 2,500 R.P.M.) in a tube with capillary bore fused on a centrifuge tube (25 ml.). The optical density and the amount of bacterial sediment were correlated in a number of preliminary experiments with gravimetric determinations (oven dry weight) of the bacterial growth. The correlation of the optical density and centrifugation data with the weight of bacterial bodies was often rendered inaccurate by the fact that the cultures exhibit marked differences in their state of dispersion depending upon the composition of the medium; thus, large clumps of bacilli or cocci are usually present in media containing soaps of fatty acids, whereas much more diffuse growths, consisting often of isolated cells, are characteristic of cultures growing in the presence of the water-soluble esters of these acids. For the sake of convenience, the yields of bacteria are expressed in mg./10 ml. of medium, although it must be emphasized that the figures correspond only to orders of magnitude and not to accurate yields.

Growth on solid media was determined by streaking the cultures diluted 1/100 on the surface of media containing 1.5 per cent agar, and measuring the diameter of well isolated colonies after 7 days' incubation at 37°C. In this case also, it must be emphasized that the colonial morphology varies greatly with the composition of the medium; the colonies are circular in outline and smooth in surface on the media containing the water-soluble esters, whereas they are irregular in contour and surface on the fatty acid media.

The Effect of Oleic Acid and Its Esters on Bacterial Growth in Liquid Media.—The cultures of micrococcus C and of tubercle bacilli grow slowly and scantily in ordinary bacteriological media (containing, for example, meat infusion, peptone, with or without glycerine or glucose); their growth is also very poor in the following semisynthetic medium which, however, is satisfactory for the development of many other bacterial species: (Details of preparation of the medium have been described elsewhere (8).)

$Na_2HPO_4 \cdot 12H_2O$	6.3 gm.
KH_2PO_4	1.0 "
Na_3 citrate $\cdot 2\ H_2O$	1.5 "
$MgSO_4 \cdot 7\ H_2O$	0.6 "

Hydrolysate of casein	1.0 gm.
Glucose	2.0 "
Yeast extract	0.2 "

Distilled water to 1,000 ml.

The effect of oleic acid and its esters on growth was tested by adding graded amounts of these substances to 5 ml. of the semisynthetic medium in test tubes 25 mm. in diameter. Only one amount (0.1 ml.) of methyl oleate was used since this substance is poorly dispersible in water and does not permit accurate sampling. The polyoxyethylene ester of oleic acid (G-2144), the polyoxyethylene derivative of sorbitan monooleate (Tween 80), and sodium oleate were diluted in H_2O. Six identical sets of media were prepared. Following autoclaving (10 minutes at 15 pounds pressure) 0.5 ml. of 5 per cent crystalline bovine serum albumin was added aseptically to three of these sets. Each of the tubes was then inoculated with 0.001 ml. of culture of micrococcus, of tubercle bacillus human strain H37S, or of avian strain TA_2.

The results presented in Table I show that growth was completely inhibited by the higher concentrations of oleate, the culture of human tubercle bacillus being the most susceptible, the avian tubercle culture next, and the culture of micrococcus C least. Esterification of the acid resulted in loss of toxicity; it is probable that the slight residual toxicity of the esters for the human tubercle bacillus is due to contamination with the free oleic acid (5). The toxic effect of the latter substance can also be completely neutralized by the addition of crystalline serum albumin to the medium.

The amount of growth given by the three cultures was scant in the control medium even in the presence of serum albumin but it increased directly with the concentration of oleate or oleic acid ester in the medium, up to the toxic level. Since the water-soluble esters (G-2144 and Tween 80) contain approximately 20 to 25 per cent oleic acid in the molecule, it appears from the results in Table I that they are somewhat more efficient than the unesterified acid in stimulating growth.

Although oleate enhances the growth of the tubercle bacilli as well as of the micrococcus C, a striking difference can be recognized between the responses of the two groups of organisms to fatty acid and albumin. It will be noticed that the amounts of oleic acid or its esters necessary to achieve a significant stimulation of growth of the acid-fast organisms are larger than those required by the micrococcus. Furthermore, we have repeatedly observed, and the phenomenon is illustrated in Table I, that larger concentrations of the fatty acid are required to initiate growth of the micrococcus C as the concentration of serum albumin becomes greater. Indeed, the slight amount of growth obtained, when this organism is cultivated in the semisynthetic casein hydrolysate medium without further addition of oleate, is completely prevented when albumin is added to the medium.[2] This inhibitory effect of albumin has not been observed with tubercle bacilli.

[2] No growth is obtained when micrococcus C is inoculated into various synthetic media free of casein hydrolysate. It is probable that the latter material is contaminated with small amounts of lipids containing the unsaturated fatty acids which are essential for the growth of the coccus.

The Effect of Glucose, Casein Hydrolysate, and Iron on Bacterial Growth in Oleate-Albumin Medium.—The basal medium described in the preceding experiment had been devised to meet the growth requirements of human tubercle bacilli; this medium contains amounts of citric acid, glucose, and casein hydrolysate sufficient to permit abundant development of a great variety of bacterial species. It will now be shown that the effect of oleate on bacterial growth can also be recognized in media of simpler composition.

TABLE I

Effect of Oleic Acid and Its Esters on Bacterial Growth in Casein Hydrolysate Liquid Medium

Lipid added to medium		Growth (mg./10 ml.) in media containing					
		No albumin			0.5 per cent crystalline albumin		
		Micrococcus C	Tubercle bacillus		Micrococcus C	Tubercle bacillus	
			Avian	Human		Avian	Human
	per cent	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
	0.01	0	0	0	5.7	1.6	2.1
	0.003	5.2	0	0	5.9	0.5	1.4
Oleic acid	0.001	2.17	0.3	0	5.1	0.3	0.9
	0.0003	1.5	0	0	3.1	0.3	0.8
	0.0001	1.1	0	0	0	0.3	0.9
Methyl oleate	0.1	1.4	1.5	0	1.9	0.9	2.4
Polyoxyethylene ester	0.1	5.4	3.1	0	5.4	4.1	3.1
of oleic acid	0.03	5.1	1.1	1.1	5.8	1.3	2.0
G-2144	0.01	5.0	0.3	0.9	5.2	0.5	0.9
	0.003	4.2	0.1	0.2	5.1	0.3	0.7
	0.001	3.1	0	0.4	0.3	0.3	0.9
Polyoxyethylene deriv-	0.1	5.1	2.5	0	5.1	3.8	3.0
ative of sorbitan	0.03	5.3	0.9	1.4	5.3	1.3	1.7
monooleate	0.01	4.8	0.3	0.7	5.7	0.3	0.8
Tween 80	0.003	3.9	0.1	0.9	5.0	0.3	0.9
	0.001	2.9	0	0.1	0	0.3	0.6
Control	—	0.3	0	0.1	0	0	0.4

The following mineral solution was distributed in 5 ml. amounts in test tubes 25 mm. in diameter:

$Na_2HPO_4 \cdot 12\ H_2O$	6.3 gm.
KH_2PO_4	1.0 "
$(NH_4)_2SO_4$	1.0 "
H_2O	1,000.0 ml.

Ferric chloride, glucose, enzymatic hydrolysate of casein, sodium stearate, sodium oleate, and serum albumin were added to the medium as described in Table II. The tubes were inoculated with 0.001 ml. of culture of micrococcus C or of avian tubercle bacillus.

The results presented in Table II indicate that the growth of the micrococcus was very scant when either glucose or oleate was absent from the medium and that it was only slightly increased by the addition of enzymatic hydrolysate of

TABLE II

Effect of Glucose, Casein Hydrolysate, and Iron on Utilization of Oleate and Stearate in Media Containing 0.5 Per Cent Serum Albumin

Glucose	Casein hydrolysate	$FeCl_3$	Oleic acid	Stearic acid	Growth (in mg./10 ml.)	
					Micrococcus C	Avian tubercle bacillus
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*
0	0	0	0.01	0	0.6	0
0.5	0	0	0.01	0	2.9	0
0	0.1	0	0.01	0	1.8	0.2
0.5	0.1	0	0.01	0	3.3	0.3
0	0	0.001	0.01	0	0.9	0
0.5	0	0.001	0.01	0	3.7	0
0	0.1	0.001	0.01	0	3.9	1.1
0.5	0.1	0.001	0.01	0	5.1	1.1
0	0	0	0.001	0	0.2	0
0.5	0	0	0.001	0	0.6	0.1
0	0.1	0	0.001	0	1.6	0
0.5	0.1	0	0.001	0	3.8	0.1
0	0	0.001	0.001	0	0.2	0.3
0.5	0	0.001	0.001	0	1.1	0.5
0	0.1	0.001	0.001	0	1.0	0.3
0.5	0.1	0.001	0.001	0	4.1	0.5
0.5	0.1	0.001	0.0001	0	0	0.5
0	0	0.001	0	0.01	0	0.8
0.5	0.1	0.001	0	0.01	0	1.2
0	0	0.001	0	0.001	0	0.3
0.5	0.1	0.001	0	0.001	0	0.6
0.5	0.1	0.001	0	0	0	0.3

casein. In the presence of 0.5 per cent glucose, 0.001 per cent oleic acid was sufficient to insure almost maximal growth under the conditions of the experiment, but growth remained poor even with the largest amount of fatty acid when glucose was omitted. It is also clear that growth is markedly dependent upon the presence of a sufficient amount of iron in the medium.

The behavior of the strain of avian tubercle bacillus was strikingly different.

Growth was scanty in the absence of the casein hydrolysate but was not significantly increased by the addition of glucose. The culture yield became significant only with the largest concentrations of oleic acid or stearic acid. Finally, it will be noted that stearic acid cannot replace oleic acid as growth factor for the micrococcus, whereas both long chain fatty acids appear equally effective in supporting the growth of the tubercle bacillus.

Effect of Different Fatty Acids on Bacterial Growth.—In order to determine whether stimulation of bacterial growth can also be obtained with other long chain fatty acids, these substances or their esters were added in different concentrations to the semisynthetic casein hydrolysate medium mentioned earlier in this report. Unfortunately, only a limited number of fatty acids and esters were available. Moreover, some of these products were impure commercial preparations. Granted these limitations, the results obtained were sufficiently striking and clear cut to warrant the following statements.

All fatty acids tested (capric, lauric, myristic, palmitic, stearic, oleic, ricinoleic, linoleic, linolenic, arachidonic) exert a bacteriostatic and bactericidal effect on the micrococcus and on all tubercle bacilli in protein-free media. Stearic acid was the least toxic of the acids tested whereas the unsaturated acids (oleic, ricinoleic, linoleic, linolenic, and arachidonic) had the most pronounced bacteriostatic and bactericidal action. The human tubercle bacilli were the most sensitive of the organisms tested; for example, inhibition of growth of small inocula of these organisms in synthetic media could be detected in concentrations of 0.00001 to 0.0001 per cent fatty acid. The avian tubercle bacilli were somewhat less sensitive (approximately 10 times), and the culture of micrococcus was much more resistant.

None of the esters exhibited any significant toxicity for the micrococcus culture; the water-soluble esters of lauric acid and palmitic acid (Tween 20, G-2144, Tween 40) exhibited an appreciable bacteriostatic and bactericidal activity on the tubercle bacilli in concentrations of 0.01 to 0.001 per cent, whereas the esters of stearic and oleic acid (Tween 60, Tween 80, G-2144) became inhibitory only in higher concentrations. In all cases, and with both cultures, the minimal inhibitory concentration became greater as the size of the inoculum increased. In all cases, also, toxicity was very much decreased or completely abolished by addition to the medium of crystalline serum albumin (0.1 to 0.5 per cent).

When tested under conditions designed to neutralize their toxic effect (*i.e.*, when used in low enough concentrations, or when tested with a large enough bacterial inoculum, or in the presence of serum albumin), the long chain fatty acids or their esters—except capric and ricinoleic acid—markedly increased the amount of growth yielded by tubercle bacilli. In the case of the micrococcus, on the contrary, only oleic, linoleic, linolenic, and arachidonic acids were effective; stimulation of growth was detectable with 1γ of these unsaturated acids

per ml. of medium and appeared to reach a maximum with 10 to 20γ per ml. The saturated fatty acids, as well as ricinoleic acid, were either completely inactive or active only in higher concentration (perhaps due to the presence of active impurities). It is of special interest that the water-soluble esters of lauric (Tween 20 and G-2124), palmitic (Tween 40), stearic (Tween 60), and ricinoleic acid (G-6486 T and G-6506 F.J.) were very ineffective in stimulating the growth of the micrococcus, whereas the two water-soluble esters of oleic acid (G-2144 and Tween 80) exhibited extremely high activity in this respect; water-soluble esters of linoleic, linolenic, and arachidonic acids were not available.

The Effect of Lipids on Bacterial Growth on Agar Media.—In addition to their effect on the density of growth developing in liquid media, the long chain fatty acids and their esters also exert a striking action on the number and size of colonies developing on agar media. This action, inhibitory or stimulatory, is dependent upon the nature of the other components of the medium, as is illustrated in the following experiments:

1.5 per cent agar (Difco) was added to the semisynthetic casein hydrolysate medium previously described; sodium oleate, ferric chloride, and crystalline bovine albumin were added as indicated in Table III. After solidification, the surface of the agar was inoculated with a loopful of a 1:100 dilution of the culture of micrococcus or of avian tubercle bacillus. The effect of the different components of the medium was determined by measuring the diameter of well isolated colonies after 7 days' incubation at 37° C. (Table III).

The results presented in Table III show that the colonies of both cultures remained extremely small in the absence of iron and of oleic acid. The tubercle bacillus failed to grow in the absence of albumin and the micrococcus was also inhibited by the larger concentration of oleic acid in the absence of the protein. In the presence of the latter substance, the colonies increased in size as the concentration of oleic acid increased from 0 to 0.0001 per cent and 0.01 per cent.

The effect of other fatty acids and of their water-soluble esters was also tested in agar media containing both iron and bovine albumin. The results presented in Table IV show that the growth of the avian tubercle bacillus was stimulated by all the fatty acids except capric, myristic, and ricinoleic acids, whereas the micrococcus responded only to the oleic, linoleic, linolenic, and arachidonic acids. It will be recalled that the water-soluble esters contain 20 to 25 per cent fatty acid; judging from the diameter of the colonies, the impression was gained that these esters were more efficient than the free acids in supporting bacterial growth on agar.

DISCUSSION

It has long been known that long chain fatty acids exert a toxic action on a number of microorganisms, the Gram-positive and acid-fast species in particular, and that their toxicity appears to be directly related to the number of un-

saturated bonds in the molecule; moreover, disappearance of toxicity in the presence of serum has also been recognized (1–3, 11–13, 16). We have observed that esterification of the fatty acids decreases or even abolishes completely their antibacterial action; thus, methyl oleate, triethanolamine oleate, and a variety of natural and synthetic phosphatides (lecithins and cephalins) do not prevent, and in fact may stimulate, the growth of tubercle bacilli. That the decrease in toxicity is not due to poor solubility of the esters is indicated by the fact that certain polyoxyethylene derivatives of oleic acid studied in this

TABLE III

Effect of Iron and Albumin on Bacterial Growth on Oleate Agar

Oleic acid	$FeCl_3$	Albumin	Size of colonies (diameter in mm.)*	
			Micrococcus C	Avian tubercle bacillus
per cent	*per cent*	*per cent*	*mm.*	*mm.*
0	0	0	<0.5	No growth
0.001	0	0	<0.5	" "
0.01	0	0	No growth	" "
0	0.001	0	<0.5	No growth
0.001	0.001	0	0.5	" "
0.01	0.001	0	No growth	" "
0	0	0.5	No growth	<0.5
0.001	0	0.5	2.0	0.5
0.01	0	0.5	4.0	0.5
0	0.001	0.5	No growth	<0.5
0.001	0.001	0.5	2.0	0.5
0.01	0.001	0.5	7.5	2.0

* Diameter measured on isolated fully grown colonies; "<0.5" indicates colonies barely visible to the naked eye; "no growth" indicates that no colonies could be detected under low power of microscope.

and earlier reports are essentially non-toxic despite the fact that they are completely dispersible in water in all proportions and that oleic acid itself is one of the most toxic of long chain fatty acids (2, 8).[3] It must be kept in mind, on the other hand, that the water-soluble, as well as the insoluble esters, can be hydrolyzed by lipases present in the medium (for example, introduced with non-crystalline serum albumin or with tissue extracts) or produced by the bacterial

[3] It is worth mentioning in this respect that the commercial preparations of polyoxyethylene derivatives of long chain fatty acids exhibit varying degrees of toxicity. However, it has been found that these commercial preparations contain a certain amount of unesterified free fatty acid (approximately 0.3 per cent in the case of Tween 80). When freed of this unreacted acid by a method described elsewhere, Tween 80 becomes essentially non-toxic to the tubercle bacillus (5).

cells themselves. In other words, it is possible that a non-toxic ester will become inhibitory as a result of saponification during incubation (5).

TABLE IV

Effect of Various Lipids on Bacterial Growth on Agar Media Containing 0.5 Per Cent Serum Albumin

Lipid added to medium	Final concentration	Size of colonies (diameter in mm.)*	
		Micrococcus C	Avian tubercle bacillus
	per cent	*mm.*	*mm.*
Capric acid	0.01	<0.5	No growth
	0.001	<0.5	<0.5
Myristic acid	0.01	<0.5	No growth
	0.001	<0.5	<0.5
Lauric acid	0.01	<0.5	1.0
	0.001	<0.5	0.5
Palmitic acid	0.01	<0.5	1.5
	0.001	<0.5	0.5
Stearic acid	0.01	<0.5	1.5
	0.001	<0.5	0.5
Oleic acid	0.01	3.0	1.5
	0.001	1.5	0.5
Ricinoleic acid	0.01	<0.5	No growth
	0.001	<0.5	<0.5
Linoleic acid	0.01	2.0	No growth
	0.001	0.5	<0.5
Linolenic acid	0.01	3.0	No growth
	0.001	0.5	<0.5
Arachidonic acid	0.01	2.5	No growth
	0.001	1.0	<0.5
Polyoxyethylene ester of Lauric acid G-2124	0.05	1.5	1.5
	0.005	<0.5	0.5
Polyoxyethylene ester of Oleic acid G-2144	0.05	6.0	2.5
	0.005	4.0	1.0

TABLE IV—*Concluded*

Lipid added to medium		Final concentration	Size of colonies (diameter in mm.)*	
			Micrococcus C	Avian tubercle bacillus
		per cent	*mm.*	*mm.*
Polyoxyethylene derivative of sorbitan monooleate Tween 80	Sorbitan monolaureate	0.05	1.5	1.5
	Tween 20	0.005	<0.5	0.5
	Sorbitan monopalmitate	0.05	<0.5	1.5
	Tween 40	0.005	<0.5	0.5
	Sorbitan monostearate	0.05	<0.5	2.0
	Tween 60	0.005	<0.5	0.5
	Sorbitan monooleate	0.05	6.0	2.0
	Tween 80	0.005	1.5	0.5
	Sorbitan monoricinoleate	0.05	<0.5	<0.5
		0.005	<0.5	<0.5
Control		0	<0.5	<0.5

* Diameter measured on isolated fully grown colonies; "<0.5" indicates colonies barely visible to the naked eye; "no growth" indicates that no colonies could be detected under low power of microscope.

Detoxification of the fatty acid can also be obtained by admixture with native serum albumin. When an adequate amount of this protein is added to an opalescent soap emulsion (at neutral pH), there occurs an immediate clearing of the emulsion suggesting the formation of a lipoprotein complex, accompanied by concomitant disappearance of toxicity. It takes approximately 40 parts by weight of albumin to achieve complete detoxification of 1 part of oleic acid. Of the many other proteins tested (globulins, lactalbumin, gliadin, edestin, gelatin, etc.) none can replace serum albumin in neutralizing the toxicity of the fatty acid; moreover, the detoxifying power of albumin is lost as soon as the integrity of the molecule is destroyed by enzymatic digestion or by heating (5).

When rendered atoxic, either by esterification or in mixture with serum albumin, a number of long chain fatty acids can enhance the growth of certain bacteria. In the experiments reported in the present paper, tubercle bacilli failed to display any clear selectivity with reference to the different fatty acids and their growth was enhanced by a number of them, saturated and unsaturated. On the other hand, oleic, linoleic, linolenic, and arachidonic acids proved to be the only substances capable of stimulating the growth of the micrococcus both in liquid media and on agar.

Tubercle bacilli and micrococcus C thus appear to differ in several respects from the point of view of their response to the growth stimulatory effect of the fatty acids. The growth of the mycobacteria is enhanced by a variety of fatty acids, saturated and unsaturated, whereas the micrococcus responds only to oleic, linoleic, linolenic, and arachidonic acids. The latter organism gives only scant or no growth when glucose or fatty acids are the only source of carbon in the medium whereas, in the presence of glucose, growth is enormously stimulated by small amounts of the unsaturated acids. In the case of the tubercle bacilli, on the other hand, early growth is not appreciably stimulated by glucose but the amount of bacterial protoplasm synthesized (within a few days) appears to be related to the amount of long chain fatty acid (irrespective of saturation or unsaturation of the molecule) present in the medium. The amounts of lipids required in this case are larger than those required for maximal growth of the micrococcus; under the conditions of the experiments, optimal growth of mycobacteria was obtained with approximately 0.01 per cent of the fatty acids. Finally, addition of albumin to media containing only minute concentrations of unsaturated acids completely inhibits the small amount of growth of the micrococcus which would have taken place in the absence of the protein and this growth inhibitory effect can be neutralized by addition of adequate amounts of oleic, linoleic, linolenic, or arachidonic acid. A similar growth inhibitory effect of albumin has not been observed in the case of tubercle bacilli. These differences are sufficiently striking to suggest that the fatty acids play very different rôles in the metabolism of the two organisms. It appears worth considering, for example, that the unsaturated acids act as catalysts in the metabolism of the micrococcus, whereas tubercle bacilli can utilize a large variety of long chain acids as metabolites in the synthesis of their protoplasm.

Many bacterial species are known to be capable of oxidizing or utilizing for their growth a variety of long chain fatty acids; it will suffice briefly to mention here a few of the known facts which have a direct bearing on the problems discussed in the present report.

Oleic acid behaves as a growth factor for *Corynebacterium diphtheriae* and *Clostridium tetani*, in concentrations of the order of 1 to 50γ per ml. of medium. Moreover, dihydroxystearic acid prepared from a sample of oleic acid of known activity was found to be unable to support growth of the diphtheria bacillus, whereas reduction of the dehydroxystearic acid back to oleic acid was accompanied by a restoration of the growth-promoting activity (4, 10). In these respects, therefore, the requirements of micrococcus C are similar to those of the diphtheria and tetanus bacillus.

It has been shown by metabolic studies in the Warburg respirometer that unsaturated acids in general, and linolenic acid in particular, inhibit oxygen uptake by tubercle bacilli and exert upon them a bactericidal effect. Stearic acid, on the contrary, exhibits no toxic effect in the same concentrations; the

toxic action of the unsaturated acids was also found to be much reduced by the addition of blood plasma to the system (2, 14). Of particular interest is the fact that oleate, stearate, and palmitate were found to be even more active than glycerol or glucose in stimulating the respiration of starved cells, oleate proving the most active of the three long chain compounds in stimulating metabolism during the first period of the experiment. However, whereas oxygen uptake remained at a high level for several hours when stearate and palmitate were used as substrate, it rapidly fell below that of the control in the preparations containing oleate. In other words, oleic acid behaved either as a stimulator or as an inhibitor of the metabolism of tubercle bacilli, depending upon the time at which the measurement of respiration was made (14). This observation can be readily interpreted in terms of the findings described in the present paper. We have seen that fatty acids exert a dual type of action on certain living cells; they are toxic but they can also stimulate metabolism and growth. Depending upon the conditions of the experiment, one effect, or the other, will dominate the picture. In the respiration experiments just referred to, the toxic effects were slow in becoming manifest because of the very large number of tubercle bacilli involved in the test; stimulation of respiration could be observed before destruction of the oxidative systems occurred following death of the bacteria.

The observations which have just been discussed suggest that the stimulatory effect of fatty acids on the metabolism and growth of microbial cells has often been missed because the test was carried out under conditions where the toxic effect interfered with respiratory processes or prevented the initiation of growth. Generalizing from the results described in the present paper, one is tempted to predict that long chain fatty acids will be found to exert stimulatory effects on many microbial species if tested under conditions where their toxicity is minimized. In the present experiments this result was achieved by using the fatty acids in the form of their water-soluble esters or in mixture with serum albumin.

Before concluding, it may be justifiable to suggest two possible applications of some of the facts discussed in the present paper. Under the proper cultural conditions, the amount of growth yielded by micrococcus C seems to be directly related to the concentration of oleic, linoleic, linolenic, or arachidonic acids present in the culture medium (between 0.00001 and 0.0001 per cent). One may hope therefore, that by skillful manipulation of the many factors which affect its growth, this organism will lend itself to the development of microbiological assay methods for certain unsaturated fatty acids. It has also been repeatedly observed that at equal concentration of fatty acid, with or without serum albumin, the water-soluble esters are much more efficient than the soaps in supporting bacterial growth; this is true for the culture of micrococcus C and for all the strains of tubercle bacilli so far tested. It would appear worthwhile to study quantitatively the rate of utilization of the fatty acids in order to determine whether one of the beneficial effects of the water-soluble esters is

to supply the long chain acids in a form more readily available to the bacterial cell.

SUMMARY

Long chain fatty acids have been found to exhibit both inhibitory and stimulatory effects on the growth of tubercle bacilli and of a certain unidentified micrococcus culture.

The toxicity of the fatty acids was much reduced or abolished by (*a*) esterification, even when the resulting product was a water-soluble ester, and (*b*) addition of crystalline serum albumin to the culture medium; other proteins tested were inactive in this respect.

Marked growth stimulation of the microorganisms studied was obtained when certain long chain fatty acids were added to the culture medium in the form of their water-soluble esters, or in admixture with adequate amounts of serum albumin.

Abundant growth of the micrococcus resulted from the addition of oleic, linoleic, linolenic, or arachidonic acid (0.0001 to 0.001 per cent) to a mineral medium containing glucose as sole source of carbon; in the case of this microbial species, none of the other substances tested could substitute for these unsaturated fatty acids.

Enhancement of growth of tubercle bacilli was obtained by adding to the medium 0.001 to 0.01 per cent of a variety of fatty acids (saturated or unsaturated) even in the absence of glucose or of any other readily available carbon compound.

These results suggest that long chain fatty acids can affect the growth of different microbial species through different metabolic channels and that, in order to study the mechanism of these metabolic and growth reactions, it is essential to use the fatty acids under conditions where they cannot manifest their toxic properties.

Addendum.—The results described in the present paper establish that the addition of crystalline serum albumin to the medium allows the growth of small inocula which would not grow in the absence of the protein; however, it does not increase appreciably the final density of the culture. Less pure preparations of the protein can, on the other hand, increase the amount of growth, as well as facilitate its initiation. Thus, we have now recognized that many samples of bovine plasma fraction V contain heat-stable impurities which can be separated from the protein, and which increase markedly the amount of growth yielded by tubercle bacilli in synthetic media.

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