APPLIED MICROBIOLOGY, Feb. 1970, p. 295-300

Vol. 19, No. 2
Printed in U.S.A.

Hydrolysis of Conjugated Bile Acids by Cell-Free Extracts from Aerobic Bacteria

DAVID W. YESAIR AND PHILIP HIMMELFARB
Arthur D. Little, Inc., Cambridge, Massachusetts 02140

Received for publication 21 August 1969

By means of an aerobic enrichment culture technique, several bacteria that hydrolyze conjugated bile acids and modify the formed deconjugates were isolated from feces of man, rat, and chicken and from soil. Hydrolase activity was intracellular and extractable, and the yield of the enzymes was increased by adding the conjugated bile acids to the culture media. The hydrolase from bacterium of human origin was stable, having a *p*H optimum at about 7.0. All bile acid conjugates were hydrolyzed linearly as a function of time.

Bile acids enter the duodenum as glycine, taurine, or both types of conjugates, but are found unconjugated in the lower intestine and feces (2, 10, 23, 27, 29). In germ-free (2, 10–12) and in antibiotic-treated animals (20), the bile acids are excreted primarily as conjugates. In several pathological conditions, for example, blind-loop (5, 19, 33), afferent-loop stasis after partial gastrectomy (8, 32), and steatorrhea (19, 34), unconjugated bile acids and microorganisms are simultaneously found in the small intestine.

Several microorganisms have been described that hydrolyze the conjugated bile acids, e.g., *Aerobacter aerogenes* (1), *Bacteroides* species (6), motile gram-negative microorganisms from several sources (7), *Aspergillus oryzae* (9), *Clostridium perfringens* (22), *Clostridium* species (25), and several species of enterococci (28). Norman and Widström (28) demonstrated that the contents of cecum and colon from rats contain enzymes that hydrolyze conjugated bile acids. Cell-free extracts that hydrolyze conjugated bile acids have been obtained from *C. perfringens* (22).

This paper describes the selection of bacteria, the preparation of cell-free extracts which hydrolyze conjugated bile acids, and the characterization of the hydrolase(s).

MATERIALS AND METHODS

Taurocholic, glycocholic, cholic, and deoxycholic acids were obtained from Mann Research Laboratories, Inc. The mixture of conjugated bile acids which was used in the growth studies was obtained from General Biochemicals. N-Z Amine-B, an enzyme-hydrolyzed casein, was purchased from Sheffield Chemical Co. Pancreatine, USP, was obtained from Nutritional Biochemical Corp.

Bacteria were isolated by an enrichment culture technique in which natural materials such as feces and soil were homogenized with water and added to flasks containing mineral salts (0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4$), 0.75% conjugated bile acid at neutral *p*H, and 1% yeast extract in tap water. The flasks were shaken aerobically for 24 to 48 hr at room temperature. Samples of these mixed cultures were transferred to a second and third flask sequentially. At each transfer, the cultures were streaked on agar plates containing conjugated bile acid as the only C source and the plates were incubated for 24 to 48 hr at room temperature.

Individual colonies were isolated from the agar plates and maintained on conjugated bile acid slants. The cultures were grown aerobically in shaken flasks containing mineral salts, 0.75% conjugated bile acids, and 0.1% yeast extract and examined for hydrolase activity. The utilization of conjugated bile salts, the production of bile acids, and the appearance of degraded bile acids were determined by thin-layer chromatography (TLC) on Silica Gel H (31); 10-μliter samples of the growth medium were taken each 24 hr for 120 hr of growth. Cell number was determined by plating samples in conjugated bile acids-agar or by measurements of optical density at 520 nm (Bausch & Lomb Spectronic-20), or by both. Hydrolase activity was assayed in both the cell-free supernatant fluid and lysed cell preparations.

Cell-free preparations of the enzyme were obtained from large batches of wet cells by grinding the cells with an equal weight of adsorption alumina [80 to 200 mesh, Fisher Scientific Co. (13)], extracting with 0.05 M phosphate buffer at *p*H 7.0, and centrifuging at 13,000 × *g* for 20 min. The supernatant fluids were dialyzed at 4 C against three changes of water and lyophilized. An equally satisfactory method of obtaining enzyme preparations from small batches of wet cells was by freeze-thawing (three times) in 10 ml of

mineral salts solution. This latter method was used to compare the activity of different cultures. One unit was defined as that amount of enzyme which hydrolyzes 7.6 mg of conjugated bile salts per hr.

Semiquantitative hydrolase activity was determined at room temperature (22 C) in enzyme preparations that contained 0.05 M phosphate buffer at *p*H 7.0 and conjugated bile salts (7.6 mg/ml). Samples (10 μliter) were taken at 15-min intervals for at least 90 min and spotted on two thin-layer (350 μm) Silica Gel H (E. Merck AG) plates. This procedure stopped the enzymatic reaction. One of the plates was chromatographed in a solvent system (toluene–HOAc–water, 5:5:1, v/v, upper phase) which was specific for separating bile acids (31). On this plate, the rate of appearance of cholic and deoxycholic acids and any degradation of the bile acids were estimated. The second TLC plate was chromatographed in a solvent system (BuOH-HOAc-water, 10:1:1, v/v) specific for separating the conjugated bile acids. With various enzyme dilutions and substrate concentrations, we were able to estimate the rate at which the conjugated bile acids were hydrolyzed to bile acids.

For the production of large quantities of one bacterium (an isolate from man) by both batch and continuous culture, we used a New Brunswick fermentor (New Brunswick Corp.). The growth medium consisted of mineral salts, 0.1% yeast extract in 0.1 M phosphate buffer at *p*H 7.0 with 0.75% N-Z Amine-B, and 1.0% conjugated bile acids. Growth conditions were: agitation at 400 rev/min with baffles, aeration at 2 liters/min with a 4-liter volume in a 7-liter fermentor, and temperature at 26 C. Continuous-flow culture was maintained at a flow rate of 340 ml/hr. Dow-Corning Antifoam A was used as needed for both batch and continuous cultures.

RESULTS

From feces of rat, chicken, and man and from soil, 16 bacteria were isolated which hydrolyzed conjugated bile salts. On the basis of Gram reaction, morphology, and hydrolase activity, we selected six pure cultures which appeared different (Table 1). All of the organisms contained extractable intracellular hydrolase(s); the bacterium isolated from rat also elaborated some extracellular hydrolase activity. One organism from chicken feces and the only culture from man showed high hydrolytic activity.

All of these bacteria hydrolyzed the conjugated bile acids completely within 24 hr (Fig. 1 and 2). As the concentration of conjugated bile acids decreased, the concentration of cholic and deoxycholic acids increased until about 11 hr and decreased thereafter with the appearance of modified bile acids (Fig. 2). In 72 hr, all of the substrate was utilized.

Because of its fast growth and high hydrolase level, the isolate (bacterium of human fecal origin) was examined in more detail. This organism grew better in the regular conjugated bile acid medium than in a medium containing 1% glucose in which marked acid production took place (Fig. 3). After the glucose-containing growth medium was buffered with phosphate (Table 2), growth in glucose was equivalent to or heavier than the growth in conjugated bile salts. However, the yield of extractable enzyme did not increase proportionately. A somewhat better yield of growth and of enzyme was obtained with N-Z Amine-B and glucose combinations, but a combination of N-Z

TABLE 1. *Bacteria capable of hydrolyzing conjugated bile salts*[a]

Source	Gram reaction	Form	Location of enzyme	Hydrolase activity[b]
Rat	−	Rod	Intra- and extracellular	++
Chicken	−	Rod	Intracellular	++
Chicken	−	Coccus	Intracellular	+
Chicken	−	Rod	Intracellular	+++
Man	−	Rod	Intracellular	+++
Soil	+	Rod	Intracellular	+

[a] Microorganisms were isolated from feces or soil, and individual colonies were grown aerobically on mineral salts, conjugated salts, and yeast extracts. Hydrolase activity was assayed for both cell-free supernatant fluid and lysed cell preparations.

[b] Evaluated from yield of bacterial cell mass and enzyme units.

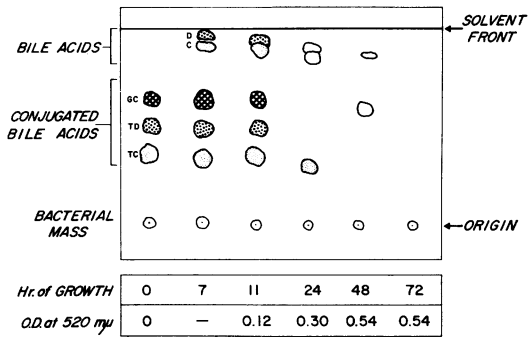

FIG. 1. *Composite illustration showing the hydrolysis of conjugated bile acids by a bacterium (an isolate from man) growing in aerobic shaken flasks. A 10-μliter amount of growth medium was spotted on Silica Gel H TLC plates and chromatographed in solvents specific for separating conjugated bile acids. Labeled and shaded areas are deoxycholic (D), cholic (C), glycocholic (GC), taurodeoxycholic (TD), and taurocholic (TC) acids. Unmarked areas are unknowns. The concentrations of conjugated bile acids decreased relative to the increasing concentration of bile acids up to 11 to 24 hr.*

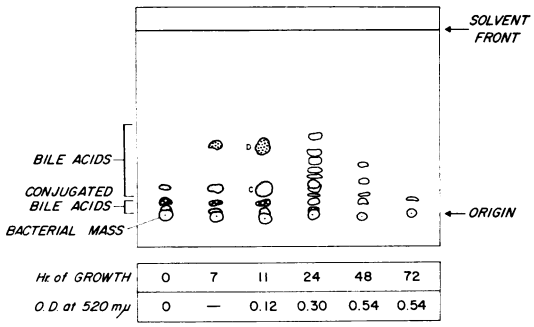

FIG. 2. *Composite illustration showing the appearance of bile acids and degradative products during growth of a bacterium (an isolate from man) in aerobic shaken flasks. A 10-μliter amount of growth medium was spotted on Silica Gel H TLC plates and chromatographed in solvents specific for separating bile acids. Labeled and shaded bile acids are deoxycholic (D) and cholic acid (C). The unlabeled areas are unknowns.*

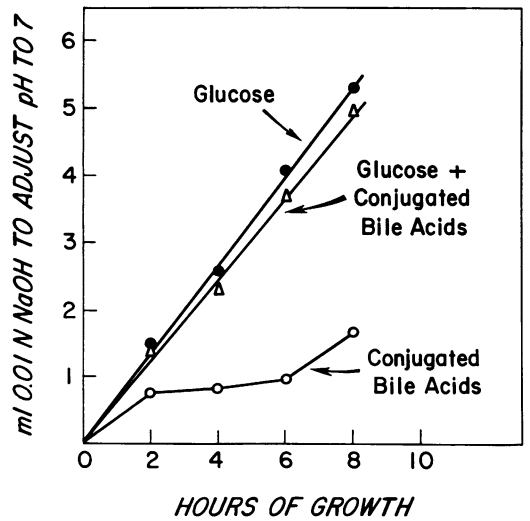

FIG. 3. *Comparative titratable acidity produced by a bacterium (an isolate from man) in glucose or in conjugated bile acids, or in both. Glucose and acid concentrations were 1.0 and 0.15%, respectively. In addition, the medium contained mineral salts and 0.1% yeast extract and was shaken aerobically at 26 C.*

Amine-B and conjugated bile acid gave the best enzyme yield. Conjugated bile acid concentrations above 1.25% and N-Z Amine-B above 1.5%, both separately and in combination, inhibited growth.

Most of the hydrolase activity in cells was liberated (Fig. 4) by freeze-thawing or grinding with alumina. The cell debris contained some hydrolase activity and also some enzymes which

TABLE 2. *Comparative growth and enzyme yield from a bacterium (an isolate from man) growing in different media*[a]

Components of medium[b] (%)			Determinations after 24 hr of growth		
Glucose	N-Z Amine-B	Conjugated bile acid	pH	Count/ml	Enzyme units[c] per 40 ml of culture
1.0			6.7	3×10^8	<1
	0.75		7.5	7×10^8	1
		0.75	7.1	2×10^8	2
1.0	0.75		6.5	10×10^8	3
	0.75	0.75	7.1	9×10^8	10

[a] The bacterium was grown for 24 hr in 40 ml of medium containing either glucose or conjugated bile salts, with and without N-Z Amine-B, or N-Z Amine-B alone. The *p*H, cell count, and yield of hydrolase units were determined.

[b] Media contain mineral salts, 0.1% yeast extract, and 0.1 M phosphate buffer (*p*H 7.0), plus additional component(s).

[c] One unit of enzyme hydrolyzes ca. 7.6 mg of bile salts in 60 min.

modify the bile acids. The activity was extractable from all organisms grown in glucose (Table 1).

A typical growth curve in a 4-liter fermentation is shown in Fig. 5. An average of 16 to 23 g of wet cells per liter was obtained in batch growth, and, in 8 days of continuous operation at 340 ml/hr, 12.9 ± 3.3 g/liter was realized. This flow rate is equivalent to a cell doubling time of 8.1 hr, which is about 2.5 times the minimum doubling time of logarithmically growing cells (Fig. 5). From 1 g (wet weight), about 100 units of enzyme could be extracted. The enzyme is stable to dialysis, lyophilization, dry storage at − 20 C for at least 3 years, and room temperature (wet and dry) for 2 days. It is denatured within 30 min at 95 C and its enzymatic activity is destroyed by pancreatic enzymes.

The hydrolysis of conjugated bile acids (surgically obtained from the gall bladders of beagles) has been evaluated for this enzyme preparation from bacterium (an isolate from man). There was no apparent denaturation of enzymatic activity by bile. The hydrolysis of conjugated bile acids was complete, and at that time any added conjugates were also hydrolyzed.

The optimum for this enzyme preparation is approximately *p*H 7.0 (Table 3); hydrolysis is decreased about 10 and 20% at *p*H 6.0 and 8.0, respectively, and is about 20% greater at 37 than at 22 C. The enzyme preparation hydrolyzes both the taurine- and glycine-conjugated bile acids linearly as a function of time. Relative rate of hy-

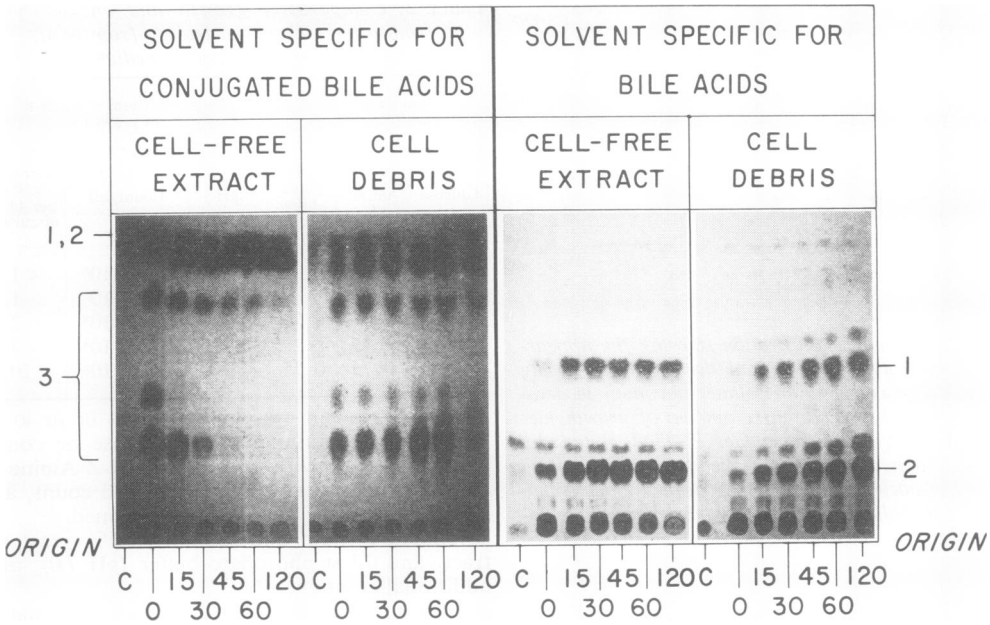

FIG. 4. *Hydrolase activity of cell-free extract and cell debris from a bacterium (an isolate from man). Samples (10 μliter) of reaction mixture were spotted on silica plates and chromatographed in solvents specific for bile acids or for conjugated bile acids. Numbers 1 and 2 designate deoxycholic and cholic acid, respectively, and 3 designates the area of the conjugates. On the abscissa, C represents the cell-free extract or cell debris before adding the conjugated bile acids, and the numbers represent the time in minutes after adding the acids.*

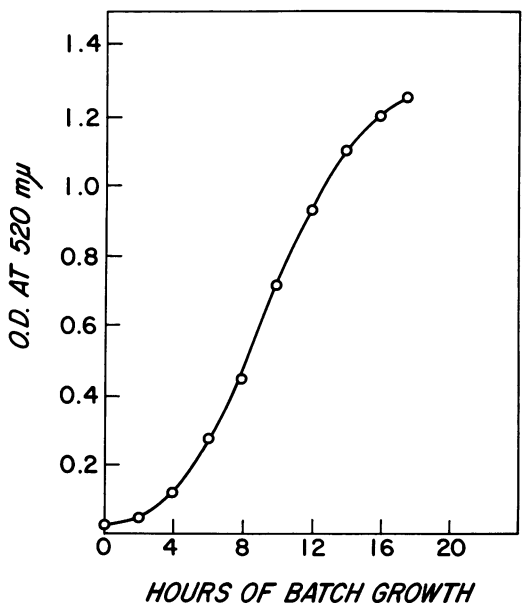

FIG. 5. *Growth curve of a bacterium (an isolate from man) in a New Brunswick fermentor. Details of batch culture conditions are described in the text.*

TABLE 3. *Effect of pH on hydrolysis of conjugated bile acids by an enzyme preparation from bacteria (an isolate from man)*

pH	Relative rate of hydrolysis
8	78
7	100[a]
6	90
5	49

[a] One unit of enzyme hydrolyzes 7.6 mg of conjugated bile salts per hr at pH 7.0 and room temperature.

drolysis has not been established, but the enzyme completely hydrolyzes both types of conjugates.

DISCUSSION

Several types of aerobic and anaerobic microorganisms have been reported which hydrolyze conjugated bile acids and modify free acids (13–15, 21, 24, 26, 30). In our study, several aerobic organisms were obtained by the enrichment techniques that hydrolyze the taurine and glycine conjugates and extensively modify the deconjugates thus formed. The hydrolase(s) from bacteria iso-

lated from man and other species was extractable from lysed cells, whereas the enzyme(s) affecting modification remained bound to the cell debris. The *p*H optimum for these hydrolases was about 7.0, similar to that reported for an extracellular enzyme(s) from *C. perfringens* (*p*H 5.8; reference 22). Our hydrolase(s) was stable to dialysis, room temperature, and cold storage, whereas the enzyme(s) from *Bacteroides* species was unstable even at 0 C (6). In all six microorganisms, the enzymes were apparently constitutive (hydrolase activity was extracted from glucose-grown cells); in the bacterium isolated from man, higher levels of enzyme per cell could be induced with conjugated bile acids.

In several pathological conditions of the small intestine (5, 8, 32, 33, 34) and in surgically created stagnant loop in animals (5, 19), free bile acids can be found. Different types of organisms have been cultured from these sources (6, 33, 34), but cultures that deconjugate bile acids have not been isolated (5). These isolated microorganisms (5) that normally grow in the presence of conjugated bile acid in these diseased conditions should be evaluated for enzyme induction because conjugated bile acids apparently can induce hydrolase synthesis.

Conjugated bile acids are important for each step in the process of triglyceride absorption: enzymatic hydrolysis by pancreatic lipase (3), micelle formation of hydrolyzed lipids (16–18), and the resynthesis of the triglyceride (4). On the other hand, bile acids inhibit the fatty acid-esterification enzymes (4) and are less efficient at micelle formation in vivo (19). If the hydrolase(s) could be protected against intestinal enzymatic digestion, the suggestion by Dawson and Isselbacher (4) and by Donaldson (5), that the microbial hydrolysis of the conjugated bile acids may be the cause of steatorrhea in the blind-loop syndrome, could be evaluated experimentally.

ACKNOWLEDGMENT

This investigation was supported by Chesebrough-Ponds, Inc., New York, N.Y.

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