The Antibacterial Activity of 3-Decynoyl-N-acetylcysteamine

INHIBITION IN VIVO OF β-HYDROXYDECANOYL THIOESTER DEHYDRASE*

(Received for publication, February 5, 1968)

LEON R. KASS

From the James Bryant Conant Laboratory, Harvard University, Cambridge, Massachusetts 02138, and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland 20014.

SUMMARY

β-Hydroxydecanoyl-thioester dehydrase is known to be the enzyme responsible for the introduction of the double bond into the unsaturated fatty acids of Escherichia coli, and is known to be vital for bacterial growth. The powerful inhibitor in vitro of the dehydrase, 3-decynoyl-N-acetylcysteamine, possesses antibacterial activity in vivo; at 10⁻⁶ M, it completely inhibits the growth of E. coli. The following evidence suggests that this growth inhibition is due to the inactivation of β-hydroxydecanoyl-thioester dehydrase. (a) Only those acetylenic compounds which block the activity of the isolated dehydrase are effective in vivo. (b) The growth inhibition is reversible by oleic but not by palmitic acid. (c) The inhibitor causes a rapid and total cessation of the formation of unsaturated acids, well before the first noticeable decline in the growth rate. (d) The inhibitor is not active against yeast or mammalian cells, cells which form their olefinic acids by a mechanism not involving the dehydrase.

A reasonable approach to the development of useful antibacterial agents might well exploit the fact that certain biosynthetic pathways in bacteria are fundamentally different from those found in animals. Knowledge of the enzymology of the unique bacterial pathways might lead to the development of specific enzyme inhibitors, which may in turn prove to be effective and specific bacteriostatic agents. The experiments described in this communication are an attempt to apply this approach to the biosynthesis of unsaturated fatty acids.

Two distinct pathways for the biosynthesis of monounsaturated fatty acids are now well established (1). In animal tissues (2, 3) (as was first shown in yeast (4), and later in protozoans (5), Euglena (6), higher plants (7), and in some bacteria (8)), long chain unsaturated fatty acids arise by direct desaturation of preformed long chain saturated acids in a reaction dependent on molecular oxygen. In certain bacteria, on the other hand, the double bond is introduced during the process of chain growth by dehydration of a cis,β,γ-decanolate intermediate (1). The cis,β,γ-decanolate thus formed is directly elongated (with retention of the double bond) to palmitoleate and cis-vaccenate, the predominant unsaturated acids found in these organisms (1, 9, 10). α,β-Dehydration of β-hydroxydecanolate leads toward saturated fatty acids by the usual chain elongation mechanism.

The enzymology of this bacterial pathway has recently been elucidated (11-13). The dehydrating enzyme, β-hydroxydecanoyl-thioester dehydrase, has been purified 1200-fold from extracts of Escherichia coli. It is responsible for the formation of cis,β,γ-decanolate, and is therefore indispensable for the biosynthesis of unsaturated fatty acids. The acetylenic analogue of β,γ-decanolate, 3-decynoyl-NAC,* was found to be a powerful and irreversible inhibitor of the isolated dehydrase (13, 14). Furthermore, when added to the complete fatty acid synthetase system (of which the dehydrase is a component), the inhibitor specifically abolished the synthesis of unsaturated fatty acids (15). Verification of the importance of the dehydrase has recently been provided by the isolation of a mutant of E. coli which lacks β-hydroxydecanoyl-thioester dehydrase. This organism cannot synthesize olefinic acids and therefore requires unsaturated fatty acids for growth (16).

Given that (a) unsaturated fatty acids are essential for the growth of E. coli (16) and (b) β-hydroxydecanoyl-thioester dehydrase is indispensable for the synthesis of olefinic acids, one might expect that the dehydrase inhibitor, 3-decynoyl-NAC, should inhibit the growth of E. coli. The present report confirms this prediction and provides evidence that this growth inhibition results in fact from the inactivation in vivo of β-hydroxydecanoyl-thioester dehydrase.

EXPERIMENTAL PROCEDURE

Materials—Escherichia coli, wild type (strain K12), was obtained from Dr. R. Martin, Salmonella typhimurium, wild type

* This work was supported in part by Grant-in-Aid HE 02477-11 from the National Institutes of Health, awarded to Professor Kourad Bloch.
and with vigorous agitation in a New Brunswick rotary shaker. plates coated with Silica Gel G impregnated with 10% AgNO₃ absorpt. maxima at 230 to 233 nip, characteristic of thioesters; tissue culture line, derived from a rat hepatoma (17). from Nuclear-Chicago. Tritium-labeled (2,5JH)-L-histidine donated by G. Helmkamp. 4-Undecynoyl-NAC was the gift all synthesized by a published procedure (13), were generously 3-nonynoic, 3-undecynoic, 3-dodecynoic, and 2-decynoic acids, purified N-acetylcysteamine thioesters of 3-decynoic, 3-octynoic, Oleic and palmitic acids, both 99+% pure, were supplied by Mann. Triton X-100 was the product of Packard. Thin layer plates coated with Silica Gel G impregnated with 10% AgNO₃ were purchased from Analtech, Inc. (Wilmington, Delaware). Growth of Bacteria—Liquid cultures were grown at 37° in Medium E (10) containing glucose (0.5%) or glycerol (0.5%) and with vigorous agitation in a New Brunswick rotary shaker. All inocula were taken from cultures growing exponentially in the same medium. Growth was followed turbidimetrically in a Beckman DU spectrophotometer at 650 rnp. An optical density of 0.100 (l.0-cm light path) corresponds to a bacterial density of 1.3 × 10⁶ cells per ml. Bacteria were also grown on solid media (2% glucose and 1.5% agar in Medium E) at 37°. The inoculum was applied as a lawn in 2 ml of liquefied 0.6% agar.

When the acetylenic compounds were used in liquid culture, they were added to the dry incubation tubes as aliquots of ethanolic solutions. The ethanol was evaporated and the residue was dissolved, with warming, in the culture medium. In experiments on solid media, the ethanolic solution was first applied to a sterile filter paper disc, the ethanol was evaporated, and the disc was then applied to the inoculated agar plate.

When fatty acids were added as supplements to an inhibited culture, they were transferred from a concentrated (1 or 5%) stock solution of the potassium salt in water Triton X-100 was used in all cases to solubilize the fatty acid salt. At the concentrations used (250 to 500 µg of Triton per ml), the detergent had no effect on the growth rate of the bacteria.

Growth of Other Organisms—Yeast was grown aerobically at 30° in liquid culture on glucose (0.5%) plus yeast nitrogen base (Difco; 0.07%). The hepatoma tissue culture cells were grown in suspension culture at 37° as described by Thompson, Tomkins, and Curran (17).

Fatty Acid Synthesis—Lipids were extracted by a modification of the method of Folch, Lees, and Sloane-Stanley (20) from cells labeled with sodium acetate-1⁴C. Samples of bacterial culture were extracted with 3 ml of chloroform-methanol (2:1) at 0°. Following centrifugation at 25,000 × g for 15 min, the chloroform layer was completely removed (2.1 ± 0.1 ml). The radioactivity of an aliquot (0.7 ml) was determined as a measure of acetate incorporation into total lipids. The remainder of the extract was transmethylated with BF₃-methanol reagent (Applied Science Laboratories, Inc., State College, Pennsylvania; 0.2 ml, 65°, 30 min), after the addition of carrier palmitic and oleic acids (25 µg each). The methyl esters were separated into saturated and unsaturated fractions by thin layer chromatography on AgNO₃-impregnated silica gel, with n-hexane-diethyl ether (9:1) as the solvent. The fatty acid ester spots were visualized under ultraviolet light after spraying the plate with dichlorofluorescein (0.2% in ethanol). The RF values for saturated and unsaturated esters were 0.55 and 0.45, respectively. The spots were scraped from the plate and counted directly in toluene scintillation solution using a Nuclear-Chicago liquid scintillation spectrometer. Percentages of unsaturated and saturated acids were calculated (taking 100% to be the sum of the counts recovered in these two spots), and the net incorporation (millimicromoles) into each was computed. A small amount of radioactivity (always less than 3% of the total) was recovered from a region having an RF of approximately 0.05 to 0.15; this radioactivity, probably representing incorporation into hydroxy acids, was ignored in the above calculations.

Protein Synthesis—Incorporation of ³H-histidine into material precipitable by hot trichloroacetic acid was taken as a measure of protein synthesis in vivo. Bacterial samples (0.1 ml) were added to 1.0 ml of 5% trichloroacetic acid. The trichloroacetic acid suspensions were heated at 90° for 10 min, poured onto Millipore filters (pore size, 0.45 µ; diameter, 25 mm), and washed with 10 ml of cold 5% trichloroacetic acid. The filters were dried and counted in toluene scintillation solution.

**RESULTS**

Effect of 3-Decynoyl-NAC on Growth of E. coli—The ability of 3-decynoyl-NAC to inhibit bacterial growth was first shown in a qualitative manner on solid media. The inhibitor, applied on a sterile disc of filter paper to a lawn of E. coli, produced a zone

![Image](http://www.jbc.org/)

**FIG. 1.** Effect of various acetylenic thioesters on the growth of E. coli. The filter paper discs contained 0.1 µmole of the following compounds: A, 3-octynoyl NAC; B, 3-nonynoyl NAC; C, 3-decynoyl NAC; D, 3-undecynoyl NAC; E, 3-dodecynoyl NAC; F, 4-undecynoyl NAC. The experiment was conducted as described under "Experimental Procedure." The photograph was taken against a black background.
of growth inhibition, seen as a clear area around the disc. A typical result is shown in Fig. 1 (Disc C).

Quantitative studies on the inhibition were then done in liquid media. Exponentially growing cells were treated with varying concentrations of inhibitor. Some of the results are shown in Fig. 2; significant inhibition was observed with 3-decynoyl-NAC at $2.5 \times 10^{-6}$ M, and complete inhibition at $1 \times 10^{-5}$ M. Intermediate concentrations impaired growth to an intermediate extent; concentrations greater than $10^{-5}$ M (up to $10^{-4}$ M) gave the same result as $10^{-5}$ M. At all concentrations tested, a lag period was observed prior to the onset of inhibition; the growth rate remained normal for nearly 2 hours after the addition of the analogue.

**Inhibitor Specificity**—Several other acetylenic derivatives were examined for antibacterial activity. As seen in Fig. 1, 3-undecynoyl-NAC and 3-dodecynoyl-NAC were effective growth inhibitors, whereas 3-octynoyl-NAC and 4-undecynoyl-NAC were inactive. Inhibition was barely detectable with 3-nonynoyl-NAC (Disc B) in this experiment, but other experiments confirm 3-nonynoyl-NAC as a weak growth inhibitor. The $\alpha,\beta$ isomer, 2-decynoyl-NAC, was without effect, as were 3-decanoyl acid and its methyl ester.

![Fig. 2. Inhibition of growth of E. coli by 3-decynoyl-NAC. At the start of the experiment, 0.1 ml of inoculum was added to 4-ml portions of glycerol medium, containing the indicated final concentrations of 3-decynoyl-NAC. Growth was followed as described under "Experimental Procedure."](image)

![Fig. 3. Reversal of inhibition by oleic acid. At the start of the experiment, 0.25 ml of inoculum was added to two 10-ml portions of glucose medium, one of which contained 3-decynoyl-NAC at $1 \times 10^{-4}$ M (INHIBITED). When growth had ceased in the inhibited culture, 2.0-ml samples of this culture were added (arrow) to three fresh tubes containing Triton X-100 (1 mg), two of which contained in addition either potassium palmitate or potassium oleate (0.4 amole). The final concentrations of the additives were Triton, 500 $\mu$g per ml, and fatty acid, $2 \times 10^{-4}$ M.](image)

The activity of these compounds as growth inhibitors is qualitatively similar to their activity on the isolated $\beta$ hydroxydecanoyl thioester dehydrase (14): NAC derivatives of the $\beta,\gamma$-monoaetylenic acids of chain length $C_9$ through $C_{12}$ were powerful inhibitors, 3-dodecynoyl-NAC was moderately active, 3-octynoyl-NAC was a very weak inhibitor, and the remaining four compounds were inactive. Some quantitative differences between the relative potencies in vivo and in vitro of the active analogues were noted: $C_9$ was much more effective in vitro, and $C_{12}$ more potent in vivo. These differences may be due to differential permeability in vivo, favoring the longer chain length compounds.

**Reversal of Inhibition by Oleic Acid**—The growth inhibition caused by 3-decynoyl-NAC is reversible by the addition of long chain unsaturated acids, but not of saturated acids (Fig. 3). Fatty acids were added (arrow) to a culture fully inhibited by 3-decynoyl-NAC ($10^{-5}$ M). Potassium oleate ($2 \times 10^{-4}$ M) reversed the inhibition, whereas potassium palmitate ($2 \times 10^{-4}$ M) was ineffective. The detergent, Triton X-100, added to solubilize the fatty acid salts, was itself not a factor in the reversal as soon in the lowest curve.

The effect of supplement concentration on reversal of inhibi-
Fig. 4. Effect of various concentrations of oleic acid on reversal of inhibition. The design and details of this experiment are identical with those described in Fig. 3, except that 16 ml starting cultures were used. At the arrow, 2.0-ml samples were removed from the inhibited culture and added to fresh tubes containing Triton X-100 (0.5 mg) and various amounts of potassium oleate to give the indicated final concentrations.

Fig. 5. Effect of 3-decynoyl-NAC on protein synthesis in vivo. At the start of the experiment, 2.1-ml samples of a culture grown to an optical density of 0.400 on glycerol medium were inoculated into two 12-ml portions of the same medium containing in addition sodium acetate-l-[^14]C (1 x 10^-5 M; 44.4 mC per mmole) and 2,5-[^3]H-n-histidine (1 x 10^-5 M; 119 mC per mmole). One of these also contained 3-decynoyl-NAC at 1 x 10^-5 M (Inhibited). Growth was followed as described under "Experimental Procedure" (data not shown, but see text and Fig. 2). At the indicated times, 0.1-ml aliquots were removed and analyzed for the incorporation of labeled histidine into protein ("Experimental Procedure"). Since some of the [^14]C-acetate present was incorporated into the trichloroacetic acid-precipitable material, double label counting was used, and the usual corrections were made to obtain the data for tritium incorporation.

Fig. 6. Effect of 3-decynoyl-NAC on fatty acid synthesis in vivo. The data shown here are the results from the experiment described in the legend to Fig. 5. At the indicated times, 0.3-ml samples were analyzed as described under "Experimental Procedure."
inhibitor during this period (Fig. 5). In contrast, a decrease in the rate of over-all fatty acid biosynthesis (incorporation of "C-acetate into lipids) was observable 45 to 60 min after inhibitor was added (Fig. 6A).

The decisive evidence for the mechanism of action of 3-decylnoyl- NAC was obtained on separation of the fatty acids of these lipids into saturated and unsaturated fractions. As seen in Fig. 6B, the inhibitor promptly and completely abolished the formation of unsaturated acids. Saturated acids were synthesized at an increased rate in the presence of inhibitor (Fig. 6C). These findings are identical with those obtained in vitro when the acetylene was added to the fatty acid synthetase system from E. coli (15).

Effect of Inhibitor on Other Organisms—S. typhimurium and P. fluorescens were as sensitive to the dehydrase inhibitor as was E. coli. In contrast, 3-decylnoyl-NAC, at a concentration which fully inhibits E. coli (10^{-5} M), had only a slight and transient effect on the growth rate of Saccharomyces cerevisiae (the doubling time, initially increased by approximately 60%, returned to normal 4 hours after the addition of inhibitor). The growth rate of the mammalian hepatoma tissue culture cells was unaffected by the rate of inhibitor.

**DISCUSSION**

β-Hydroxydecanoyl-thioester dehydrase is indispensable for the synthesis of unsaturated fatty acids in E. coli (15), and mutations resulting in the loss of this enzyme are lethal (16). Therefore, compounds which specifically inhibit the dehydrase in vitro should be antibacterial agents in vivo, if they can enter the bacterial cell. The studies reported in this paper show that 3-decylnoyl-NAC, a potent inhibitor of the dehydrase in vitro, has the predicted antibacterial activity. Inhibition of bacterial growth by 3-decylnoyl-NAC appears to be due to its effect on β-hydroxydecanoyl-thioester dehydrase because: (a) only those acetylenic compounds which show inactivation of the dehydrase in vitro are effective in vivo; (b) the growth inhibition is reversible by unsaturated fatty acids (oleic) and not by saturated acids (palmitic); (c) the inhibitor causes a rapid and complete cessation of the formation of unsaturated fatty acids well before the first noticeable decrease in the growth rate; and (d) the acetylenic analogue is not active against yeast or mammalian cells, which form their unsaturated acids by the oxygen-dependent desaturation pathway.

The presence of a lag period preceding the appearance of growth inhibition deserves some comment, especially in view of the almost immediate effect of the inhibitor on the synthesis in vivo of olefinic acids. This lag is best explained by assuming that the inhibited bacteria contain a sufficient "reserve" of unsaturated acids to sustain some additional growth. However this reserve is limited, since the cells can undergo only one to two doublings after addition of the inhibitor. One may infer that the amount of unsaturated acids produced by uninhibited bacteria is not greatly in excess of the minimal physiological requirement and, therefore, that the activity of this biosynthetic pathway is under careful regulation.

As indicated above, the synthesis of saturated fatty acids was increased several-fold in the presence of inhibitor, despite the prompt abolition of unsaturated acid formation. An identical result has been reported in vitro for the addition of 3-decylnoyl- NAC to the fatty acid synthetase system from E. coli; the formation of unsaturated long chain acids was completely abolished at 10^{-6} M inhibitor, and this block was accompanied by a compensatory 5-fold increase in the formation of saturated acids (15). These observations are explained by the fact that β-hydroxydecanoyl-thioester dehydrase, both in vivo and in vitro, would be expected to cause the observed diversion of β-hydroxydecanoyl-toward saturated long chain products.

**Acknowledgments—**These experiments were conceived and initiated while I was a member of Professor Bloch's laboratory, and it is a pleasure to acknowledge his encouragement and helpful advice. I wish to thank George Helmkamp and Dr. Bob Rando for their generous gifts of acetylenic compounds, Dr. Giovanni Ames for helpful discussions, Drs. Fred Valeriote and Max Gottesman for a critical reading of the manuscript, and Dr. Gordon Tomkins for making available the facilities of his laboratory. The idea for these experiments was generated in one of many stimulating discussions with Dr. David Brock.

**REFERENCES**

1. **B**loch, K., **B**aronowsky, P., **G**oldfine, H., **L**ennartz, W., **L**ight, R., **N**orris, A., and **S**cheuerbrandt, G., Fed. Proc., 20, 921 (1961).
14. **H**elmkamp, G. M., **J**e, R. R., **R**andu, R. R., **B**rook, D. J. H., and **B**loch, K., 243, 3299 (1968).
The Antibacterial Activity of 3-Decynoyl-N-acetylcysteamine: INHIBITION IN VIVO OF β-HYDROXYDECANOYL THIOESTER DEHYDRASE

Leon R. Kass


Access the most updated version of this article at http://www.jbc.org/content/243/12/3223

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/243/12/3223.full.html#ref-list-1