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Inhibitory Action of Fatty Acids on the Growth of *Neisseria gonorrhoeae*

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Fatty acids of various chain lengths (C_1 to C_{24}) were examined for their effects on growth, oxygen consumption, and in vitro reduced nicotinamide adenine dinucleotide oxidase activity of *Neisseria gonorrhoeae* CS-7. The growth inhibition caused by saturated fatty acids increased with increasing chain length to a maximum with palmitic acid (C_{16}). Stearic acid (C_{18}) and longer saturated fatty acids showed little inhibition of growth. However, unsaturated fatty acids of chain length C_{16} to C_{20} were inhibitory. Similar inhibition was observed with *Bacillus subtilis* and a deep rough mutant of *Salmonella typhimurium*. Wild-type *S. typhimurium* and *Pseudomonas aeruginosa* were more resistant to medium-chain (C_7 to C_{10}) fatty acids and completely resistant to long-chain (C_{12} to C_{18}) fatty acids. Thus, sensitivity of *N. gonorrhoeae* to long-chain fatty acids appears to be related to the permeability of the outer membrane. Growth inhibition by short-chain (C_1 to C_6) fatty acids was pH dependent; inhibition of growth increased with decreasing pH. Saturated fatty acids inhibited oxygen consumption by log-phase cells of *N. gonorrhoeae*. This inhibition increased with increasing chain length to a maximum observed with myristic acid (C_{14}). Whereas stearic acid (C_{18}) had little effect upon oxygen consumption, unsaturated C_{18} fatty acids were inhibitory. An in vitro inhibition of reduced nicotinamide adenine dinucleotide oxidase activity by saturated (C_1 to C_{12}) and unsaturated (C_{16} to C_{20}) fatty acids was also observed. Although the inhibitory concentrations were generally higher than those required to inhibit growth or oxygen consumption, an inhibition of electron transport may be partially responsible for the observed growth inhibition.

The sensitivity of *Neisseria gonorrhoeae* to free fatty acids in agar has been known since the work of Ley and Mueller in 1946 (27). Despite the widespread acceptance of this observation, a detailed study of the fatty acid sensitivity of *N. gonorrhoeae* has not been carried out, with the possible exception of the work of Walstad et al. (48). These workers studied the production of growth inhibitory substances by *N. gonorrhoeae* that inhibited other strains of gonococci or meningococci. These inhibitors were identified as long-chain free fatty acids and phospholipids, which were released into the medium during growth. The inhibition of the growth of *N. gonorrhoeae* on agar by selected fatty acids was also studied. A recent report of phospholipase A activity in the outer membrane of *N. gonorrhoeae* (40) supports the hypothesis that, during growth, free fatty acids are released by a stepwise degradation of phosphatidylethanolamine in the outer membrane.

The ubiquitous nature of fatty acids in agar (27), spent growth media (3, 48), human vaginal secretions (29), and animal tissue and plasma (21, 25) indicates that a more in-depth study of the effects of fatty acids on *N. gonorrhoeae* is warranted. The present report describes the effect of saturated and unsaturated fatty acids of various chain lengths on the growth, oxygen consumption, and reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase activity of *N. gonorrhoeae* CS-7. The fatty acid sensitivity of several gram-positive and gram-negative bacteria is also reported.

MATERIALS AND METHODS

Organisms. *N. gonorrhoeae* CS-7 (type 4), *Salmonella typhimurium* DB-21, *S. typhimurium* TA1535, and *Pseudomonas aeruginosa* PS-7 have been previously described (30, 32, 34). These organisms were maintained as previously described (30, 32). *Bacillus subtilis* was obtained from the culture collection of the Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, and was maintained on Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.) slants stored at 4°C.

Inocula and cultural conditions. Stock cultures of *N. gonorrhoeae* CS-7 were prepared by growing the

organism in 2 liters of basal medium (30) at 37°C in a gyratory shaker. Cultures were harvested during the late exponential phase of growth (150 Klett units [KU]), centrifuged at 4,000 × *g* for 10 min at 4°C, and suspended in Trypticase soy broth (BBL) containing 20% glycerol. One-milliliter portions were transferred to sterile screw-top vials, frozen in a dry ice-acetone bath, and stored at −70°C. Starter cultures were prepared by inoculating 2 ml of the thawed stock culture into 25 ml of basal medium and incubating the mixture for 1 h at 37°C in a gyratory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Nepheloflasks (300-ml flasks containing 50 ml of basal medium) were inoculated with a sufficient number of bacteria (ca. 1.5 ml) to achieve an initial turbidity of 25 KU (7×10^7 colony-forming units per ml).

For all other bacteria, a 1% (vol/vol) inoculum of a suspension of cells from an overnight culture was used to inoculate the growth medium. Incubation was at 37°C in a New Brunswick gyratory shaker.

Chemicals and fatty acids. All fatty acids were obtained from Sigma Chemical Co., St. Louis, Mo., and were the highest grade available. Formic, acetic, and propionic acids were purchased as the sodium salts. All others were in the free acid form. Short-chain fatty acids (C_1 to C_6) were dissolved in a sufficient volume of water to prepare either 5 or 2.5 M solutions, adjusted to pH 6.5 to 7.0, and sterilized by filtration. At the pH values used for growth (6.5 to 7.2), a large percentage of these short-chain fatty acids were present in the ionized form. However, because growth inhibition in other organisms has been shown to be due to the undissociated acid (19, 26) and for the sake of consistency, we have referred to all fatty acids as the undissociated acids. Fatty acids containing 7 or more carbons were dissolved in 95% ethanol (for growth studies) or 100% dimethyl sulfoxide (for oxygen uptake studies). The final concentration of solvent in the growth medium was less than 0.5% for ethanol and 3.3% for dimethyl sulfoxide; these concentrations did not inhibit growth or oxygen uptake.

$NADH_2$ was also purchased from Sigma Chemical Co. All other chemicals were of analytical grade and were obtained from standard commercial sources.

Miscellaneous measurements. Turbidity was measured by Klett-Summerson colorimetry at 540 nm. Protein was assayed by the method of Lowry et al. (28), with bovine serum albumin as the standard.

Enzyme assays. $NADH_2$ oxidase was measured by following the oxidation of $NADH_2$ at 340 nm. The reaction mixture contained 10 mM sodium phosphate buffer (pH 7.2 or 6.5), 0.15 mM $NADH_2$, 3 mM $MgCl_2$, and water and membrane preparation to a final volume of 0.5 ml. Decrease in the absorbance at 340 nm was followed in a Beckman model 25 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 25°C.

Oxygen uptake. Cultures of *N. gonorrhoeae* CS-7 were grown to 50 KU, and 3-ml samples were transferred to an oxygen electrode chamber (model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio) with constant stirring. After a 2-min incubation period to insure consistent oxygenation and temperature equilibrium, the rate of oxygen uptake was followed polarographically at 37°C. Fatty acids were added to the cells prior to the 2-min incubation period.

Preparation of cell membranes. Late-exponential-phase cultures were harvested by centrifugation (4,000 × *g* for 10 min) at 4°C, washed twice, and suspended in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid. Cells were disrupted by sonic oscillation (Bronwill Biosonik IV, Bronwill Scientific Inc., Rochester, N.Y.) in an ice bath for 5 min in 30-s pulses, each followed by a 2-min cooling period. The resulting suspension was centrifuged (12,000 × *g* for 10 min) to remove unbroken cells and debris. The supernatant was then centrifuged at 100,000 × *g* for 90 min (type 30 rotor, Beckman model L5-65 ultracentrifuge). The membrane pellet was washed once and suspended in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid.

RESULTS

Effect of fatty acids on the growth of *N. gonorrhoeae* CS-7. Addition of saturated fatty acids (17 carbons or less) to the medium at time of inoculation markedly inhibited the growth of *N. gonorrhoeae* CS-7. In general, inhibition was concentration dependent (Fig. 1), and the concentration required to inhibit growth decreased as the chain length of the fatty acid increased. The relationship between the concentration of fatty acid needed to cause a 50% inhibition of growth (IC_{50}) and the chain length of the fatty acid is shown in Fig. 2. Except for a reversal of this trend with propionic (C_3), butyric (C_4), and valeric (pentanoic; C_5) acids, the IC_{50} decreased with increasing chain length until a minimum IC_{50} of 5.8 μM was obtained with palmitic (hexadecanoic; C_{16}) acid. Heptadecanoic acid (C_{17}) was significantly less inhibitory (IC_{50} = 75 μM), whereas stearic (octadecanoic; C_{18}), nonadecanoic (C_{19}), arachidic (eicosanoic; C_{20}), behenic (docosanoic; C_{22}), and lignoceric (tetracosanoic; C_{24}) acids had no inhibitory effect upon growth.

The effect of unsaturated fatty acids on the growth of *N. gonorrhoeae* CS-7 is shown in Table 1. Unlike the C_{18} or greater saturated fatty acids, unsaturated fatty acids (C_{16} to C_{20}) were all inhibitory. The IC_{50} values for these unsaturated fatty acids were all in the range of 5 to 11 μM and were similar to the minimum IC_{50} observed with saturated fatty acids. With unsaturated fatty acids of 16 to 20 carbons, the chain length, the number and position of double bonds, and the steric isomer of the fatty acid appeared to introduce little variability in the degree of inhibition. Nervonic acid (*cis*-15-

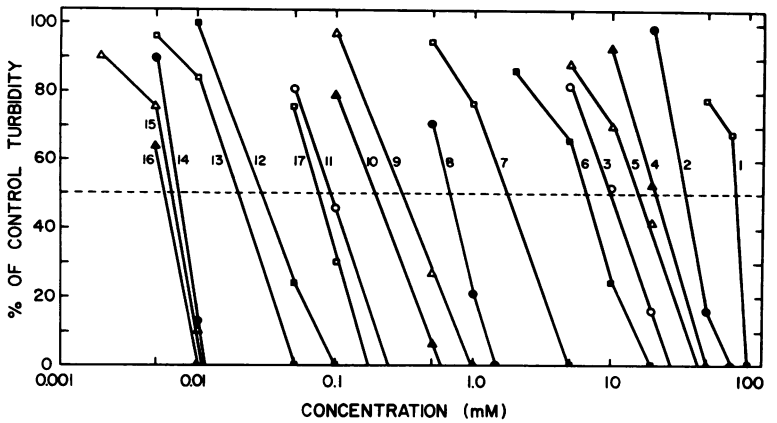

FIG. 1. *Effect of fatty acids (C_1 to C_{17}) on the growth of N. gonorrhoeae CS-7. Numbers adjacent to each line indicate the fatty acid chain length.*

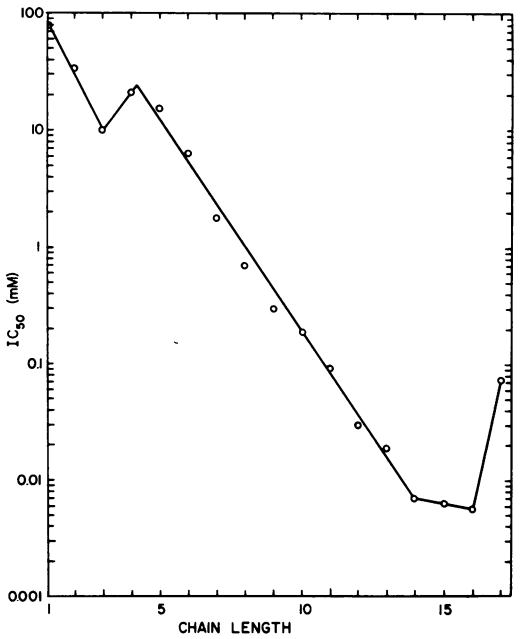

FIG. 2. *Relationship between the fatty acid concentration that inhibits growth by 50% (IC_{50}) and the fatty acid chain length.*

tetracosanoic acid; C_{24}), however, had no inhibitory effect on the growth of *N. gonorrhoeae*. This fatty acid was considerably less soluble than the other unsaturated fatty acids tested, which may have influenced its inhibitory effect.

The effect of several fatty acid derivatives and other lipids on the growth of *N. gonorrhoeae* CS-7 was examined (Table 2). Methyl esters of palmitic and stearic acids and the 12-hydroxy derivative of oleic (ricinoleic) acid had IC_{50} values similar to those of palmitic, stearic, and oleic acids, respectively. The IC_{50} for stearyl alcohol and the branch-chain fatty acids, isobutyric and isovaleric acids, was also similar to their respective parent compounds. Isostearic acid (16-methyl-heptadecanoic acid), however, was extremely inhibitory, with an IC_{50} value similar to that of the unsaturated fatty acids. The dicarboxylic acids, adipic (hexanedioic; C_6) and pimelic (hepanedioic; C_7), were 4 to 13 times less inhibitory than caproic (hexanoic; C_6) and heptanoic (C_7) acids, respectively.

Comparison of growth inhibition by fatty acids between *N. gonorrhoeae* and other bacterial species. Sheu and Freese (42) reported that the outer membrane protects gram-negative bacteria against inhibition by long-chain fatty acids, whereas gram-positive bacteria are more sensitive. The inhibition of growth of selected gram-positive and gram-negative bacteria by saturated fatty acids (C_2 to C_{16}) is shown

TABLE 1. *Inhibition of growth and $NADH_2$ oxidase activity of N. gonorrhoeae by unsaturated fatty acids*

Fatty acid	Chain length	IC_{50} (mM)	
		Growth	$NADH_2$ oxidase
Palmitoleic	16:1 (*cis*-9)	0.007	ND[a]
Palmitelaidic	16:1 (*trans*-9)	0.008	0.10
Petroselinic	18:1 (*cis*-6)	0.006	ND
Oleic	18:1 (*cis*-9)	0.005	0.20
Elaidic	18:1 (*trans*-9)	0.011	ND
cis-Vaccenic	18:1 (*cis*-11)	0.009	ND
trans-Vaccenic	18:1 (*trans*-11)	0.007	ND
Linoleic	18:2 (*cis*-9, *cis*-12)	0.005	0.20
Arachidonic	20:4 (5,8,11,14)	0.007	0.10
Nervonic	24:1 (*cis*-15)	NI[b]	ND

[a] Not determined.
[b] Non-inhibitory within limits of solubility (IC_{50} greater than 0.5 mM).

TABLE 2. *Effect of fatty acid derivatives and other lipids on the growth of N. gonorrhoeae CS-7*

Compound	Chain length	IC_{50} (mM)
Methyl ester of fatty acids		
Palmitic	16	0.009
Stearic	18	NI[a]
Hydroxy fatty acid		
Ricinoleic	18:1	0.007
Branch-chain fatty acid		
Isobutyric	4	12
Isovaleric	5	7.2
Isostearic	18	0.007
Dicarboxylic acid		
Adipic	6	86
Pimelic	7	7.4
Alcohol		
Stearyl	18	NI

[a] Non-inhibitory within limits of solubility (IC_{50} greater than 0.5 mM).

in Fig. 3. The gram-positive *B. subtilis* (Fig. 3B) was sensitive to both short (C_2 to C_6)- and long (C_{12} to C_{16})-chain fatty acids. The IC_{50} concentrations for each fatty acid were similar to those observed for *N. gonorrhoeae* CS-7 and decreased with increasing chain length to a minimum of 5 μM for myristic (tetradecanoic; C_{14}) acid. Palmitic acid (C_{16}) was significantly less inhibitory for *B. subtilis* (IC_{50} = 120 μM) than for *N. gonorrhoeae* (IC_{50} = 5.8 μM); stearic acid (C_{18}) was not inhibitory.

P. aeruginosa (Fig. 3A) and *S. typhimurium* DB-21 (Fig. 3C) were as sensitive to the short-chain (C_2 to C_4) fatty acids as was *N. gonorrhoeae*. However, both organisms were increasingly less sensitive than *N. gonorrhoeae* to fatty acids of increasing chain length. The IC_{50} for caproic (hexanoic; C_6) acid was ca. 5-fold higher for *P. aeruginosa* than for *N. gonorrhoeae*; the IC_{50} for capric (decanoic; C_{10}) acid was 76-fold higher. Neither *P. aeruginosa* nor *S. typhimurium* DB-21 was sensitive to fatty acids longer than 10 carbons. The outer mem-

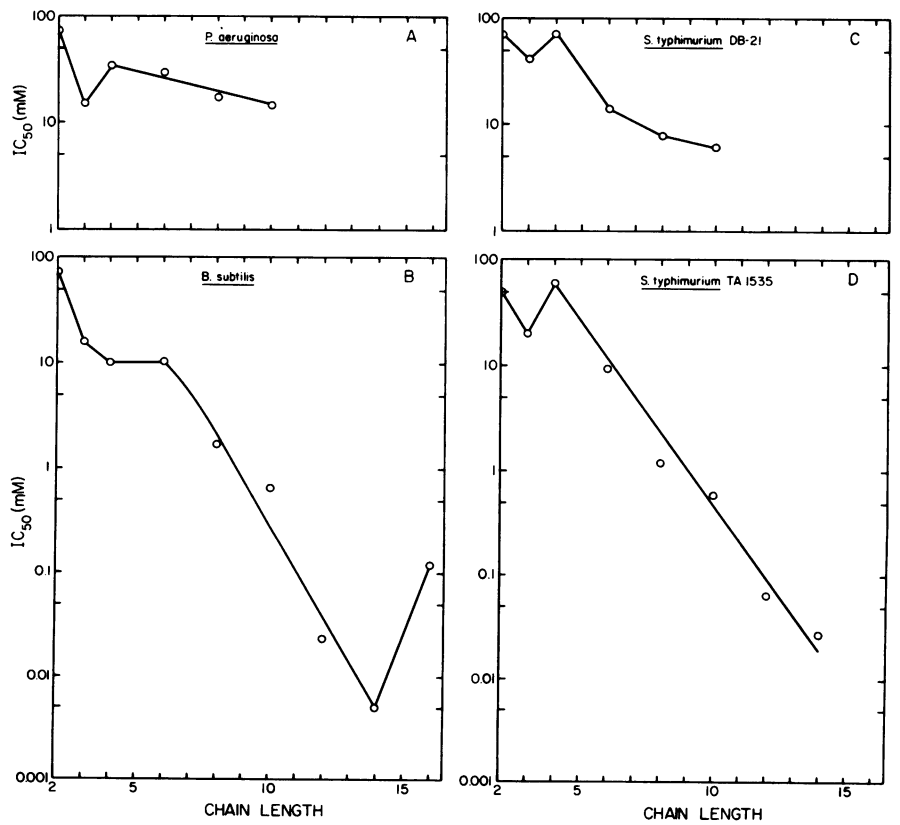

FIG. 3. *Comparison of the growth inhibition by fatty acids (C_2 to C_{16}) for (A) P. aeruginosa, (B) B. subtilis, (C) S. typhimurium DB-21, and (D) S. typhimurium TA1535.*

brane mutant *S. typhimurium* TA1535 (Fig. 3D), which lacks a complete lipopolysaccharide layer, was sensitive to both short- and long-chain fatty acids. The IC_{50} decreased with increasing chain length, similar to that for *N. gonorrhoeae*. However, *S. typhimurium* TA1535 was somewhat less sensitive to the longer-chain fatty acids. Maximum inhibition was observed with myristic acid (C_{14}; IC_{50} = 27 μM). Palmitic (C_{16}) and stearic (C_{18}) acids were not inhibitory.

Effect of pH on growth inhibition of *N. gonorrhoeae* CS-7 by short-chain (C_1 to C_6) fatty acids. Short-chain fatty acids are weak acids whose growth inhibitory properties for other organisms increase when the pH is lowered from neutrality (19, 26). Thus, growth inhibition appears to be due to the undissociated acid. It was of interest to determine whether pH influenced the growth inhibition of *N. gonorrhoeae* by short-chain fatty acids.

Initial studies were carried out in basal medium buffered with 0.05 M tris(hydroxymethyl)aminomethane-maleate at the desired pH. The results (Table 3) indicate that the pH of the medium markedly affected the inhibition of gonococcal growth by short-chain fatty acids. Decreasing the pH increased the inhibitory properties of the fatty acid. Acetic (C_2) and butyric (C_4) acids showed the most dramatic effect. Concentrations of acetic (34 mM) and butyric (21 mM) acids, which gave only slight inhibiton at pH 7.5, gave almost complete inhibition at pH 6.5. Propionic and caproic acids gave similar results, although they were more inhibitory at the higher pH. At pH values less than 6.5 (5.5 and 6.0), the growth rates of *N. gonorrhoeae* CS-7 were too slow to provide useful data.

IC_{50} concentrations at pH 6.5 were determined for C_1 to C_6 fatty acids in basal medium adjusted to pH 6.5. These values and the IC_{50} values obtained with basal medium at pH 7.2 are compared in Table 4. In all cases, the fatty acids were 1.5 to 2.7 times more inhibitory at pH 6.5 than at 7.2.

Effect of fatty acid addition during exponential growth of *N. gonorrhoeae* CS-7. The effect of fatty acids on actively growing cells was examined. The addition of representative short (acetic; C_2)-, medium (caprylic; C_8)-, and long (myristic; C_{14})-chain fatty acids to mid-logarithmic-phase cultures (50 KU; ca. 2×10^8 colony-forming units per ml) produced an immediate, concentration-dependent growth inhibitory effect (Fig. 4). The concentration of fatty acid necessary to completely inhibit growth (IC_{100}) of actively growing cells was significantly higher than the IC_{100} for these fatty acids when added at zero time. The IC_{100} values for acetic, caprylic, and myristic acids at 50 KU (175, 7, and 0.04 mM, respectively) were three- to fivefold greater than the IC_{100} values when added at zero time (Fig. 1). This difference may reflect the increased number of cells per ml in the logarithmic-phase cultures.

TABLE 3. *Effect of pH on the growth inhibition of N. gonorrhoeae CS-7 by short-chain fatty acids*

Fatty acid	Concn[a] (mM)	Turbidity (% of control)		
		pH 6.5	pH 7.0	pH 7.5
Acetic	34	5	29	76
Propionic	10	19	24	45
Butyric	21	0	10	86
Caproic	6.5	0	20	58

[a] The concentration for each fatty acid was the IC_{50} at pH 7.2.

TABLE 4. *Comparison of growth inhibition of N. gonorrhoeae CS-7 by short-chain fatty acids*

Fatty acid	IC_{50} (mM)	
	pH 6.5	pH 7.2
Formic	30	80
Acetic	14	34
Propionic	6.6	10
Butyric	11	21
Valeric	9.5	16
Caproic	2.4	6.5

The inhibitory effect of acetic acid on *N. gonorrhoeae* CS-7 was reversible. Actively growing cells (50 KU) inhibited by 175 mM acetic acid (IC_{100} = 175 mM) for up to 3 h resumed normal growth when centrifuged (3,000 $\times$ *g*, for 5 min) and suspended in fresh medium without acetic acid (Fig. 5). Caprylic and myristic acids, however, appeared to inhibit irreversibly. Actively growing cells (50 KU) exposed to IC_{100} concentrations of caprylic and myristic acids for only 15 min were unable to resume normal growth when suspended in fresh medium (data not shown).

Inhibition of oxygen consumption. A reduction in the rate of oxygen consumption due to the presence of fatty acids has been observed for *B. subtilis* (41). It was of interest to know whether the inhibition of gonococcal growth by fatty acids could be correlated with an inhibition in the rate of oxygen consumption. The addition of fatty acids to mid-logarithmic-phase cultures (50 KU) actively oxidizing glucose produced a concentration-dependent inhibition of oxygen consumption. The IC_{50} concentrations for fatty acids C_1 to C_{16} are depicted in Fig. 6. In

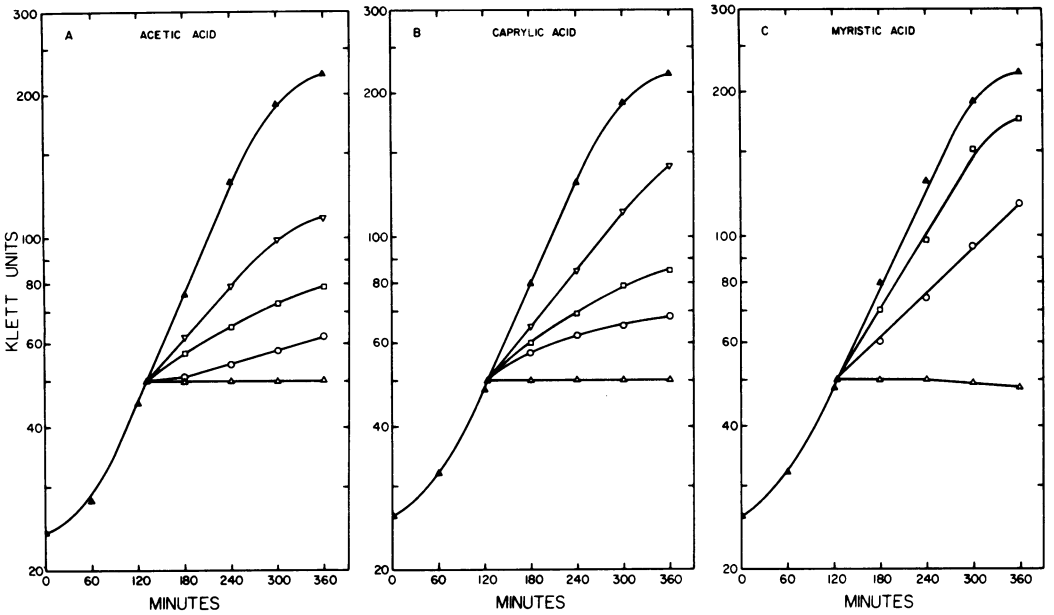

FIG. 4. *Effect of fatty acid addition during the exponential growth of N. gonorrhoeae CS-7. Fatty acids were added at 50 KU. (A) Acetic acid: ▽, 50 mM; □, 75 mM; ○, 125 mM; △, 175 mM. (B) Caprylic acid: ▽, 4 mM; □, 6 mM; ○, 7 mM; △, 8 mM. (C) Myristic acid: □, 0.030 mM; ○, 0.035 mM; △, 0.040 mM.*

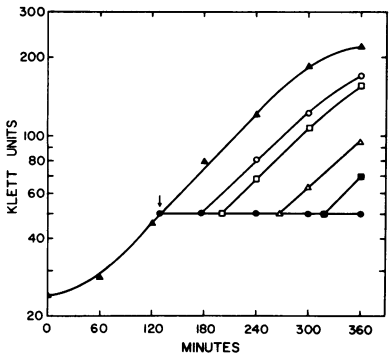

FIG. 5. *Reversal of acetic acid inhibition of N. gonorrhoeae CS-7. Arrow indicates time of addition of 175 mM acetic acid. At various times, cells were harvested by centrifugation and suspended in fresh medium. Symbols: ▲, control (no acetic acid); ○, 45 min postaddition; □, 85 min postaddition; △, 135 min postaddition; ■, 185 min postaddition; ●, not suspended.*

general, the fatty acid concentration necessary to inhibit oxygen consumption decreased with increasing chain length to a minimum of 0.042 mM for myristic acid (C_{14}). Palmitic acid (C_{16}) was inexplicably higher at 0.35 mM. Stearic (C_{18}) and arachidic (C_{20}) acids were not inhibitory. However, the C_{18} unsaturated fatty acids, elaidic (*trans*-9-octadecenoic) and oleic (*cis*-9-octadecenoic) acids, were inhibitory (data not shown). Except for a few variations, this relationship was similar to the inhibition of growth indicated in Fig. 2. The concentrations necessary to inhibit oxygen consumption of actively growing cells were similar to those necessary to inhibit growth. At subinhibitory or inhibitory levels, none of the fatty acids tested stimulated oxygen consumption by cell suspensions of *N. gonorrhoeae* CS-7.

Inhibition of $NADH_2$ oxidase. The fatty acid inhibition of in vitro $NADH_2$ oxidase activity in isolated membranes of *N. gonorrhoeae* CS-7 was examined. Saturated fatty acids with chain lengths of C_1 to C_{12} exhibited a concentration-dependent inhibition of enzyme activity. The IC_{50} concentrations at pH 6.5 and 7.2 for these fatty acids are depicted in Fig. 7. Except for propionic (C_3), butyric (C_4), and valeric (C_5) acids, the IC_{50} values decreased with increasing chain length. C_1 to C_6 fatty acids were more inhibitory at pH 6.5, although the pattern of inhibition was similar to the inhibition observed at pH 7.2. The observed concentrations for inhibition of $NADH_2$ oxidase with C_1 to C_{10} fatty acids were in the same range as those observed for inhibition of growth (Fig. 2) and oxygen consumption (Fig. 6). However, with lauric acid (dodecanoic acid; C_{12}) the minimum IC_{50} value (1.2 mM) was ca. 10-fold higher than the IC_{50} for inhibition of oxygen consumption. The inhibition caused by fatty acids of longer

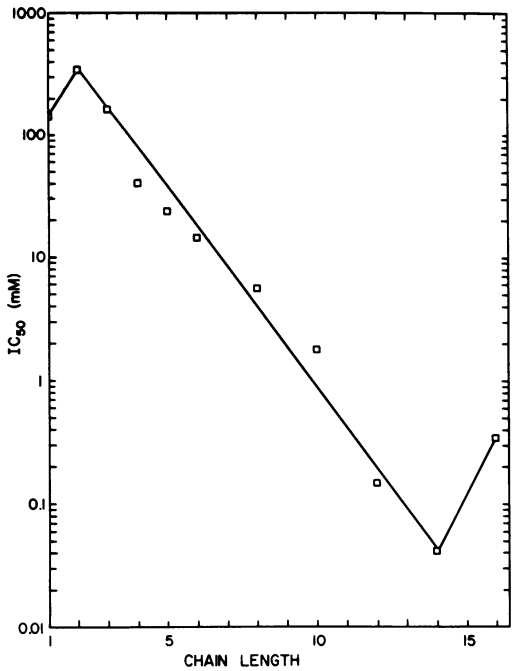

FIG. 6. *Relationship between the fatty acid concentration that inhibits oxygen consumption by 50% (IC_{50}) and the fatty acid chain length.*

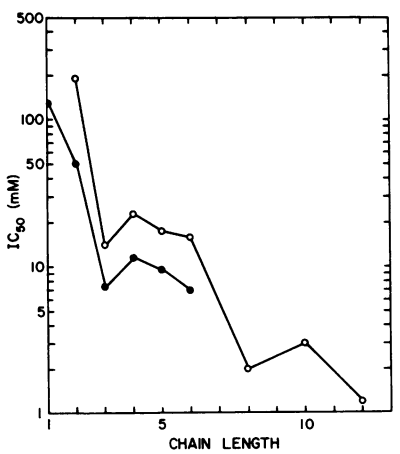

FIG. 7. *Relationship between the fatty acid concentration that inhibits $NADH_2$ oxidase activity by 50% (IC_{50}) and the fatty acid chain length. Symbols: ○, pH 7.2; ●, pH 6.5.*

chain length (myristic, palmitic, and stearic acids) was inconsistent due to their insolubility at concentrations necessary for inhibition. The unsaturated fatty acids, palmitelaidic ($C_{16:1}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and arachidonic ($C_{20:4}$), were inhibitory, with IC_{50} values in the range of 0.1 to 0.2 mM (Table 1). Although these unsaturated fatty acids were more inhibitory than saturated fatty acids of the same chain length, the IC_{50} values were still considerably higher (at least 10-fold) than the concentrations needed to inhibit growth (Table 1).

DISCUSSION

Gram-positive bacteria can often be differentiated from gram-negative bacteria on the basis of their sensitivity to fatty acids (23, 35, 42). Although sensitivity to short-chain (C_1 to C_6) fatty acids is commonly observed with both gram-positive and gram-negative bacteria (4, 19, 26, 43), sensitivity to long-chain (C_{12} or greater) fatty acids is usually found only with gram-positive organisms (23, 35, 42). However, there have been several reports that showed long-chain fatty acid sensitivity for certain strains of *Escherichia coli* (10, 22, 45), *Haemophilus pertussis* (36), and *Pasteurella pestis* (9). The present investigation shows that *N. gonorrhoeae* is an atypical, gram-negative organism with respect to long-chain fatty acid sensitivity.

Sheu and Freese (42) postulated that the outer membrane of gram-negative bacteria serves as a permeability barrier to protect the cell from inhibition by long-chain fatty acids. Rough, outer membrane mutants of *E. coli* and *S. typhimurium* lacking a complete lipopolysaccharide were increasingly more sensitive to long-chain fatty acids than were the parent strains. We confirmed these results in our study with the deep rough mutant *S. typhimurium* TA1535 and the wild-type strain DB-21. However, *N. gonorrhoeae* CS-7 was more sensitive to long-chain fatty acids than the outer membrane mutant of *S. typhimurium*, which exhibited a pattern of inhibition similar to the gram-positive *B. subtilis*. *N. gonorrhoeae* CS-7 was 20-fold more sensitive than *B. subtilis* to palmitic acid (C_{16}). It appears that the outer membrane of the gonococcus is extremely permeable to fatty acids. Atypical sensitivities to other compounds such as antibiotics (39), detergents (39), dyes (15, 30), and steroid hormones (30, 33) have also been reported for *N. gonorrhoeae* and are attributed to an increased permeability of the outer membrane.

N. gonorrhoeae CS-7 was inhibited by both saturated and unsaturated fatty acids. This inhibition was observed by measuring growth (turbidity), oxygen consumption, or in vitro $NADH_2$ oxidase activity in isolated membranes. The inverse relationship between inhibitory concentrations and chain length is a phenomenon that has been observed in other systems (11, 38, 42). At the present time, we have no explanation for the reversal of this

trend with propionic, butyric, and valeric acids, although the same phenomenon was seen with inhibition of $NADH_2$ oxidase (but not with oxygen consumption).

Straight-chain, saturated fatty acids with 18 carbons or more did not inhibit *N. gonorrhoeae*. These fatty acids may be too large to pass through even the gonococcal outer membrane. However, unsaturated fatty acids of the same chain length (C_{18} to C_{20}) were extremely inhibitory. Isostearic acid, which differs from stearic acid only in a methyl branch on the end of the molecule opposite the carboxyl group, also possesses potent inhibitory properties. The reason for the increased inhibition of isostearic acid and the unsaturated fatty acids is unclear. The introduction of a double bond into the chain, in addition to causing a conformational change in the molecule, causes a decrease in its melting point and an increase in its solubility in the growth medium (14). There appears, however, to be no direct relationship between the melting point and the inhibitory properties of these compounds. Undecanoic acid (C_{17}) has a lower melting point than palmitic acid (C_{16}), yet is significantly less inhibitory. The methyl esters of stearic acid and nervonic acid, a C_{24} unsaturated fatty acid, have no inhibitory properties. On the basis of melting points alone, one would predict inhibition from both of these compounds.

The lack of inhibition may be due to the insolubility of these longer saturated fatty acids. Although long-chain fatty acids form soluble micelles above a certain critical concentration (unique for each fatty acid), the binding to, or incorporation into, the cell membranes appears to be a property of the monomeric form of the molecule (18, 46). The concentration of monomers of stearic or longer saturated fatty acids may be too low to elicit the inhibitory effect. Introduction of a double bond or methyl branch results in an increased monomeric solubility (14) and an increased inhibitory effect. This does not necessarily exclude the possibility that the non-inhibitory, long-chain saturated fatty acids may find no compatible areas of the membrane to which they can bind. Introduction of a double bond or methyl group would then introduce a conformational change in the molecule such that binding and inhibition could occur. The degree of binding of radiolabeled fatty acids to isolated membranes of *N. gonorrhoeae* could prove or disprove this possibility; however, this work has not been done to date.

The inhibitory nature of the methyl ester of palmitic acid suggests that the presence of a free carboxyl group is unnecessary for the inhibitory activity of long-chain fatty acids. This is contrary to the findings of Walstad et al. (48) and Kodicek and Worden (23). The introduction of a hydroxyl group to the hydrocarbon chain of oleic acid (ricinoleic acid) had no effect on the inhibitory properties. Branch-chain fatty acids (isobutyric and isovaleric acids) were as inhibitory as corresponding straight-chain compounds. This observation has also been noted by Samson et al. (38) for short-chain fatty acid inhibition of yeast metabolism. Dicarboxylic acids (adipic and pimelic) were significantly less inhibitory than the corresponding chain length single carboxylic acids. The presence of a polar group at both ends of the hydrocarbon chain may interfere with the binding of the molecule to hydrophobic regions of the membrane.

The effect of pH on inhibition by short-chain fatty acids has been reported by several authors (5, 19, 20, 26, 38) who found that inhibitory activity increases as the pH is lowered. This observation has led to the conclusion that the undissociated molecules are responsible for toxicity (12, 19, 26). We have confirmed this observation with short-chain fatty acid inhibition of *N. gonorrhoeae*. Growth, oxygen consumption (unpublished data), and $NADH_2$ oxidase activity were inhibited to a greater extent at pH 6.5 than at 7.2.

The mechanism of inhibition of *N. gonorrhoeae* by fatty acids is still not completely understood. The literature contains a large and diverse collection of reported effects of fatty acids, including hemolytic activity (25); inhibition of phosphate uptake in yeast (2, 38), adenosine triphosphate-inorganic phosphate exchange (6, 50), and bacterial sporulation (17); selective inhibition of enzymes involved in lipogenesis in *Arthrobacter cystallopietes* (11); and an uncoupling of oxidative phosphorylation in isolated animal mitochondria (1, 21, 37, 50) and bacteria (12, 41, 43). Oxygen uptake, $NADH_2$ oxidase activity, and the immediate inhibitory effect on the growth of exponentially growing cells (Fig. 4) indicate that uncoupling oxidative phosphorylation, blocking electron transport, or both are major mechanisms by which fatty acids inhibit *N. gonorrhoeae*.

The role of long-chain fatty acids (particularly unsaturated fatty acids) in the uncoupling of oxidative phosphorylation in mitochondria has been well documented (1, 21, 37, 50). Oleic and other unsaturated fatty acids stimulate latent adenosine triphosphatase activity of fresh rat liver mitochondria and cause a complete loss of adenosine 5'-diphosphate respiratory control (1, 37). It has been postulated that the

ionizable group of low pk_a value and the hydrophobicity of the hydrocarbon chain are important for inhibitory activity (49). These properties would be consistent with the theory that uncouplers dissolve in the membrane and act as circulating carriers, conducting protons across the membrane barrier (31). Weinbach and Garbus (49) have suggested that compounds which uncouple oxidative phosphorylation bind to membrane proteins, leading to conformational changes in the protein structure and loss of biological activity. The binding of fatty acids to proteins, such as serum albumin, is well known (7, 13, 47). Hardesty and Mitchell (16) reported that binding of fatty acids to mammalian cytochrome *c* causes conformational changes in the protein structure as well as altered biological activity.

The mechanism of fatty acid inhibition in bacteria has been studied by Sheu et al. (43) and Freese et al. (12). The inhibition of *E. coli* (12) and *B. subtilis* (12, 43) by fatty acids was caused by the uncoupling of substrate transport and oxidative phosphorylation from the electron transport system. Whereas saturated fatty acids (C_2 to C_{10}) had only uncoupling activity, unsaturated fatty acids also had a partial inhibition of electron transport. In the present study, both saturated and unsaturated fatty acids inhibited oxygen consumption in whole cells of *N. gonorrhoeae* CS-7. This effect could be due to either an inhibition of electron transport or a depletion of electron donors. Since we have observed an inhibition of $NADH_2$ oxidase activity, we can assume that inhibition of electron transport is at least partially responsible for the growth inhibition. The mechanism of inhibition of these fatty acids may be similar to that of steroid hormones, which inhibit oxygen consumption (unpublished data) and $NADH_2$ oxidase activity (33) in *N. gonorrhoeae*. The concentrations of fatty acids necessary to inhibit $NADH_2$ oxidase were, however, generally higher than those required to inhibit oxygen uptake or growth. Long-chain fatty acids that uncouple oxidative phosphorylation inhibit mitochondrial electron transport when added at higher concentrations (8, 44). A similar uncoupling at low fatty acid concentrations may also occur in *N. gonorrhoeae*. Studies to verify this possibility are in progress. An inhibition of other enzyme systems cannot be eliminated at this time. The lack of agreement between the inhibitory concentration of palmitic acid (C_{16}) on growth (Fig. 2) and oxygen consumption (Fig. 6) suggests that the observed inhibition on growth may result from more than one site.

The results of the present study support the earlier results of Walstad et al. (48) for fatty acid inhibition of *N. gonorrhoeae*, but we observed inhibition at considerably lower concentrations. However, this may be expected since we used only liquid media and they used a double-overlay method on agar plates.

Palmitic acid (C_{16}) and unsaturated $C_{16:1}$ and $C_{18:1}$ fatty acids were the major free fatty acids detected in cultures of *N. gonorrhoeae* (48). The present study confirms that these fatty acids are extremely inhibitory to the growth of *N. gonorrhoeae* CS-7. The production of self-inhibitory fatty acids by the gonococcus during growth (48) may explain its relatively short survival when grown in artificial media. This must be taken into account when performing extended growth studies. Sensitivity to free fatty acids may also partially explain the interference of *N. gonorrhoeae* growth by other bacterial species (24). In addition, the presence of fatty acids in vaginal secretions (29) and in animal tissue and plasma (21, 25) suggests the possibility that these compounds may influence in vivo growth of this organism.

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