Microbial Conversion of Linoleic and Linolenic Acids to Unsaturated Hydroxy Fatty Acids[1]

S. Koritala* and **M.O. Bagby**

Oil Chemical Research, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

The conversion of oleic acid to 10-hydroxystearic acid with resting cells of *Nocardia cholesterolicum* (NRRL 5767) has been previously reported. These same microorganisms also convert linoleic and linolenic acids to 10-hydroxy-12*c*-octadecenoic and 10-hydroxy-12*c*,15*c*-octadecadienoic acids, respectively. The reaction occurs best at 35°C and a pH of 6.5. Under optimum conditions, 75–80% of the unsaturated fatty acid substrate is converted to the corresponding hydroxy acid. The hydroxy products were characterized by gas chromatography, gas chromatography-mass spectrometry, nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy. Other microorganisms that successfully converted these substrates include another strain of *Nocardia cholesterolicum* (NRRL 5768) and *Nocardia* sp. (NRRL 5636).

KEY WORDS: Linoleic acid, linolenic acid, microbial conversion, unsaturated hydroxy fatty acids.

The harnessing of microorganisms to produce useful chemical intermediates from fats and oils is increasingly being investigated. Unlike traditional chemical processes, which require extreme temperatures and pressures, microbial conversions take place under mild conditions and, in some instances, the products are formed stereoselectively. At the National Center for Agricultural Utilization Research (Peoria, IL), we have instituted a program to explore the utilization of microorganisms for conversion of soybean oil into useful products (1). We have previously shown (2) that *Nocardia cholesterolicum* converts oleic acid to 10-hydroxy stearic acid. We have extended the studies to linoleic and linolenic acids, and the results are reported here.

EXPERIMENTAL PROCEDURES

Materials. Linoleic and linolenic acids (purity >99% by gas chromatography (GC)) were purchased from NuChek Prep Inc., Elysian, MN. All solvents used were ACS grade and were obtained from commercial sources. Yeast extract, malt extract and dextrose were from Difco Laboratories, Detroit, MI.

Fermentation. Microorganisms used in this study were obtained from the Northern Regional Research Center's (NRRL) Culture Collection. Yeast-malt extract broth, prepared by dissolving 4 g dextrose, 4 g yeast extract and 10 g malt extract in 1 L of water, was sterilized, inoculated and shaken at 29°C on a rotary shaker at 150 rpm. At the start of the exponential growth phase, which commenced at 17 h, 0.25 mL oleic acid was added as an inducer, and growth was continued for another 24 h. The wet cells were isolated by centrifugation at 7000 rpm for 10 min.

Bioconversions were carried out in narrow-mouth bottles (25 mL) by resuspending the wet cells (1.7 g; dry wt 20%) in 10 mL of 0.05 M sodium phosphate buffer (pH varied). Linoleic or linolenic acid (0.175 g) was added, the bottles were capped with rubber septa and the air was replaced by nitrogen. Anaerobic conditions are necessary to inhibit further reaction of hydroxy acids to their corresponding keto acids (2). The reaction was carried out by shaking (200 rpm) in a constant-temperature bath held at 35°C.

Analysis. After the reaction, samples were extracted three times with equal volumes of diethyl ether. The combined ether extracts were washed with distilled water, dried over anhydrous magnesium sulfate, and filtered. The solvent was removed on a rotary evaporator. For GC analysis, 2–3 mg of fatty acids were converted to methyl esters with diazomethane. Esters were separated on an SPB-1 (Supelco Inc., Bellefonte, PA) column (15 m × 0.32 mm) installed in a Hewlett-Packard (Avondale, PA) 5890 GC, which was temperature programmed from 180°C (1 min) to 200°C at 2°/min. Peak areas were integrated on a Hewlett-Packard 3392A electronic integrator. Gas chromatography-mass spectrometry (GC-MS) of trimethyl silyl derivatives were performed on a Hewlett-Packard 5790 with a mass selective detector.

NMR spectra were obtained in deuterated chloroform solution with a Bruker WM-300 spectrometer (Rheins, Tepten, Germany) operating at 75.5 MHz. Infrared spectra were obtained in CS_2 solution on a Perkin-Elmer (Norwalk, CT) 1750 Fourier Transform spectrometer.

Purification of products. Methyl esters of hydroxy fatty acids were purified by silicic acid chromatography. Six grams of silica gel (60–200 mesh, J. T. Baker Inc., Phillipsburg, NJ) were packed into a 1/2-in. diameter column and eluted with hexane (100 mL) to remove most of the unreacted fatty acid esters. Elution with 100 mL hexane:ether (50:50) gave products of 93–95% purity. Rechromatography on a fresh silica column yielded 99% pure hydroxy esters.

RESULTS AND DISCUSSION

Hydroxylation. The progress of converting linoleic acid to 10-hydroxy-octadecenoic acid is shown in Figure 1. As the concentration of linoleic acid decreased, the amount of hydroxy product increased. In GC analysis, these two components were accompanied by 2–3% of several unidentified peaks. The amount of product increased with time up to 8 h and then tapered off. The conversion of oleic acid to 10-hydroxy stearic acid (2) was complete in about 8 h and required only 70 mg of cells (dry wt) to complete the reaction. With linoleic acid, however, five times the amount of cells was needed, and the conversion was only about 71% after 24 h.

[1]Presented at the 82nd Annual meeting of the American Oil Chemists' Society, Chicago, IL, May 12–15, 1991.

*To whom correspondence should be addressed at National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, IL 61604.

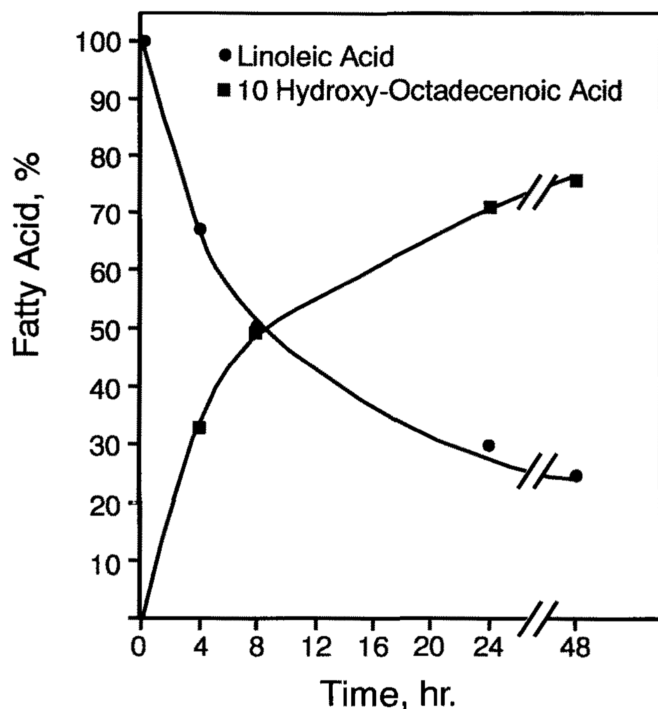


FIG. 1. Time course for the production of 10-hydroxy-octadecenoic acid by resting cells of *Nocardia cholesterolicum* (NRRL 5767). 1.7 g wet cells (0.34 g dry wt) in 10 mL of 0.05 M sodium phosphate buffer, pH 6.5 and 175 mg linoleic acid at 35°C.

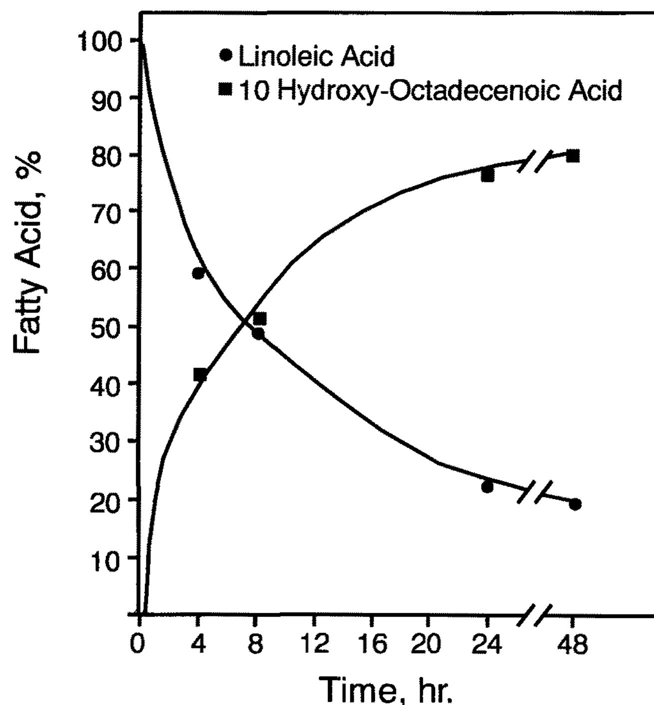


FIG. 3. Time course for the production of 10-hydroxy-octadecenoic acid by resting cells of *Nocardia* sp. NRRL 5636. Reaction conditions same as Figure 1.

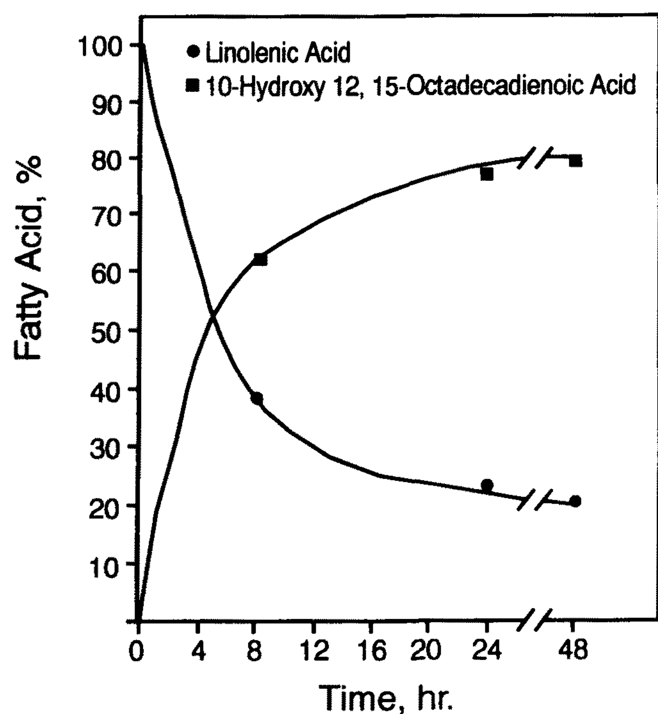


FIG. 2. Time course for the production of 10-hydroxy-octadecadienoic acid by resting cells of *Nocardia cholesterolicum* (NRRL 5767). 1.7 g wet cells in 10 mL of 0.05 M sodium phosphate buffer, pH 6.5 and 175 mg linolenic acid at 35°C.

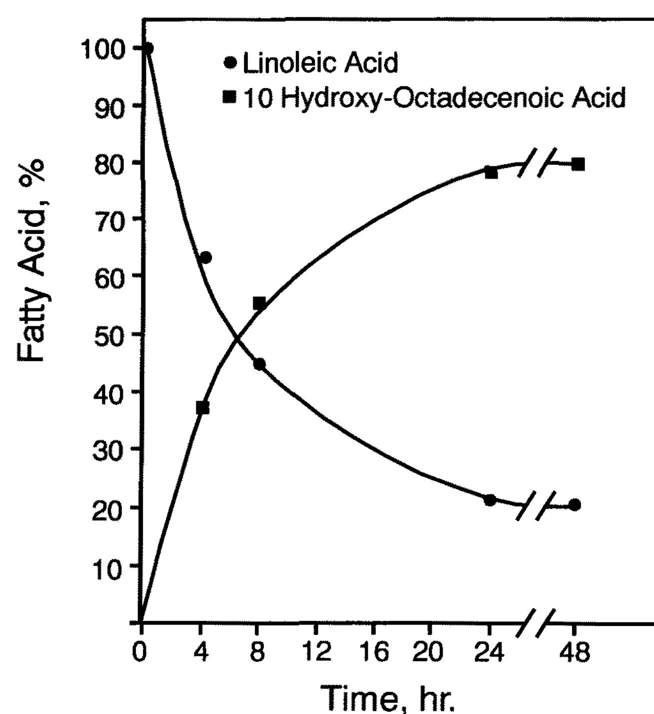


FIG. 4. Time course for the production of 10-hydroxy-octadecenoic acid by resting cells of *Nocardia cholesterolicum* (NRRL 5768). Conditions same as in Figure 1.

The conversion of linolenic acid to 10-hydroxy-octadecadienoic acid (Fig. 2) was also followed in a similar fashion. The reaction tapered off after 8 h and essentially stopped after 24 h. A slightly higher conversion rate (77%) was achieved. This incomplete conversion was probably due to product inhibition. However, the cells at 8-h reaction grew profusely when transferred to a yeast extract-malt extract agar plate, but no viable cells were found at 24-h reaction.

In addition to NRRL 5767, two other microorganisms (*Nocardia* sp. NRRL 5636 and *Nocardia cholesterolicum* NRRL 5768) gave similar conversions of linoleic acid, as shown in Figures 3 and 4. Both organisms gave slightly higher yields of hydroxy acid (77 and 78%) in 24 h than did NRRL 5767.

Figures 5 and 6 indicate that the optimum conditions for temperature and pH of the reaction are 35°C and 6.5, respectively. The pH optimum was the same as that for oleic acid conversion (2), whereas the temperature optimum with oleic acid was between 35 and 45°C.

Structure of hydroxy products. From previous studies (2) on the mechanism of hydroxylation, it is to be expected that both linoleic and linolenic acids will form hydroxy acids by hydration of the double bond. Because the enzyme (hydratase) is stereospecific, 10-hydroxy acids are expected, with the remaining double bonds unaffected. Thus, 10-hydroxy-12*c*-octadecenoic and 10-hydroxy-12*c*,15*c*-octadecadienoic acids should form from linoleic and linolenic acids, respectively.

During gas chromatography, hydroxy compounds eluted prior to 10-hydroxy-stearic acid, indicating unsaturation. After hydrogenation of the reaction products, the elution time of the hydroxy compounds coincided with that of 10-hydroxystearic acid.

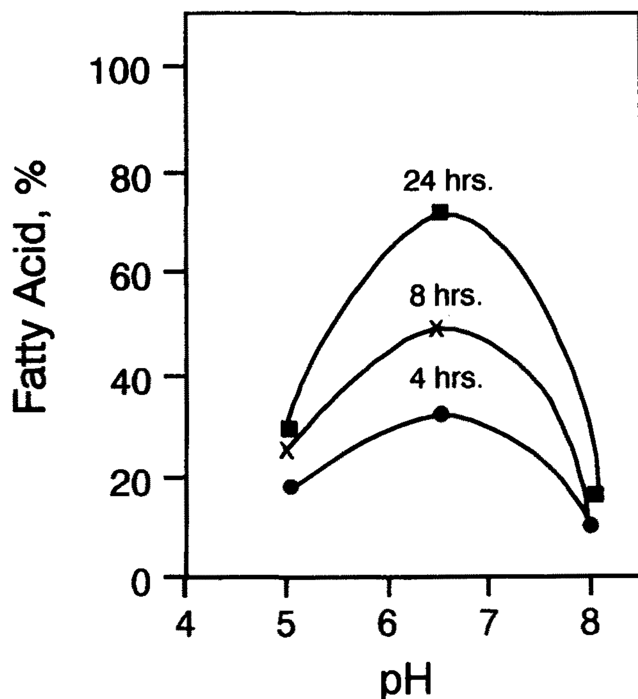


FIG. 5. Effect of pH on the formation of 10-hydroxy-octadecenoic acid by resting cells of *Nocardia cholesterolicum* (NRRL 5767). Conditions same as Figure 1, except pH was varied.

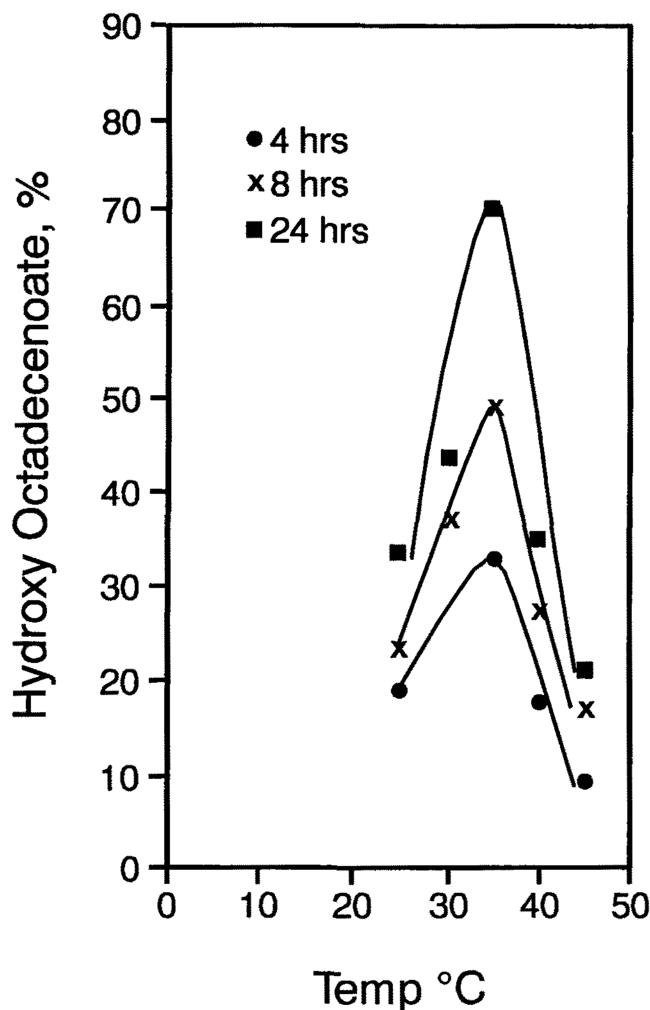
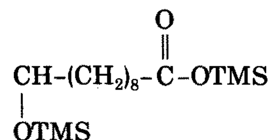


FIG. 6. Effect of temperature on the formation of 10-hydroxy-octadecenoic acid by resting cells of *Nocardia cholesterolicum* (NRRL 5767). Conditions same as in Figure 1, except temperature was varied.

Pure methyl esters of hydroxy acids were prepared by silicic acid chromatography. Methyl 10-hydroxy-12*c*-octadecenoate ($[\alpha]_D^{24} = -6.87$, methanol) and methyl 10-hydroxy-12*c*,15*c*-octadecadienoate ($[\alpha]_D^{24} = -4.39$, methanol) were better than 99.5% pure by gas chromatography. Infrared spectra of hydroxy esters derived from linoleic and linolenic acids showed the absence of *trans* unsaturation. GC-MS of hydroxy acids, as their trimethylsilyl (TMS) derivatives, from both linoleic and linolenic acids gave the fragment



with an *m/e* of 331, which corresponds to hydroxyl at the 10-position.

The purified hydroxy methyl esters from linoleic and linolenic acids were also subjected to ^{13}C NMR analyses. The chemical shifts assigned (Table 1) are consistent with

TABLE 1

Chemical Shifts for Selected Carbons of Unsaturated Fatty Acid Methyl Esters

Carbon number	10-Hydroxy-12*c*-octadecenoate	10-Hydroxy-12*c*,15*c*-octadecadienoate
4–8	28.9–29.4	28.9–29.4
9	36.6	36.7
10	71.2	71.2
11	35.2	35.2
12	125.1	125.4[a]
13	132.9	131.9[a]
14	27.2	24.7
15	29.4	126.7[a]
16	31.3	130.9[a]
17	22.4	20.4

[a]Chemical shifts not assigned among these carbons.

the proposed chemical structure. The allylic carbon shifts indicate *cis* configuration of the double bonds. The large chemical shift at carbon 11 is due to the influence of both the double bond at 12–13 and the hydroxyl at carbon 10. The difference in chemical shift at allylic carbon 14 between the two compounds is due to the influence of two double bonds in one case and only one double bond in another.

The unsaturated hydroxy acids of this study have potential for industrial uses. For example, the 10-hydroxy-12*c*-octadecenoic acid is an isomer of ricinoleic acid, an industrial chemical used in paints, lubricants and plastics. Whether these new acids can find use in industry will depend in part upon improving the yields from the present 75 to 80% so as to produce these acids more economically.

ACKNOWLEDGMENTS

GC-MS by R. D. Plattner and NMR by Dr. David Weisleder.

REFERENCES

1. Koritala, S., C.W. Hesseltine, E.H. Pryde and T.L. Mounts, *J. Am. Oil Chem. Soc. 64*:509 (1987).
2. Koritala, S., L. Hosie, C.T. Hou, C.W. Hesseltine and M.O. Bagby, *Appl. Microbiol. Biotechnol. 32*:299 (1989).

[Received September 9, 1991; accepted March 12, 1992]