CONVERSION OF LONG-CHAIN UNSATURATED FATTY ACIDS TO HYDROXY ACIDS BY HUMAN INTESTINAL BACTERIA

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PLATE VII

THE presence of long-chain hydroxy fatty acids, mainly 10-hydroxystearic acid, in human faeces was reported by James, Webb and Kellock in 1961. Faecal hydroxy fatty-acid levels have been shown to be greatly increased in some patients with steatorrhoea (Gompertz and Sammons, 1963; Webb, James and Kellock, 1963; Kim and Spritz, 1968*a*; Kellock *et al.*, 1969; Soong *et al.*, 1972), and it was postulated that because of its chemical similarity to ricinoleic acid, the active principle of castor oil, hydroxystearic acid (OHSA) may have a similar cathartic action and contribute to the diarrhoea often associated with steatorrhoea (James *et al.*, 1961). It was suggested (James *et al.*, 1961; Gompertz and Sammons, 1963; Kim and Spritz, 1968*b*) that the faecal OHSA was produced from dietary fat by intestinal bacteria. Some *Pseudomonas* spp. can convert oleic and other unsaturated fatty acids to OHSA and other hydroxy-acids *in vitro* (Wallen, Benedict and Jackson, 1962; Davis *et al.*, 1969), and Thomas (1972) has reported the in-vitro conversion of oleic acid to OHSA by type cultures of eight species of bacteria that can be found in the gut.

In the present study bacteria isolated from the faeces of normal human subjects and patients with high and low faecal OHSA levels were screened for their ability to produce hydroxyacids from unsaturated fatty acids. An attempt was also made to relate the numbers of active bacteria and the amount of OHSA in the faeces.

MATERIALS AND METHODS

Bacteriological methods

Preservation of specimens. From a freshly passed stool, 0.5 g of faeces was thoroughly mixed with 4.5 ml of glycerol broth (Crowther, 1971), the suspension frozen in solid carbon dioxide and stored at -20°C until cultivated.

Cultivation of specimens. To prevent loss of the very oxygen-sensitive non-sporing anaerobic bacteria, which are the dominant organisms in faeces (Eggerth and Gagnon, 1933; Drasar, 1967; Gorbach *et al.*, 1967), the initial procedures were performed in an anaerobic cabinet (Drasar and Crowther, 1971). Several ten-fold dilutions of the faecal suspension were prepared in oxygen-free Brain-Heart Infusion Broth (Oxoid) containing 0.05% (w/v) cysteine hydrochloride; 0.1 ml of appropriate dilutions were seeded on plates

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of Reinforced Clostridial Agar (Oxoid) within the cabinet and the plates transferred to an anaerobic jar for incubation. The dilutions were then transferred to the open bench and seeded on a series of selective and non-selective media to enumerate enterobacteria, enterococci, clostridia, lactobacilli and veillonellae (table I).

Bacteria were assigned to broad groups on the basis of colonial morphology on selective media, and Gram staining.

Sources of strains studied. The bacteria from the faeces of normal people were those isolated during studies on the intestinal bacteria and bowel cancer (Aries *et al.*, 1969; Hill *et al.*, 1971). Other bacteria were isolated from the faeces of patients in the Gastroenterology Department of the Central Middlesex Hospital during the course of this study.

Storage of bacteria for metabolic study. Isolated colonies were subcultured and stored for use in metabolic studies. Bifidobacteria and *Bacteroides* spp. were stored at -20°C in Robertson's Cooked Meat Medium (Southern Group Laboratories) containing 10%

TABLE I
Media and conditions for the isolation and enumeration of faecal bacteria.

Organisms	Medium*	Incubation of plates	
		Atmosphere	Time (days)
Total anaerobes	Reinforced clostridial agar with 1% (w/v) liver digest and 10% (v/v) horse blood	AN	4
<i>Bacteroides</i> spp.		AN	4
Bifidobacteria		AN	4
Clostridia	Willis and Hobbs' agar	AN	3-4
Lactobacilli	Rogosa's agar (L)	90% CO ₂	3
Veillonellae	Rogosa's agar (V)	AN	3-4
Enterobacteria	MacConkey's agar	O	1
Enterococci	Azide agar, MacConkey's agar	O	1

* Drasar and Crowther (1971).
AN = Anaerobic; O = aerobic.

(v/v) sterile glycerol. Clostridia were stored at room temperature in Robertson's Cooked Meat Medium and enterobacteria and enterococci were kept at room temperature on Dorset Egg Slopes (Southern Group Laboratories).

Incubation with fatty acids. Method A. In preliminary experiments organisms were inoculated into Reinforced Clostridial Medium containing 0.5% (w/v) sodium taurocholate (Koch-Light Laboratories) and 1% (v/v) oleic acid (British Drug Houses). However, the BDH oleic acid contained an impurity that caused analytical problems. *Method B.* Because Robertson's meat broth contains approximately 1% of fatty acids found to be readily available for bacterial metabolism, an attempt was made to use this medium for a screening test. However, the complexity of the analysis involved precluded its routine adoption. *Method C.* The most satisfactory analytical results were obtained by the use of pure oleic acid (Sigma Chemical Corp., 99%). This was added to peptone-yeast broth (Cato *et al.*, 1970) at a concentration of 1 mg per ml and was dispersed by sonic treatment of the mixture for 1 min. before autoclaving. Twenty ml of this medium was seeded with the centrifuged deposit from 25 ml of an overnight broth culture of enterobacteria or enterococci, or with 0.5 ml of deposit from the liquid part of a Robertson's meat culture of anaerobes. Cultures were incubated for 72 hours, the anaerobes in an atmosphere of hydrogen : carbon dioxide 9 : 1. In some experiments oleic acid was replaced by palmitoleic or linoleic acid (both Sigma Chemical Corp., 99%). Palmitoleic acid was added in the same way as oleic, but linoleic acid was added aseptically to the medium after this was autoclaved and then dispersed by shaking.

FIG. 1.—Two-stage thin-layer chromatogram of fatty acids from bacterial cultures in peptone-yeast-oleic acid medium; nos. 1 and 19, uninoculated; nos. 5 and 17, reference standard (12-hydroxystearic acid, oleic acid, and cholesterol); nos. 2, 3, 4, 10, 11, 14, 15, 16 and 18, enterococci; nos. 6 and 7, clostridia; nos. 12 and 13 bifidobacteria.

Biochemical methods

Analysis of incubation mixture. After incubation, the cultures were transferred to 250-ml polypropylene bottles; 20 ml of 30% aqueous KOH and 80 ml of ethanol were added and the mixture was heated for 30 min. in a waterbath at 80°C. The samples were diluted with 40 ml of water, cooled, acidified with HCl and the fatty acids extracted with 50 ml of toluene. The toluene extract was evaporated and the residue examined by thin-layer chromatography (TLC) of free acids or by gas-liquid chromatography (GLC) of the methyl esters prepared by treatment of the residue with freshly prepared ethereal diazomethane (de Boer and Backer, 1954). Some samples were examined by both methods. After incubation-methods A and B it was necessary to perform an additional separation, either solvent partition or silicic-acid chromatography before the extracted fatty acids were examined by GLC. In samples containing linoleic acid, precautions were taken to prevent autoxidation, by conducting the procedures under nitrogen whenever possible.

Determination of the OHSA content of faecal fat. Fatty acids were extracted from 5–10 g samples of faeces by the method of van de Kamer (van de Kamer, Huinink and Weyers, 1949) except that the solvent was toluene, as recommended for hydroxy acids (Jover and Gordon, 1962). Portions of the toluene extract containing about 100 mg of fatty acids were dried and treated with freshly prepared ethereal diazomethane (de Boer and Backer, 1954). The ether was evaporated off, the residue redissolved in heptane, and the fatty-acid composition determined by GLC of the methyl esters.

Gas-liquid chromatography. GLC of fatty-acid methyl esters was performed on a Pye Series-104, Model-4, isothermal gas chromatograph equipped with a flame-ionisation detector. A 152 cm × 4 mm glass column packed with 3% E.30 on 100–120 mesh Diatomite C-Q (packed column supplied by Pye-Unicam, Ltd) was used at a temperature of 200°C. The percentage composition of fatty-acid methyl-ester mixtures was calculated as peak height multiplied by retention time to estimate the relative area under the peak of each component of the chromatogram (Horning *et al.*, 1964). This method of calculation gave satisfactory results with standard mixtures containing normal and hydroxy fatty-acid methyl esters, and gave the same results as triangulation for faecal fatty-acid mixtures (Pearson, 1972).

Thin-layer chromatography. Fatty-acid mixtures were separated by TLC on 20 × 20 cm plates of silica gel (Silica Gel MFC without binder, Hopkin and Williams) in layers 0.25 mm thick. The plates were dried at room temperature, stored over calcium chloride, and activated for 1 hour at 110°C just before use. Samples were dissolved in heptane-chloroform 1 to 1 (v/v) or 1 to 2 (v/v) to a concentration of 10 mg per ml of fatty acids and 10 µl of this solution were spotted on a plate. Because the toluene extracts contained neutral lipid components, probably sterols, a two-stage development of the plate was necessary to give a clear separation of normal acids, hydroxy acids, and neutral lipids.

Chromatograms were first developed to a height of 14–15 cm with diethyl ether saturated with 25% aqueous ammonia—about 200 to 1 (v/v). The solvents were evaporated off by leaving the plate in a fume cupboard for about 30 min. and a second development was made to a height of 9–11 cm with heptane-diisopropyl ether-acetic acid 20 to 80 to 5 by volume. The solvents were removed as before and the fatty acids visualised by spraying the cold plate with freshly prepared ethanolic phosphomolybdic acid 10% (w/v) and heating at 110°C for at least 20 min. A mixture of 12-hydroxystearic acid (Sigma Chemical Corp.), oleic acid (Sigma), and cholesterol (Koch-Light Laboratories) was used for reference.

Acetylation. Hydroxy fatty-acid esters were acetylated by the rapid perchloric acid catalysed acetylation method (Fritz and Schenk, 1959).

Determination of carbon numbers. Carbon numbers were determined as described by Woodford and van Gent (1960).

Column chromatography. Samples of up to 100 mg mixed fatty acids dissolved in toluene were fractionated on a 1-cm diameter 2.5 g silicic acid ("Unisil" 200–235 mesh, Clarkson Chemical Co., distributors Kodak Ltd) column. Non-polar acids were eluted with 100 ml of 5% diethyl ether in heptane and polar acids with 100 ml of 30% diethyl ether in heptane.

RESULTS

Identification of hydroxystearic acid

OHSA from faecal fat and from extracts of bacterial cultures was identified by comparing its properties with those of 12-hydroxystearic acid. During GLC on E. 30, the retentions relative to stearate were virtually the same—1.87 for the faecal and bacterial OHSA and 1.85 for the 12-hydroxystearate; for the acetoxy esters the relative retentions were also very close—2.34 and 2.37

FIG. 2.—Comparison of the visual assessment of the amount of hydroxystearic acid in fatty-acid mixtures on thin-layer chromatograms (TLC) and the percentage found by gas-liquid chromatography (GLC) on E.30; ± = barely visible; + = clearly visible, small; ++ = clearly visible, larger; +++ = intense, large; ++++ = very intense, as large as oleic-acid spot.

respectively. On thin-layer chromatograms the mobilities were the same and they behaved similarly on a silicic acid column.

Some typical TLC separations are shown in fig. 1. The percentage of OHSA present in the extracted fatty acids could be approximately assessed by visual examination of the chromatograms. The comparison of this assessment with the percentage found by GLC in 31 samples is shown in fig. 2. There was a reasonable agreement between the visual assessment and the GLC estimation. Examination of the extracted fatty acids by TLC was thus able to give a rough quantitative assessment of the percentage of OHSA present; the limit of detection was about 2% with a load of 100 µg. In samples for which TLC gave an equivocal result the amount of OHSA was checked by GLC.

Comparison of incubation methods

The amount of OHSA detected in different cultures varied, and organisms were classified as having low, moderate, or high activity, depending on the proportion of oleic acid converted to OHSA under the test conditions. The basis of this assessment is shown in table II.

TABLE II
*Basis for assessment of the oleic-acid hydrating activity of
intestinal bacteria in vitro*

Grade of activity	Basis for assessment by		
	method A; percentage of oleic acid converted to OHSA	method B; percentage of oleic acid converted to OHSA	method C; approximate intensity of OHSA spot on thin-layer plate (see fig. 2)
Low	<2	1-5	+
Moderate	2-5	5.1-10	++
High	>5	>10	+++

TABLE III
Comparison of methods for detecting hydroxystearic acid production by bacteria

Bacteria, and strain number	Hydroxystearic acid production* by		
	method A	method B	method C
Enterococci			
EC9	3	5	+
EC10	2	7	+
EC11	8	17	...
EC13	3	15	...
EC15	...	17	+++
Cr.SB1	...	+	++
Clostridia			
CC63	2	+	...
CL20A	0	...	++
CC270	0	...	-
CC393	0	...	-
Enterobacteria			
EBC157	0	...	-
EBC249	0	...	-
Do.EBM4	...	+	++

* Numerals indicate percentage of oleic acid converted, estimated by GLC; symbols -, +, ++, and +++ indicate amount of OHSA detected by TLC; ... = not done.

Eleven strains of bacteria were tested by two of the methods used and two strains by all three methods. Good agreement between methods was obtained with 12 of the 13 strains tested (table III).

Conversion of oleic acid to OHSA by intestinal bacteria

One hundred and four strains from normal subjects and 103 strains from 20 samples of faeces from 15 patients with gastro-intestinal disorders were tested. Of the 207 strains, 94 were able to convert oleic acid to OHSA.

All the groups of bacteria studied possessed strains able to form OHSA (table IV). The most active group was the enterococci; 98% of these were active and most had moderate or high activity. The enterobacteria and three

TABLE IV
Production of hydroxystearic acid by bacteria isolated from faeces

Bacteria	Number of strains tested	Number of strains forming OHSA				Percentage forming OHSA
		Low activity	Moderate activity	High activity	Total	
<i>Bacteroides</i> spp.	34	5	0	1	6	18
Bifidobacteria	47	12	3	0	15	32
Clostridia	22	4	6	1	11	50
Enterobacteria	52	4	4	3	11	21
Enterococci	52	11	26	14	51	98

groups of anaerobes tested—*Bacteroides* spp., bifidobacteria, and clostridia—all contained some active strains. The activity of these was somewhat lower than that of the enterococci, but some moderately active strains were found in all these groups. The low activity found among the strict anaerobes may have been partly due to growth conditions being suboptimal. One of the strains of *Clostridium* from a patient's faeces had the highest activity found, converting over 70% of the oleic acid to OHSA.

TABLE V
Production of hydroxy acids from unsaturated fatty acids by bacteria from faeces

Bacteria	From linoleic acid		From palmitoleic acid	
	Number tested	Number forming hydroxy acid	Number tested	Number forming hydroxy acid
<i>Bacteroides</i> spp.	4	0	6	0
Bifidobacteria	6	0	1	1
Enterococci	4	0	4	4

Two strains of enterococci from normal subjects also formed OHSA when incubated under anaerobic conditions.

The formation of hydroxyacids from other unsaturated fatty acids by intestinal bacteria

The bacteria incubated with linoleic and palmitoleic acids included some strains known to convert oleic acid to OHSA. None of the organisms tested produced an unsaturated hydroxy acid from linoleic acid, but five out of six strains that produced OHSA also formed a polar product from palmitoleic acid (table V).

This product migrated to the same position as OHSA on a TLC plate and was eluted by 30% ether in heptane from a silicic acid column. When the methyl ester was subjected to GLC on E. 30, the retention time was that expected

TABLE VI

Carbon numbers of straight-chain saturated fatty acids and their derivatives

Parent acid	Carbon number of the methyl ester of		
	normal acid	hydroxy acid	acetoxy acid
Stearic	18	19.8	20.6
Palmitic	16	17.8	18.5

for an hydroxy-palmitic acid. Acetylation increased the retention time and therefore the carbon number by the same amount as those of OHSA on acetylation (table VI). This indicates strongly that the product was an hydroxy-palmitic acid with the hydroxyl group on one of the carbon atoms from 5 to 13 (Tulloch, 1964). Although the position of the hydroxyl group was not determined, in the only other report of an hydroxypalmitic acid formed from palmitoleic acid by a micro-organism this was stated to be 10-hydroxy-palmitic (Davis *et al.*, 1969). It is therefore assumed that the product formed by intestinal bacteria is probably 10-hydroxypalmitic.

TABLE VII

Production of hydroxystearic acid by bacteria from faeces with normal and raised hydroxystearic acid level

Bacteria	Strains from faeces with				P χ^2
	normal OHSA		raised OHSA		
	Number tested	Percentage forming OHSA	Number tested	Percentage forming OHSA	
<i>Bacteroides</i> spp.	18	22	16	13	Not significant 0.05
Bifidobacteria	35	40	12	8	
Clostridia	20	55	2	0	...
Enterobacteria	25	4	27	37	<0.01
Enterococci	33	100	19	95	Not significant
Totals	131	48	76	41	Not significant

The relationship between faecal OHSA level and bacterial flora

The proportion of active strains among bacteria from faeces with a normal OHSA level, i.e., less than 4% of the total faecal fatty acids (Pearson, 1972; Wiggins *et al.*, in prep.) was not significantly different from that among bacteria from faeces with a raised OHSA level. However, there were some differences

among the different groups of bacteria (table VII). There was apparently a smaller proportion of active strains among the bifidobacteria from faeces

FIG. 3.—Relationship between faecal hydroxystearic acid level and bacterial count; ● total anaerobes; ▲ enterococci; — geometric mean.

FIG. 4.—Relationship between faecal hydroxystearic acid level and bacterial count; ● enterobacteria; ▲ clostridia; — geometric mean.

with a raised OHSA level. The only group that had a larger proportion of active strains in faeces with a raised OHSA level was the enterobacteria. *Bacteroides* spp. and enterococci showed no difference and too few clostridia were tested for valid comparison.

OHSA determinations and bacterial counts were performed on 21 freshly passed specimens of faeces from patients with gastro-intestinal disorders. At each level of faecal OHSA there was considerable variation in the counts of clostridia, lactobacilli, veillonellae, enterobacteria and total anaerobes and relationship with OHSA level could not be demonstrated (figs. 3-5).

FIG. 5.—Relationship between faecal hydroxystearic acid level and bacterial count; ● lactobacilli; ▲ veillonellae; — geometric mean.

DISCUSSION

The ability to form hydroxystearic acid (OHSA) from oleic acid is widely distributed among the strictly and facultatively anaerobic intestinal bacteria. The results obtained with organisms freshly isolated from human faeces are in broad agreement with those previously obtained with type cultures (Thomas, 1972). The 26 strains tested by Thomas were mostly clostridia and non-sporing anaerobes, and the proportion of active strains found in those groups are similar to those reported here.

The mechanism of OHSA formation by intestinal bacteria is probably similar to that described for a *Pseudomonas* strain by Wallen *et al.* (1962). The reaction in that organism was shown to be a stereospecific addition of the elements of water across the double bond of oleic acid to give 10-D-hydroxystearic acid (Shroepfer, 1966).

Intestinal bacteria hydrating oleic acid are also able to hydrate palmitoleic acid which, like oleic, possesses a 9-10 *cis* double bond. The *Pseudomonas* studied by Davis *et al.* (1969) was also able to perform this reaction, but it has not previously been reported in intestinal bacteria. Unlike the *Pseudomonas*, however, none of the intestinal bacteria tested was able to hydrate linoleic acid to form an unsaturated hydroxyacid similar to ricinoleic acid.

Hydration of palmitoleic acid, and possibly shorter-chain acids with a 9-10 *cis* double bond, may account for the small amounts of shorter-chain polar acids often found in faecal fat (Pearson, 1972).

In faeces with a high OHSA level, we failed to find an increase in either the proportion of active strains in most groups of bacteria able to hydrate oleic acid, or an increase in the total numbers of bacteria in those groups; this indicates that the increased faecal-OHSA levels found in some patients with steatorrhoea (Webb *et al.*, 1963; Kim and Spritz, 1968a; Kellock *et al.*, 1969; Soong *et al.*, 1972) cannot be ascribed to changes in the faecal flora. Other factors that may be responsible are an increase in available substrate, and the time for which this is in contact with the colonic flora. Evidence that these factors are concerned in the increase in faecal OHSA is provided by reports that there is some correlation between OHSA level and both faecal fat (Pearson, 1972; Wiggins, Cummings and Pearson, in prep.) and transit time (Wiggins *et al.*, in prep.).

SUMMARY

Two hundred and seven strains of bacteria from human faeces were screened for their ability to convert oleic acid to hydroxystearic acid *in vitro*. Ninety-four strains were able to perform this reaction. All but one of the enterococci tested were active, and some active strains were also found among *Bacteroides* strains (18%), bifidobacteria (32%), clostridia (50%), and enterobacteria (21%). Oleic acid-hydrating bacteria were also able to hydrate palmitoleic but not linoleic acid. Relationship could not be demonstrated between faecal hydroxystearic acid level and the number of bacteria in the faeces able to produce this acid.

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