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Effect of 2-Alkynoic Acids on In Vitro Growth of Bacterial and Mammalian Cells

WATTANA KONTHIKAMEE,[1] JOHN R. GILBERTSON,[1]* HERMAN LANGKAMP,[2] AND HERMAN GERSHON[3]

Departments of Pharmacology and Physiology[1] and Microbiology,[2] School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and Boyce Thompson Institute for Plant Research at Cornell University, Ithaca, New York 14853[3]

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3-Decynoyl-*N*-acetylcystamine is known to inhibit the in vitro growth of *Escherichia coli* but not of yeasts or mammalian cells. Neither the free acid nor the 2 positional isomer is active (L. R. Kass, J. Biol. Chem. **243**:3223–3228, 1968). Other studies have shown that 2-hexadecynoic acid is fungitoxic whereas most of the shorter chain isomers are inactive (H. Gershon and L. Shanks, Can. J. Microbiol. **24**:591–597, 1978). Since these studies suggested that positional or chain length isomers of the acetylenic acids may selectively inhibit the growth of microorganisms, the effect of the alkynoic acids on the in vitro growth of gram-positive and gram-negative bacteria was evaluated. 2-Hexadecynoic acid was found to be the most active species. This acid was bacteriostatic for all gram-positive bacteria tested. The acid was readily taken up by the treated cells and incorporated into the phospholipid fraction. When added to the culture medium, 2-hexadecynoic acid inhibited the growth of HeLa cells, but when mixed with an equivalent amount of palmitic acid, growth inhibition was not observed.

In bacteria, the acyl moieties of the different classes of phospholipids are characterized by the absence of polyunsaturated fatty acids (3, 9, 16). Alteration of the composition of the acyl chains of the membrane phosphatides results in a change in the physical properties of the membranes, as well as the function of constituent proteins; thus, it is not surprising that linoleic and linolenic acids act as antimicrobial agents when incorporated into culture medium (1, 10, 12).

Further evidence indicating that the antimicrobial activity of the fatty acids reflects a change in the physical properties of the plasma membrane is the observation that some acetylenic fatty acids are active antimicrobial agents (4, 11, 13).

In this instance, the antimicrobial activity varied with the derivative of the acid employed, the chain length of the molecule, and position of the acetylenic bond (11, 13). The growth of gram-negative bacteria, *Escherichia coli*, but not yeast is inhibited by the *N*-acetylcystamine derivative of the 3-ynoic acids, 9 to 11 carbon atoms in chain length. The acetylenic derivative inhibits β-hydroxy decanoylthioester dehydrase, preventing biosynthesis of unsaturated fatty acids in the affected bacterium (13). A relative strict specificity exists in that the free acid or the isomers having the acetylenic bond at carbons 2 or 4 are inactive (13). In contrast, the 9-ynoic acid of similar chain length supports the growth of *E. coli* (14). These observations, coupled with the demonstration that the 2-ynoic acids, 14 or 16 carbon atoms in chain length, are fungistatic (7), suggest that the position of the acetylenic bond may convey a microbial species specificity. Since certain bacterial species are considered to be uniquely involved in the development of the diseases of the mouth (6, 17, 19; A. C. R. Crawford, S. S. Socransky, E. Smith, and R. Phillips, J. Dent. Res. **56B**:275, B120, 1977), the possibility that specific isomers of the ynoic acids might selectively inhibit the growth of a different class of microorganism is attractive to us. This report describes the effect of the 2-ynoic acids on the in vitro growth of specific gram-positive and gram-negative microorganisms.

MATERIALS AND METHODS

Organisms. Gram-positive cocci: *Streptococcus mutans* ATCC 25175, *Streptococcus sanguis* ATCC 10556, *Streptococcus mitis* ATCC 6249, *Streptococcus salivarius* ATCC 13419-2, and *Staphylococcus aureus* ATCC 25923 and (penicillin resistant). Gram-negative cocci: *Neisseria flava* (wild type), *Neisseria gonorrhoeae* (wild type), and *Neisseria meningitidis* (wild type). Gram-positive bacilli: *Actinomyces viscosus* m-100, *Clostridium butyricum* ATCC 6015, and *Lactobacillus brevis* ATCC 14869. Gram-negative ba-

cilli: *Bacteroides fragilis* (wild type), *E. coli* ATCC 25922, *E. coli* B. American P+, and *Pseudomonas aeruginosa* (wild type). HeLa cells were used.

Media. Gram-positive cocci were grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.). Gram-negative cocci were grown in Trypticase soy broth (BBL). Gram-positive and gram-negative bacilli were grown in fluid thioglycolate medium (BBL). *L. brevis* was cultured in Rogosa SL broth (Difco Laboratories, Detroit, Mich.). HeLa cells were cultured in Eagle basal medium (Difco) enriched with 10% calf serum and the antibiotics penicillin G (100 μg/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.025 μg/ml) (Sigma Chemical Co., St. Louis, Mo.).

Chemicals. The 2-alkynoic acids of chain lengths 4 to 9, 14, and 16 carbon atoms were obtained as reported previously (7). Palmitic acid was purchased from Applied Science Laboratories, State College, Pa. The solvents used in the chromatography procedures have been described elsewhere (5). Tissue culture Hanks solution was purchased from GIBCO Laboratories, Grand Island, N.Y.

Growth assay. Bacterial growth as turbidity of the culture medium was evaluated with a Klett-Summerson colorimeter equipped with a no. 66 filter. After the initial inoculation and at appropriate time intervals thereafter, the cultures were mixed on a Vortex mixer for 5 s, and turbidity was determined. Each assay was run in quadruplicate on two separate occasions. The values presented are the means ± standard deviation of the individual assays. The initial inocula, 10^9 colony-forming units, were added to 7 ml of culture medium. All inocula were obtained from seed cultures grown to stationary phase in the respective media. Cultures were incubated at 37.5°C.

Effect of 2-alkynoic acids on the growth of *S. mutans*. Samples of 2-alkynoic acids of chain lengths from 4 to 16 carbon atoms were dissolved in 95% ethanol to provide stock solutions that were 0.1, 1, 5, 10, and 20 μM. In the growth assay, 30-μl samples were removed from the stock solutions and injected from a microsyringe into 7 ml of the culture media. The minimal inhibitory concentration of 2-hexadecynoic acid for *S. mutans* was established as 28.6 μM. This concentration was employed to compare the growth inhibitory effects of the several alkynoic acids against *S. mutans*. Preparation of the treated culture media, inoculation of *S. mutans*, and the method of assessing bacterial growth were the same as those described above.

Effects of 2-hexadecynoic acid treatment on the fatty acid composition of *S. mutans*. Bacteria were grown at 37.5°C for 18 h in Todd-Hewitt broth and the same media containing 2.4 μM 2-hexadecynoic acid. Cells were harvested by centrifugation ($10^4 \times g$ for 15 min in a Sorvall RC 2-B centrifuge) and washed three times with Hanks balanced salt solution. The final cell pellet was drained and weighed. Lipids were extracted from the pellet twice with chloroform-methanol 2:1 (vol/vol), and the residue was concentrated to dryness in a rotary evaporator at 37°C (5). The lipid residue was blanketed with nitrogen, dissolved in benzene, and stored at −60°C before lipid analysis.

The total lipid extract was weighed and resolved into three fractions, neutral lipids, glycolipids, and phospholipids, by chromatography on silicic acid (18). The neutral lipid fraction was separated into its constituent classes by thin-layer chromatography on silica gel plates (8). The free fatty acid fraction was eluted from the silica gel, and the acids were methylated and identified by gas-liquid chromatography (15). In the phospholipid fraction, the esterified fatty acids were transmethylated and identified by gas-liquid chromatography (15). Identification of the individual methyl esters was based on their relative retention times as compared with known standards on polar and nonpolar columns and confirmed by mass spectrometry.

Effect of 2-hexadecynoic acid on growth of cultured HeLa cells. HeLa cells were cultured at 37.5°C in Eagle medium containing 10% calf serum and added antibiotics at 37°C for 24 h. An inoculum of 1.8×10^6 cells was plated and incubated for 2 h before the addition of fresh media containing concentrations of albumin-bound 2-hexadecynoic acid (10, 30, 61, 122, 244, 328, and 488 μM). Parallel cultures containing palmitic acid (67, 133, and 266 μM) and equimolar concentrations of 2-hexadecynoic and palmitic acids (67, 133, and 266 M) were prepared. Control cultures were prepared by adding the same volume of the albumin solution containing no additional fatty acids. At the end of the incubation interval, calls were harvested by trypsinization, and the DNA content of each culture flask was quantitated (2).

RESULTS

Bacteria growth studies. Figure 1 depicts the growth response of an initial inoculum, 10^9 colony-forming units, of *S. mutans* in Todd-Hewitt broth containing graded concentrations of 2-hexadecynoic acid. With this microorganism, minimal growth was observed at an acid concentration of 14.3 μM. The growth response of the other bacterial species was determined in a similar manner. The minimal inhibitory concentration of 2-hexadecynoic acid with these several species of microorganisms is presented in Table 1. The effect of palmitic acid on the growth of each of these microorganisms was assessed at the minimal inhibitory concentration established for 2-hexadecynoic acid and at twice this concentration. In all instances, the addition of palmitic acid at either concentration did not alter the growth response of the bacteria from that observed with untreated cultures.

The growth response of *S. mutans* was also evaluated in the simultaneous presence of palmitic acid and 2-hexadecynoic acid. In this instance, the concentration of palmitic acid in the culture media was 28.6 μM, whereas that of the 2-hexadecynoic acid was 4.2 μM. At this molar ratio of palmitic and 2-hexadecynoic acids of approximately 7:1, the growth inhibition was the same as that observed in the presence of the acetylenic acid alone (data not shown).

Growth inhibitory effects of 2-alkynoic acids of varying chain lengths. The growth inhibitory effects of 2-alkynoic acids having chain lengths of 4 to 9, 14, and 16 carbon atoms were evaluated at a concentration of 28.6 μM. Growth was assessed as before with *S. mutans*. At this

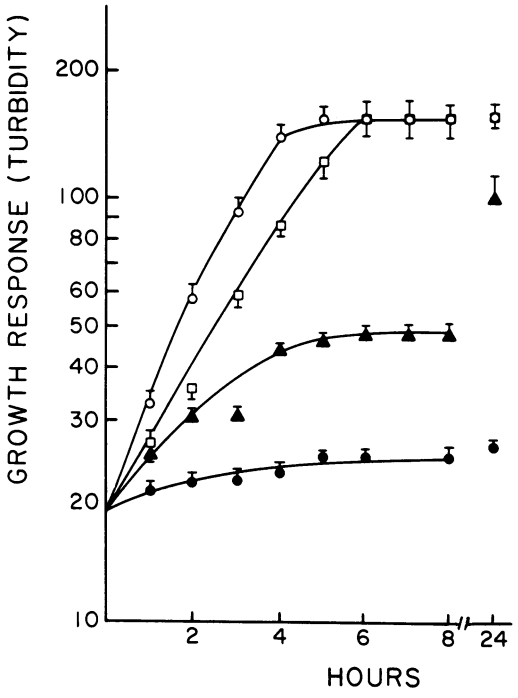

FIG. 1. Effect of various concentrations of 2-hexadecynoic acid on the growth *S. mutans*. Samples (10^9 colony-forming units) were removed in quadruplicate from an overnight bacterial culture and incubated at 37°C in Todd-Hewitt broth containing 2-hexadecynoic acid at the following micromolar concentrations: ○, none; □, 1.4; ▲, 4.2; ●, 14.3. The values plotted are the average turbidity readings (Klett units) from the individual cultures. The bars represent 1 standard deviation.

concentration, no effect on bacterial growth was noted with 2-alkynoic acids, 9 carbon atoms or shorter in chain length. In the presence of 2-tetradecynoic acid, the reduction of turbidity of the culture media after 8 and 24 h averaged 12% of the control; in the presence of 2-hexadecynoic acid, no growth was noted.

Composition of free fatty acids and phospholipid fatty acids of *S. mutans* grown in the presence and absence of 2-hexadecynoic acid. The composition of the free fatty acids and phospholipid fatty acids from *S. mutans* grown in the presence and absence of 2-hexadecynoic acid is presented in Table 2. It is apparent that the free fatty acids present in the untreated bacteria are primarily saturated, varying in chain length from 14 to 19 carbon atoms. Hexadecenoic and octadecenoic acids were the only unsaturated acids noted.

The esterified fatty acids from the phospholipids of the untreated bacteria were of chain length similar to those noted in the free fatty acid fraction. The only exceptions were that dodecanoic and docosenoic acids were present in the phospholipids but not in the free fatty acid fraction, whereas the odd chain fatty acids, hepta- and nonadecanoic acids, were not noted in the phospholipids. In the same lipid fractions extracted from bacteria treated with 2-hexadecynoic acid, the acetylenic acid accounts for a large proportion of the total fatty acids present. The presence of this fatty acid in both lipid fractions indicates that 2-hexadecynoic acid was taken up from the culture medium by the treated cells and was utilized in the biosynthesis of complex lipids.

TABLE 1. Minimal inhibitory concentration observed for several different bacterial species grown in the presence of 2-hexadecynoic acid

Bacteria	MIC[a] (μM)
Gram-positive cocci	
S. mutans	28.6
S. sanguis	21.4
S. mitis	21.4
S. salivarius	21.1
S. aureus	21.4
S. aureus (penicillin resistant)	56.8
Gram-negative cocci	
N. flava	21.4
N. gonorrhoeae	5.0
N. meningitidis	5.0
Gram-positive rod	
A. viscosus	14.2
L. brevis	NE[b]
C. butyricum	4.2
Gram-negative rod	
B. fragilis	21.4
E. coli	NE
E. coli B. American P+	NE
P. aeruginosa	NE

[a] The minimal inhibitory concentration (MIC) is the media concentration of 2-hexadecynoic acid at which no change in turbidity of the culture occurred over a 24-h interval.

[b] NE, No growth inhibition.

Effect of 2-hexadecynoic acid on growth of HeLa cells. 2-Hexadecynoic acid, when present in the culture medium in increasing concentrations, progressively inhibits the growth of cultured HeLa cells. However, if both palmitic and 2-hexadecynoic acid were present together, at the same concentration, inhibition of cellular growth was not noted (Table 3).

The effect of exposure to 2-hexadecynoic acid on the fatty acid composition of the cellular phospholipids was also evaluated. In the phospholipid fraction from both treated and untreated HeLa cells, palmitic, stearic, oleic, linoleic, and arachidonic acids accounted for at least 93% of the total fatty acids detected. The only differ-

TABLE 2. Composition of the free fatty acids and phospholipid fatty acids of *S. mutans* after exposure to 2-hexadecynoic acid[a]

Carbon chain[b]	Free fatty acids (wt %)				Phospholipid fatty acids (wt %)			
	Control		Treated		Control		Treated	
	I	II	I	II	I	II	I	II
12:0	ND[c]	ND	ND	ND	2.5	2.8	2.0	ND
14:0	2.1	2.4	ND	2.2	6.6	8.6	3.2	2.2
16:1	2.5	2.0	ND	4.4	7.8	9.6	5.3	5.3
16:0	12.3	14.9	4.5	11.6	30.9	37.4	22.8	19.9
16:ynoic	ND	ND	90.2	52.9	ND	ND	28.1	32.3
17:0	16.3	15.2	ND	ND	ND	ND	ND	ND
18:1	24.6	26.8	2.6	16.6	30.2	21.4	17.8	26.2
18:0	35.9	30.4	1.8	12.3	11.3	6.7	7.5	5.0
19:0	8.2	7.9	ND	ND	ND	ND	ND	ND
20:1	ND	ND	ND	ND	8.3	13.5	10.4	8.2

[a] The values are from two separate experiments (I and II) in which *S. mutans* was grown in Todd-Hewitt broth containing 2.4 μM 2-hexadecynoic acid at 37.5°C for 18 h.

[b] Number of carbon atoms:number of double bonds. Ynoic refers to 2-hexadecynoic acid. Identification was based on the mobility of the methyl esters relative to standards during gas-liquid chromatography on a polar SP-2340 column and a nonpolar column OV-101 and confirmed by mass spectrometry.

[c] ND, Not detected.

ence noted in fatty acid composition was a decrease in the arachidonic acid content from 16.5 ± 3.7 to 10.6 ± 3.1% in the untreated as compared with the treated cells. In contrast to the results noted with *S. mutans*, 2-hexadecynoic acid was not detected as a constituent fatty acid in the alkynoic acid-treated cells.

DISCUSSION

The results presented in Fig. 1 demonstrate that the growth of *S. mutans* decreases in proportion to the concentration of the 2-hexadecynoic acid in the culture medium. That this inhibition of growth was not unique to *S. mutans* was indicated by the results presented in Table 1. 2-Hexadecynoic acid was shown to inhibit the growth of a number of gram-positive microorganisms as well as some gram-negative species. The lack of growth inhibition noted with *E. coli* was similar to that reported before, where the 2 positional isomer of either the free acid or the *N*-acetylcystamine derivative did not inhibit the growth of this bacterium (13). In contrast to the results reported elsewhere for the gram-negative bacteria (13), the free acid was an effective inhibitor of the gram-positive bacteria. A further difference from the results obtained previously was the chain length specificity of the acetylenic acid. In this instance, 2-hexadecynoic acid was the most active species, whereas with the 3-alkynoic acids, the most active antibacterial species varied in chain length from 9 to 11 carbon atoms (13).

The compositions of the free fatty acids and acyl moieties from the phospholipids of *S. mutans* were relatively similar. However, the presence of species having an odd chain length in the free fatty acid fraction and eicosenoic acid in the phospholipid fraction indicates a specificity with respect to the fatty acids that are utilized for complex lipid biosynthesis in this bacterium. The exogenous 2-hexadecynoic acid was readily absorbed and utilized in the synthesis of phospholipids by *S. mutans*. This was indicated by the qualitative composition of the free fatty acids and acyl moieties of the phospholipids isolated from cells grown in the presence of 2-hexadecynoic acid. In these cells, the acetylenic

TABLE 3. Effect of 2-hexadecynoic acid on growth of HeLa cells

Acid and concn (μM)	*n*[a]	Absorbance
0	12	0.154 ± 0.021
Alkynoic acid		
10	2	0.144
30	2	0.122
61	2	0.125
122	4	0.088 ± 0.024
244	4	0.033 ± 0.009
328	2	0.010
488	4	0.008 ± 0.002
Palmitic acid		
67	2	0.148
133	2	0.152
266	4	0.140 ± 0.008
Alkynoic acid + palmitic acid		
67	2	0.169
133	2	0.176
266	4	0.137 ± 0.030

[a] *n*, Number of independent observations.

fatty acid accounted for a major proportion of the fatty acids present in each lipid fraction. That 2-hexadecynoic acid was a component of the phospholipids also indicates that this moiety was activated to the acyl coenzyme A thioester and acts as a substrate for the acyl coenzyme A phospholipid acyl transferase of these cells. The acetylenic acid does not alter the utilization of endogenous fatty acids in the biosynthesis of phospholipids by the treated cells. This was indicated by the observation that correction of the composition of the acyl moieties in the phospholipid fraction for the proportion of hexadecynoic acid present yields a distribution of fatty acids similar to that noted in the untreated cells. However, the observation that the odd-chain-length fatty acids were not present in the free fatty acids of the treated cells suggests that the 2 positional isomer may cause a change in fatty acid biosynthesis, an event noted before (13, 20).

That the inclusion of 2-hexadecynoic acid in the culture medium inhibited the growth of HeLa cells suggests that the acetylenic acid would be toxic to mammals. However, the observation that the simultaneous addition of palmitic acid to the culture medium prevents this growth inhibition suggests that this response would not occur in animals consuming a normal diet. Essentially, this response has been noted before in rats consuming a nearly fat-free diet supplemented with 2-hexadecynoic acid (21).

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LITERATURE CITED

1. **Berde, C. B., H. C. Andersen, and B. S. Hudson.** 1980. A theory of the effects of head-group structure and chain unsaturation on chain melting transition of phospholipid dispersion. J. Am. Chem. Soc. **19**:4276–5293.
2. **Burton, K.** 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
3. **Cronan, J. E., and R. P. Vagelos.** 1972. Metabolism and function of membrane phospholipids in *Escherichia coli*. Biochim. Biophys. Acta **265**:25–60.
4. **Endo, K., G. M. Helmkamp, and K. Block.** 1970. Mode of inhibition of β-hydroxydecanolythioester dehydrase by 3-decynoyl-N-acetylcystamine. J. Biol. Chem. **245**:4293–4296.
5. **Folch, J., M. Lees, and G. H. Sloane-Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **266**:497–509.
6. **Genco, R. J., R. T. Evans, and S. Elkson.** 1969. Review of dental research in microbiology with emphasis on periodontal disease. J. Am. Dent. Assoc. **78**:1016–1037.
7. **Gershon, H., and L. Shanks.** 1978. Antifungal properties of 2-alkynoic acids and their methyl esters. Can. J. Microbiol. **24**:591–597.
8. **Gilbertson, J. R., R. C. Johnson, R. A. Gelman, and C. Buffenmyer.** 1972. Further studies on the natural occurrence of free fatty aldehydes in bovine cardiac muscle. J. Lipid Res. **13**:491–499.
9. **Goren, M. B.** 1972. Mycobacterial lipids: selected topics. Bacteriol. Rev. **36**:33–64.
10. **Greenway, D. L. A., and K. G. H. Dyke.** 1979. Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. J. Gen. Microbiol. **115**:233–245.
11. **Helmkamp, G. M., R. R. Rando, D. J. H. Brock, and K. Block.** 1968. β-Hydrodecanoyl thioester and dehydrase. J. Biol. Chem. **245**:3229–3231.
12. **Kabara, J. J., D. M. Swieczkowski, A. J. Conley, and J. P. Truant.** 1972. Fatty acids and derivatives as antimicrobial agents. Antimicrob. Agents Chemother. **2**:23–28.
13. **Kass, L. R.** 1968. The antibacterial activity of decynoyl-N-acetylcystamine. J. Biol. Chem. **243**:3223–3228.
14. **Lands, W. E. M., J. B. Ohlroggs, J. R. Robinson, R. W. Sacks, J. A. Barve, and F. D. Gunstone.** 1977. Quantitative effects of unsaturated fatty acids in microbial mutants. Biochim. Biophys. Acta **486**:451–461.
15. **Naccarato, W. F., J. R. Gilbertson, and R. A. Gelman.** 1974. *In vivo* and *in vitro* biosynthesis of free fatty alcohols in *Escherichia coli* K-12. Lipids **9**:419–424.
16. **O'Leary, W. M.** 1974. Microbial lipids, p. 185–221. *In* J. B. Kwapinski (ed.), Molecular biology. John Wiley & Sons, New York.
17. **Orland, F. J., J. R. Blayney, R. W. Harrison, J. A. Reyniers, P. C. Trexler, M. Wagner, H. A. Gordon, and T. D. Luskey.** 1954. The use of germ free animal techniques in the study of experimental dental caries. J. Dent. Res. **33**:147–174.
18. **Rauser, G., G. Koutchevsky, G. Simon, and G. J. Nelson.** 1967. Quantitative analysis of brain and spinach leaf lipids employing silicic acid column chromatography and acetone elution of glycolipids. Lipids **2**:37–40.
19. **Socransky, S. S., and A. C. R. Crawford.** 1977. Current therapy in dentistry, p. 1–19. *In* H. M. Goldman, H. W. Gilmore, W. B. Irby, and R. E. McDonald (ed.), Recent advances in the microbiology of periodontal disease, vol 6. C. V. Mosby Co., St. Louis.
20. **Upreti, G. C., M. Matocha, and R. Wood.** 1981. Effect of 2-hexadecynoic acid on cultured 7288 Hepatoma Cells. Lipids **16**:315–322.
21. **Wood, R., T. Lee, and H. Gershon.** 1979. Effect of methyl 2-hexadecynoate on hepatic fatty acid metabolism. Lipids **15**:141–150.