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Long-Chain Fatty Acid Inhibition of Growth of *Streptococcus agalactiae* in a Chemically Defined Medium[1]

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ABSTRACT

WILLETT, NORMAN P. (University of Pennsylvania School of Veterinary Medicine, Kennett Square, Pa.), AND GUY E. MORSE. Long-chain fatty acid inhibition of growth of *Streptococcus agalactiae* in a chemically defined medium. J. Bacteriol. **91**:2245–2250. 1966.—A chemically defined medium was developed for *Streptococcus agalactiae* which supported growth comparable to that obtained in complex medium. The effects of long-chain fatty acids on growth of the organisms were determined turbidimetrically. The order of activity of the fatty acids was dependent upon whether complete inhibition or median response (50% inhibition point) was used as a parameter of activity. When complete inhibition of growth was used as a measure, the degree of unsaturation of C_{18} acids enhanced antimicrobial activity. However, when the median response was used as an index, this order was reversed. Increase in carbon chain from C_{12} to C_{18} did not correlate with either complete inhibition or median response points. Antimicrobial activity of unsaturated and saturated fatty acids was reversed by bovine serum albumin and other compounds, suggesting a bacteriostatic action.

Previous studies by Murphy (14) suggested that the "keratin-like" material found in the bovine teat canal plays a significant role in susceptibility to mastitis. This material, which lines and partially occludes the first 8 to 10 mm of the teat canal, if removed, will render previously resistant animals uniformly susceptible to experimental challenge with *Streptococcus agalactiae*.

The teat canal "keratin" has been shown to contain a significant lipid fraction (1, 21). The antimicrobial properties of fatty acids as well as their growth-stimulating properties have been well documented by Kodicek (9), Nieman (15), and others (8, 16, 23). In general, gram-positive organisms were found to be more sensitive to these compounds than gram-negative bacteria. *S. agalactiae* and other gram-positive organisms were markedly inhibited by linoleic acid, and the effect could be reversed by cholesterol, but only with lower concentrations of acid (8). Adams (1) investigated the antistreptococcal activity of bovine teat canal "keratin" from a qualitative view without emphasizing the quantitative aspects.

The "self-disinfecting" power of skin was associated with medium chain-length free fatty acids by Burtenshaw (4). Recently, the role of fatty acids in the protective mechanisms of skin was thoroughly reviewed by Rothman and Lorincz (18) and placed in proper perspective along with other chemical, physical, and physiological defense systems.

It is the purpose of this study to investigate the effect of known fatty acids on the growth of *S. agalactiae* in a chemically defined medium, and thus to provide a base line for further investigations on the teat canal "keratin," as well as to help explain the pathogenicity of this organism on a biochemical level. As a prerequisite, a chemically defined medium must be developed which could support growth approaching that in complex media. The advantages of working with a medium of known chemical composition are self-evident. Nieman (15), Arai (2), and Demain (6) have shown that trace amounts of fatty acids found in vitamin-free casein acid-hydrolysate and other constituents of bacteriological culture medium markedly influenced bacterial growth.

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MATERIALS AND METHODS

The culture used was *S. agalactiae* (Cornell Strain 48) which conforms to the criteria as given in *Bergey's Manual of Determinative Bacteriology*. Cultures were maintained on blood-agar slants kept at 4 C and were transferred at 1-month intervals. Log-phase inocula were prepared by serially passing the organism three successive times in Brain Heart Infusion (BBL), once at 24 hr and the last two times at 6-hr intervals. The culture was then centrifuged, washed twice with sterile physiological saline, and resuspended in saline to give an optical density (OD) of 0.80 or approximately 75×10^6 to 100×10^6 colony-forming units per ml.

Defined medium in 10-ml amounts was dispensed aseptically into test tubes with Morton closures, and 0.1 ml of inoculum was added.

Reagent-grade fatty acids (Calbiochem and Sigma Chemical Co., St. Louis, Mo.) were dissolved in redistilled 95% ethyl alcohol and were added in 0.1-ml amounts at zero time; concentrations were expressed as micrograms per milliliter. A 0.1-ml amount of ethyl alcohol was added to control tubes without fatty acid. Tubes were incubated at 37 C for 24 hr and OD was read at 550 mμ on a Spectronic-20 colorimeter. Plate counts of the inocula were made with Brain Heart Infusion Agar (BBL). Culture purity was determined by streaking on bovine blood-agar plates. Relative concentrations of bacteria expressed as per cent growth of control were calculated according to the method of Toennies and Gallant (19), and dose-response curves were plotted on log probability paper. Use of this method compensates for deviations from Beer's Law and also provides a convenient technique to measure relative bacterial concentrations in the presence of a drug as compared with a control grown in its absence.

All glassware was acid-cleaned with a 50:50 mixture of sulfuric and nitric acids, and all reagents were made up with double-distilled water. Extreme care was taken not to contaminate the glassware and closures with extraneous sources of fatty acid.

RESULTS

The composition of the chemically defined medium is shown in Table 1. The medium is based on that of Mergenhagen and Scherp (13), although a number of modifications have been made. These included addition of cysteine and glutamine, increased vitamin and salt concentrations, and an increase in *p*H. Details on the development of the medium and the nutritional requirements of the organism will be presented elsewhere. Results of a typical growth curve are shown in Fig. 1. Growth compared favorably with that obtained in Brain Heart Infusion broth. The log phase was complete in less than 6 hr with OD readings of 0.60 to 0.70. After completion of the log phase, OD remained essentially constant until at least 24 hr. This was also true of the experiments with fatty acids. The final *p*H in defined medium was 4.3 to 4.4, which usually resulted in a rapid decrease in viable cells after completion of the log phase.

TABLE 1. *Chemically defined medium* for Streptococcus agalactiae C-48*

Compound	Final concn	Compound	Final concn
	μg/ml		μg/ml
L-Alanine	250	K_2HPO_4	1,000
L-Arginine	320	KH_2PO_4	1,000
L-Aspartic acid	500	$FeSO_4 \cdot 7H_2O$	10
DL-Asparagine	100	$MnCl_2$	25
L-Cystine	600	NaCl	10
L-Cysteine HCl†	600	$ZnSO_4 \cdot 7H_2O$	10
L-Glutamic acid	400	$MgSO_4 \cdot 7H_2O$	80
L-Glycine	200	$NaHCO_3$	500
L-Glutamine†	100	Glucose	10,000
L-Histidine	320		
L-Leucine	200	Guanine	10
L-Lysine	320	Xanthine (Na salt)	10
L-Isoleucine	200	Uracil	10
DL-Methionine	200	Adenine	10
L-Phenylalanine	200		
L-Proline	200	Nicotinic acid	10
DL-Serine	400	Ca pantothenate	10
L-Tryptophan	200	Pyridoxal HCl	10
L-Threonine	200	Thiamine HCl	0.6
L-Tyrosine	200	Riboflavine	1
L-Valine	200	Biotin	0.2
		Folic acid	0.02

* Medium adjusted to *p*H 7.4 and sterilized by filtration.

† Cysteine and glutamine added just prior to use, final *p*H adjusted to *p*H 7.6 with NaOH.

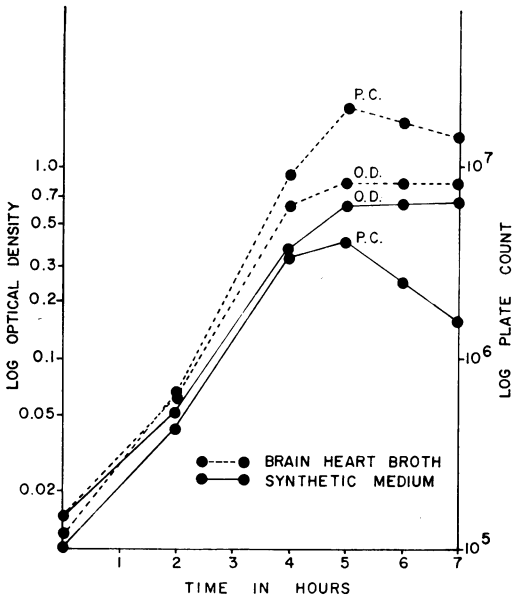

FIG. 1. *Comparison of growth curves in synthetic medium and Brain Heart Infusion broth. Optical density (OD) read at 550 mμ on Spectronic-20 colorimeter. Plate counts (PC) made with Brain Heart Infusion agar.*

TABLE 2. *Concentration of long-chain fatty acids necessary for complete inhibition of growth*

Unsaturated*	Concn	Saturated*	Concn
	μg/ml		μg/ml
Linoleic (18:2)...	5.0	Myristic (14:0) ..	10.0–11.0
Linolenic (18:3)..	5.0	Palmitic (16:0) ..	25.0
Oleic (18:1).....	10.0	Lauric† (12:0).	>40.0
		Stearic† (18:0).	>40.0

* Numbers in parentheses represent ratio of total chain length to number of double bonds.

† Relative ranking based on final *p*H in growth tube.

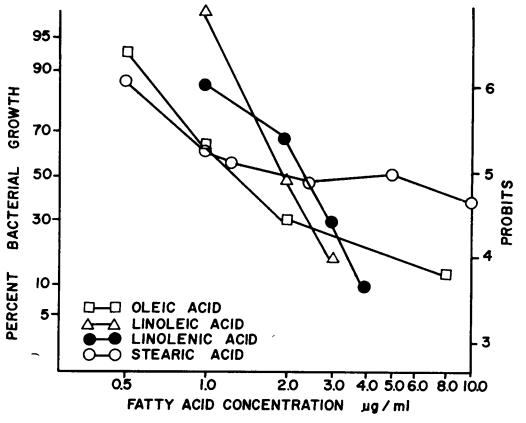

FIG. 2. *Effect of unsaturation of C_{18} fatty acids on growth of Streptococcus agalactiae in a chemically defined medium. Results expressed as percentage of bacterial growth in tube with fatty acid absent. OD read at 550 mμ on Spectronic-20 colorimeter.*

The first experiments with the fatty acids were performed with the intention of finding the dose which completely inhibits growth (Table 2). The degree of unsaturation apparently greatly enhanced inhibitory activity. Linoleic and linolenic were the most active with no differences noted between them, followed by oleic and stearic acids, in that order. On the other hand, increase of chain length from 12 to 18 carbons did not correlate directly with an increase in antimicrobial activity of the saturated fatty acids. Myristic acid was the most active, followed by palmitic, lauric, and stearic, in that order.

After plotting the dose-response curves, it was noted that the slope and median responses were characteristic for each particular acid. The slope of a dose-response curve is determined by the particular combination of characteristics of the chemical and of the organism. Median response is that concentration of chemical that permits growth one-half as great as that obtained in the same period in the absence of a drug (7).

The results in Fig. 2 demonstrate the effect of unsaturation on the relative activity of several 18-carbon acids. Each point represents the average value obtained from several experiments. Oleic, linoleic, and linolenic acids, which have one, two, and three double bonds, respectively, had approximately similar slopes over most of their length, whereas the slope for stearic acid was extremely flat and paralleled that of the other acids only at lower concentrations.

The median responses or 50% inhibition points are shown in Table 3. Paradoxically, with the exception of stearic, the order of activity by this parameter was almost exactly opposite that necessary for complete inhibition.

The effect on the antimicrobial activity of increasing carbon chain length from 12 to 18 carbons is shown in Fig. 3. The slopes for myristic and lauric acid appeared to be relatively parallel, whereas lauric acid tailed off with a flat slope similar to palmitic and stearic acids at concentrations over 10 μg ml. The slopes for palmitic and stearic acids were approximately the same as the other acids for rather low concentrations, and then became almost horizontal. The median responses of these curves are shown in Table 4.

The reversal of unsaturated fatty acid inhibi-

TABLE 3. *Median response* of long-chain fatty acids as a function of unsaturation*

Fatty acid	Concn
	μg/ml
Oleic (18:1)	1.3
Stearic (18:0)	1.8
Linoleic (18:2)	2.0
Linolenic (18:3)	2.4

* Concentration necessary for 50% inhibition.

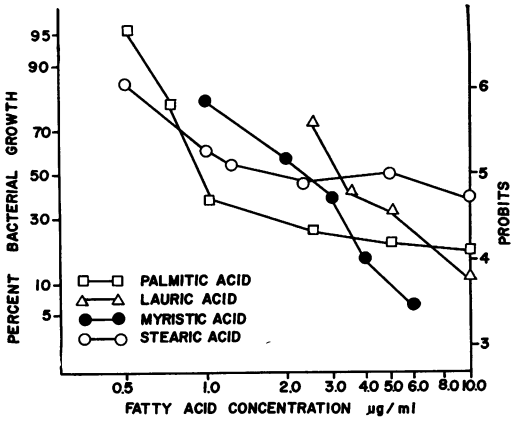

FIG. 3. *Effect of increasing carbon chain length on growth of Streptococcus agalactiae in a chemically defined medium. Results expressed as percentage of bacterial growth in tube with fatty acid absent. OD read at 550 mμ on Spectronic-20 colorimeter.*

tion by bovine serum albumin and other surface-active agents, as shown in Table 5, agreed with that reported for other gram-positive organisms (9). Ergosterol was inactive at the concentration used, but this does not preclude a reversal at higher concentrations. Reversal of saturated fatty acid activity, however, is contrary to that previously reported, and, although generally less in magnitude than in the case of the unsaturated acids, followed the same pattern of reversal (10).

DISCUSSION

The relative antimicrobial activity of the fatty acids necessary for complete inhibition of *S. agalactiae* differed qualitatively and quantitatively from those previously reported (1). Exceptions to this were myristic and linoleic acids, whose relatively high antibacterial activity was confirmed in this study. Discrepancies can be explained in part by the rigorous experimental conditions used in this work. On the other hand, results fell into the general pattern of inhibition observed by investigators for gram-positive organisms other than streptococci (9, 10). This

TABLE 4. *Median response* of long-chain fatty acids as a function of chain length*

Fatty acid	Concn
	μg/ml
Palmitic (16:0)	0.9
Stearic (18:0)	1.8
Myristic (14:0)	2.4
Lauric (12:0)	3.2

* Concentration necessary for 50% inhibition.

TABLE 5. *Effect of antagonists on fatty acid inhibition of Streptococcus agalactiae**

Antagonist	Linoleic	Myristic
	%	%
Fatty acid alone	10.3	8.0
Cholesterol†	17.8	19.0
Ergosterol†	9.3	8.0
DL-α-Tocopherol†	32.7	4.0
Bovine serum albumin (0.1%)‡	101.9	60.0
β-Lactoglobulin (0.003%)‡	18.6	11.0
Lecithin†	45.8	32.0

* Results are expressed as percentage of growth in tube without fatty acid or antagonist.

† Final concentration of antagonist equivalent to final concentration of respective fatty acid, i.e., linoleic, 4 μg/ml; myristic, 8.0 μg/ml.

‡ Final concentration of antagonist as stated in table. Fatty acid concentrations identical with remainder of experiment.

included the observation of the enhanced antimicrobial activity correlating with increasing unsaturation observed by Kodicek (10) in the case of *Lactobacillus casei.*

The use of dose-response curves is extremely valuable in quantitating either antimicrobial or stimulatory activity. This is borne out by the change in order of relative activity when the median response point, rather than complete inhibition, is used as a measure of activity. Care must be taken to specify at what point in a dose-response curve inhibition is measured. Comparative activities of compounds will not be as significant if they are not measured at points where the slopes of the dose-response curves are similar. It can be seen that greater than 50% of the activity of acids such as palmitic and stearic occurred at comparatively low concentrations and then leveled off. This fact could not have been elucidated without the aid of a dose-response curve. In addition, similar slopes and shapes of curves suggest similar modes of action, thus giving valuable information for correlating chemical structure with biological activity.

A number of explanations are possible for the sudden breaks in the dose-response curves. The

limited solubility of palmitic and stearic acids may be a factor; however, these acids were completely soluble at concentrations up to 10 μg/ml, which included the highest levels in the dose-response curve. Secondly, due to *p*H change and changes in concentration of metabolites, it cannot be assumed that the organisms are in a constant environment; therefore, the sensitivity of the organism will also change. Changes in slopes may represent altered growth rates of survivors resistant to fatty acid. Another factor to be considered is the release of cellular material containing fatty acids or compounds antagonistic to acids, or both, with resultant auto-intoxication or detoxification. Finally, according to Rahn (17), the fact that the streptococci form long chains results in a nonlogarithmic order of death, and survivor curves will be concave downwards.

Reversal of fatty acid inhibition by serum albumin and other compounds suggests that inhibition is of bacteriostatic rather than bactericidal nature. Maxcy and Dill (12) recently showed that fatty acids absorbed onto the surface of various species of streptococci can be eluted with weak alkali, suggesting a surface phenomenon which could be reversed more easily with consequent bacteriostasis. Tomarelli et al. (20) have reported the presence of factors antagonistic to fatty acid inhibition in human and cow's milk. Thus, the presence of these factors could conceivably play a major part in the pathogenesis of the disease, relegating the fatty acids to a secondary role.

Fatty acids and lipids possess a wide spectrum of biological activity as related to disease, in addition to antimicrobial activity. Included among these are antigenicity (10), bacterial stimulation (16), enzyme inhibition (3), inhibitory (5) and stimulating (11) effects on leukocytes, and local tissue reactions (22).

The significance of the lipid fraction in the teat canal "keratin" remains to be evaluated. Studies are being carried out in our laboratories to characterize this fraction by gas chromatography and to determine whether the analysis can be correlated with susceptibility to mastitis and the known sensitivity of the organism to fatty acids.

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