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HYDROXY FATTY ACIDS OF *AZOTOBACTER AGILIS*

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SUMMARY

Approximately half of the fatty acids recovered after alkaline hydrolysis of the bound lipid of *Azotobacter agilis* were hydroxy acids. The hydroxy acids were isolated by gas–liquid chromatography and were identified as 3-hydroxydecanoic, 3-hydroxydodecanoic and 2-hydroxydodecanoic acids by the retention volume of the methyl ester in gas–liquid chromatography, by infrared spectum, and by identification of the products of oxidation by permanganate. The chain length of β-hydroxy fatty acids was confirmed by dehydration–hydrogenation, which gave the expected *n*-saturated acids.

INTRODUCTION

IKAWA *et al.*[1] isolated D-3-hydroxytetradecanoic acid which constituted approx. 25 % of the total fatty acids contained in a tumor-necrotizing substance isolated from *Escherichia coli*. Hydroxy fatty acids were also found in the hydrolytic products of a lipo-polysaccharide of Salmonella[2]. Bacteria have been reported to contain substantial amounts of bound lipid which could not be extracted with solvents[3]. This paper reports the occurrence and characterization of three hydroxy fatty acids which are major components of the bound lipid of *Azotobacter agilis*.

EXPERIMENTAL

Growth and extraction of cells

A. agilis (*A. vinelandii* strain O) was grown aerobically in BURK'S synthetic medium[4]. The cells were centrifuged soon after the end of exponential growth and were washed by centrifugation from distilled water. The free lipids were removed by extracting the cells twice with warm ethanol and twice with 30 % methanol–chloroform (30:70, v/v). The residue was dried to a constant weight at 60° and 15 cm Hg.

The residue after extraction of free lipid was hydrolyzed in 3 N NaOH in methanol at refluxing temperature for 2 h. The mixture was cooled, acidified, and filtered through Celite; the residue was washed 4 times with 20-ml portions of diethyl ether. The combined filtrates were concentrated *in vacuo* at 50°.

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Analysis of fatty acids

The methyl esters of fatty acids were prepared by heating in anhydrous methanol with 0.1N HCl at 60° for 1 h. The methanolic solution was concentrated *in vacuo*, diluted with water, and extracted with several portions of diethyl ether. The ether solution was dried with anhydrous Na_2SO_4.

Methyl esters of hydroxy fatty acids were separated from other lipids by chromatography on a 1.6 × 60 cm column containing 12 g of silicic acid (Mallinkrodt Chemical Works, 100 mesh). Fractions were eluted with 12 column volumes of (I) diethyl ether–petroleum ether (4:96, v/v), (II) diethyl ether–petroleum ether (20:80) (III) diethyl ether, (IV) methanol–diethyl ether (25:75). Fraction II contained the esters of hydroxy acids.

Methyl esters were determined by gas–liquid chromatography on 0.25 in × 5 ft or 0.25 in × 8 ft columns of diethylene glycol–succinate polyester (25:75), on firebrick (Wilkens Instrument and Research, Inc.) at 187° or 189° using helium as the carrier gas. The effluent was monitored with a 4-filament katharometer (Loe Engineering Company) and a 1-mV recorder.

Individual esters were isolated from a preparative, 0.75 in × 5 ft column of diethylene glycol–succinate polyester (25:75) on firebrick. 20–70 mg of the desired esters were collected. The esters were separated from the products of thermal decomposition of the liquid phase in gas–liquid chromatography by chromatography on silicic acid[3]. The methyl esters of hydroxy fatty acids were recovered by eluting with 20 % diethyl ether in petroleum ether.

Synthesis of β-hydroxy acids

Methyl esters of racemic 3-hydroxydecanoic and 3-hydroxydodecanoic acids were synthesized by the REFORMATSKY reaction[5]. After fractional distillation, the ester was saponified with 2 N KOH in 95 % ethanol at refluxing temperature for 45 min. The solution was chilled in an ice bath, and the crystalline potassium salt of the hydroxy acid was recovered by filtration. The salt was washed once with cold ethanol and dissolved in a small volume of water. The cold, aqueous solution was acidified with HCl, and the insoluble hydroxy acid was recovered by filtration. The acid was recrystallized from petroleum ether (3-hydroxydecanoic acid, m.p. 55–57°; 3-hydroxydodecanoic acid, m.p. 68.5–69.5°, uncorrected).

Synthesis of α-hydroxy acids

2-Bromoacyl bromides were prepared by reaction of fatty acids of the desired chain length with Br_2 and PBr_3 (see refs. 6, 7). The 2-bromoacyl bromide was converted to the methyl ester by reaction with anhydrous methanol, and the 2-bromo ester was heated in aqueous formamide to yield the 2-hydroxy ester[8]. The methyl ester of the 2-hydroxy acid was saponified in 2N KOH in 95 % ethanol at refluxing temperature for 45 min. The alcoholic solution was diluted with water and acidified with HCl. The 2-hydroxy acid was extracted with diethyl ether. 2-Hydroxytetradecanoic acid and 2-hydroxydodecanoic acid (m.p. 72–73°) were crystallized from petroleum ether and purified by recrystallization from petroleum ether. The 2-hydroxy acids of lower molecular weight failed to crystallize. The methyl esters were formed, and the esters were purified by chromatography on a column of silicic acid. The

methyl esters of 2-hydroxyoctanoic acid and 2-hydroxydecanoic acid were eluted with diethyl ether–petroleum ether (20:80).

Permanganate oxidation of hydroxy acids

5–15 mg of hydroxy acid was dissolved in 8 ml acetone containing 0.25 ml of saturated aqueous $NaHCO_3$; 100 mg of powdered $KMnO_4$ was added in 4 equal portions over a period of 30 min[9]. The solution was heated to boiling for 30 sec after each addition of $KMnO_4$. After the addition of $KMnO_4$, the solution was boiled for 5 min and concentrated *in vacuo*. The concentrate was diluted with 4 ml water, acidified, and extracted with diethyl ether. The fatty acids were extracted from the ether solution with 2 ml of saturated $NaHCO_3$. The aqueous extract was acidified, and the fatty acids were again extracted into ether. The solution was dried with anhydrous Na_2SO_4, concentrated to an oil, and esterified with acidic methanol.

Desaturation–hydrogenation of β-hydroxy acids

5–15 mg of hydroxy acid was mixed with 40 mg P_2O_5 and 2 ml anhydrous benzene[10]. The mixture was heated at refluxing temperature for 1 h. 2 ml of water was added cautiously after cooling the reaction mixture. The aqueous phase was separated and washed twice with 1.5 ml benzene. The carboxylic acids in the benzene phase were extracted with 2–4 ml of saturated aqueous $NaHCO_3$. The aqueous solution was acidified with dilute HCl and extracted with diethyl ether. The ether solution was evaporated to dryness.

The products of dehydration were hydrogenated in 2 ml of methanol at 1 atm of H_2 with a catalyst of 5 % Pt on charcoal for 2 h at 40°. The resulting saturated acids were esterified with acidic methanol.

RESULTS

Bound lipid

The lipids of *A. agilis* extractable into organic solvents before hydrolysis comprised 10.4 % of the dry weight of the cells. After alkaline hydrolysis and acidification, an additional 2.6 % of the dry weight was extractable by diethyl ether (bound lipid).

The products of hydrolysis of the bound lipid gave an intense absorption in the infrared at 1700–1750 cm^{-1}, indicating a high concentration of fatty acids. The products were esterified, and the methyl esters were separated from other lipids by chromatography on a column of silicic acid (Table I).

TABLE I

CHROMATOGRAPHY OF METHYL ESTERS FROM THE BOUND LIPIDS OF *A. agilis* ON SILICIC ACID

	*Eluting solvent**	*Per cent recovered*
I	Diethyl ether–petroleum ether (4:96)	36.6
II	Diethyl ether–petroleum ether (20:80)	31.9
III	Diethyl ether	13.7
IV	Methanol–diethyl ether (25:75)	18.8

* The eluting solvents were prepared volume for volume; 12 times the volume of silicic acid was used to collect each fraction.

Identification of hydroxy fatty acids

Fraction I (Table I) gave an infrared spectrum typical of fatty acid methyl esters with an intense ester carbonyl absorption at 1740 cm^{-1}. Fraction II also absorbed strongly at 1740 cm^{-1} but showed an additional band at 3500 cm^{-1} not given by Fraction I. The infrared spectrum, together with the polar characteristics of Fraction II, suggested that this fraction contained methyl esters of hydroxy fatty acids. Fractions III and IV contained only small amounts of esters.

Gas–liquid chromatography of Fraction I (Fig. 1a) demonstrated the presence of methyl esters of the saturated and mono-unsaturated fatty acids previously identified in the phospholipids of *A. agilis*[3]. Fraction II (Fig. 1b) contained three major components which differed in retention volume from the esters of saturated and mono-unsaturated fatty acids (Fig. 2). The retention volume of these three components was not altered by hydrogenation. Component A has a retention volume identical with that of either methyl 2-hydroxyundecanoate or methyl 3-hydroxydecanoate. Component B has a retention volume similar to that of methyl 2-hydroxydodecanoate or

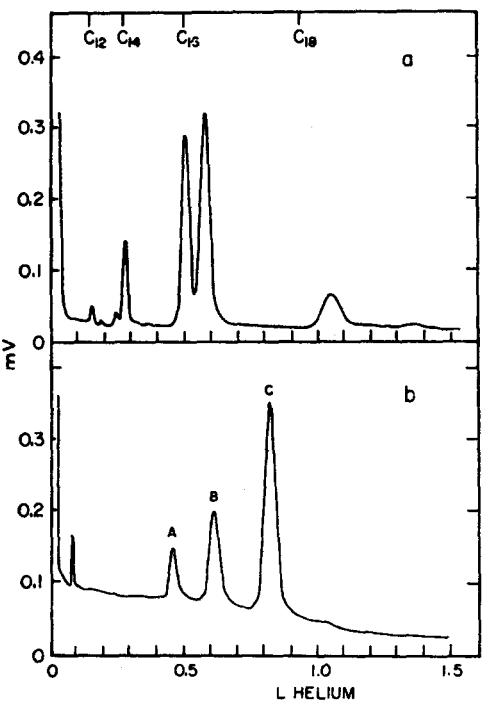

Fig. 1. Gas–liquid chromatograms of methyl esters from the bound lipids of *A. agilis*. a, fraction I (Table I); b, fraction II (Table I). The retention volumes of the methyl esters of saturated fatty acids are indicated. The column was 0.25 in × 5 ft of diethylene glycol–succinate polyester (25:75) on firebrick, operated at 189°; flow rate, 89.6 ml He/min; detector current, 95 mA.

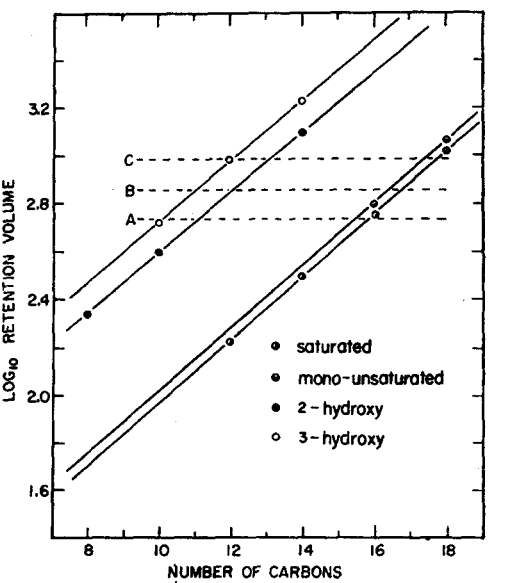

Fig. 2. Retention volumes in gas–liquid chromatography of methyl esters of *n*-saturated, *n*-mono-unsaturated, 2-hydroxy, and 3-hydroxy monocarboxylic acids. The logarithm of the retention volume in millilitres measured from the air peak is plotted as function of the number of carbon atoms. A, B, and C indicate the retention volumes of three major components in Fig. 1b. The column was 0.25 in × 5 ft of diethylene glycol–succinate polyester (25:75) on firebrick, operated at 187°; flow rate, 96.8 ml He/min; detector current, 95 mA.

methyl 3-hydroxyundecanoate. Component C has a retention volume identical with that of the methyl ester of either 2-hydroxytridecanoic acid or 3-hydroxydodecanoic acid.

Identification of components A and C

Components A, B, and C were recovered separately from preparative gas–liquid chromatography and purified by chromatography on silicic acid. The infrared spectra component A (Fig. 3b) and component C (Fig. 3c) were essentially identical with the spectrum of the methyl ester of synthetic 3-hydroxydodecanoic acid (Fig. 3a). The spectra of free acids recovered by saponification of components A and C were also similar to the spectrum of 3-hydroxydodecanoic acid and distinct from the spectrum of α-hydroxy acids.

Components A and C were oxidized with permanganate, and the products of oxidation were esterified. The resulting methyl esters were determined by gas–liquid chromatography (Table II). The products of oxidation of synthetic 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid were similarly determined. Component A and 3-hydroxydecanoic acid yielded methyl octanoate as the principal product. Component C and 3-hydroxydodecanoic acid yielded methyl decanoate as the principal product.

The identity of Component A with the methyl ester of 3-hydroxydecanoic acid and of Component C with the methyl ester of 3-hydroxydodecanoic acid was further confirmed by dehydration with P_2O_5 and catalytic hydrogenation of the resulting products (Table III). The methyl ester of decanoic acid was identified as the principal product of both component A and methyl 3-hydroxydecanoate. The methyl ester of dodecanoic acid was identified as the principal product of both Component C and methyl 3-hydroxydodecanoate.

TABLE II

PRODUCTS OF THE OXIDATION OF COMPONENTS A AND C WITH ALKALINE PERMANGANATE

Reactant	*Products*				
	Methyl heptanoate (%)	*Methyl octanoate (%)*	*Methyl nonanoate (%)*	*Methyl decanoate (%)*	*Methyl undecanoate (%)*
Component A	6.9	61.2	6.5	18.5	6.9
3-Hydroxy-decanoic acid	7.5	92.5	0	0	0
Component C	0	1.7	7.5	75.3	10.0
3-Hydroxydodecanoic acid	0	1.2	10.6	83.6	0

TABLE III

PRODUCTS OF DEHYDRATION–HYDROGENATION OF COMPONENTS A AND C

Reactant	*Products*	
	Methyl decanoate (%)	*Methyl dodecanoate (%)*
Component A	88.8	0.4
3-Hydroxydecanoic acid	>99	0
Component C	15.4	84.5
3-Hydroxydodecanoic acid	3.3	96.7

Identification of Component B

Component B was saponified, and the free acid was recovered. The infrared spectrum of the free acid from component B (Fig. 4) was essentially identical with the spectrum of 2-hydroxydodecanoic acid. The carbonyl absorptions of component B and of 2-hydroxydodecanoic acid are at 1750 cm^{-1}. This shift to higher frequencies is characteristic of α-hydroxy acids.

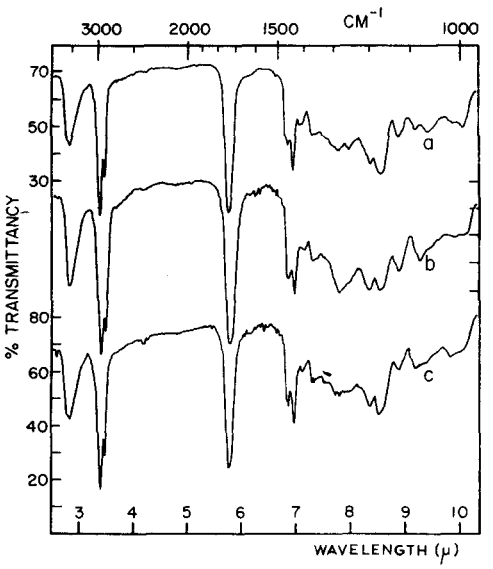

Fig. 3. Infrared spectra of: a, synthetic methyl 3-hydroxydodecanoate; b, component A; c, component C. The samples were spread as a film on the surface of discs of fused KBr.

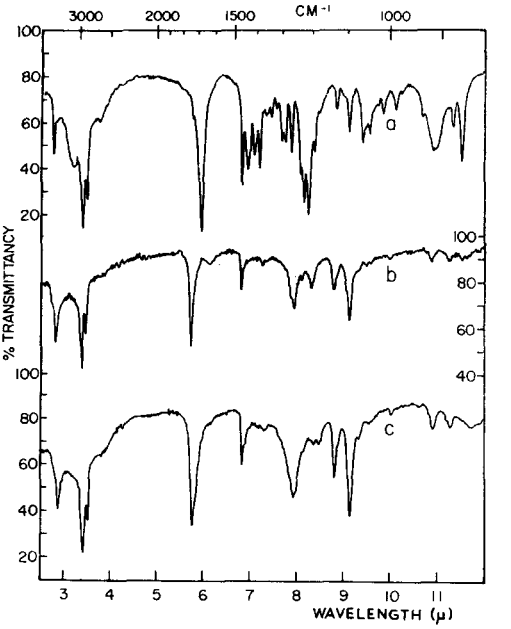

Fig. 4. Infrared spectra of: a, synthetic 3-hydroxydodecanoic acid; b, synthetic 2 hydroxydodecanoic acid; c, the free acid from saponification of component B. The sample and powdered KBr were ground to a homogeneous mixture and fused.

Component B was further identified as methyl 2-hydroxydodecanoate by oxidation with permanganate (Table IV). The principal products of oxidation of an α-hydroxy acid with permanganate are saturated fatty acids with 1 and 2 fewer carbon atoms. The principal products of oxidation of both Component B and 2-hydroxydodecanoic acid were methyl undecanoate and methyl decanoate.

DISCUSSION

HOFMANN *et al.*[11,12] defined bound lipids to include all fatty acids which were liberated upon hydrolysis of an acetone–ether-insoluble residue of cells. A survey of Gram-positive lactobacilli indicated that about 80 % of the lipids were bound and that the fatty acids of the bound lipid were similar to fatty acids from extractable lipids. In *A. agilis* only 20 % of the lipids were bound. Approximately half of the fatty acids recovered from the bound lipid was found to be hydroxy fatty acids, yet hydroxy fatty acids cannot be detected in the lipids extractable from cells with ethanol and methanol–chloroform[3].

Hydroxy fatty acids have been found in highly polar lipids of other Gram-negative bacteria[1,2]. The hydroxy acid from an endotoxin of *E. coli* was mainly 3-hydroxytetradecanoic acid[13]. Hydroxy fatty acids have been found in covalent linkage with polar compounds. BERGSTRÖM *et al.*[14] and JARVIS AND JOHNSON[15] found D-3-hydroxydecanoic acid in a rhamnolipid produced by pseudomonads. CARTWRIGHT[9,10] found a D-3-hydroxydecanoic acid as an amide of serine in serratamic acid from Serratia.

TABLE IV

PRODUCTS OF THE OXIDATION OF COMPONENT B WITH ALKALINE PERMANGANATE

Reactant	*Products*			
	Methyl octanoate (%)	*Methyl nonanoate (%)*	*Methyl decanoate (%)*	*Methyl undecanoate (%)*
Component B	3.7	3.4	40.6	52.5
2-Hydroxydodecanoic acid	0.7	4.5	41.0	54.0

More recently, serratamic acid was isolated as a heterocyclic dimer in the form of serratamolide[16]. 3-Hydroxydecanoic, 3-hydroxydodecanoic, and 3-hydroxy-5-dodecenoic acids were found in the lipid from *S. marcescens*[17], but the location and binding of hydroxy acids within cells were not specified. Since hydroxy fatty acids are found in bound lipids of Gram-negative bacteria, questions arise as to whether Gram-positive organisms possess similar hydroxy fatty acids or whether the hydroxy fatty acids are localized exclusively in surface structures as endotoxic lipo-polysaccharides which seem to be unique to Gram-negative bacteria.

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