Hydrolysis of Lithocholate Sulfate by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa was found to be able to hydrolyze bile salt sulfate. This property was observed when lithocholate sulfate was substituted for the sulfur source in the culture medium. The addition of $MgSO_4$ to the medium inhibited the hydrolysis of the bile salt sulfate.

Until a short time ago, bile salts were thought to be metabolic end products of cholesterol. Palmer (4) first demonstrated that lithocholate was transformed to a 3α-sulfate ester and showed that bile contained 40 to 75% lithocholate sulfate. In healthy subjects, the urinary concentrations of sulfated and nonsulfated bile salts are quite low (8). However, Stiehl et al. (8) and Makino et al. (3) demonstrated that in subjects with various diseases (notably, extrahepatic obstruction, hepatitis, cirrhosis, and liver metastasis) there was a marked increase in the amount of sulfated bile salts excreted in the urine. These results suggest that the sulfation of bile salts might be a mechanism used by the liver to eliminate potentially hepatotoxic substances, since it is known that lithocholate and its conjugates can cause liver damage (6). In spite of the possible significance of bile salt sulfation, methods of analysis are tedious and time consuming (7, 8). The procedure itself involves 108 h of acid hydrolysis (solvolysis) to cleave the sulfate ester bond. After cleavage, the method requires an additional 16 h of reflux in alkaline solution to hydrolyze the methyl ester formed during the acid hydrolysis. The entire procedure requires many solvent extractions and column chromatographic separations. Using these lengthy and difficult methods, the recovery has been shown to be low (64.6 ± 7.9%), and the reproducibility is poor (coefficient of variance 12.2%) (7). The present paper reports the ability of *Pseudomonas aeruginosa* to hydrolyze the bile salt sulfate ester bond. Isolation and purification of this bacterial enzyme will significantly improve the methodology of bile salt sulfate analysis.

Sulfated lithocholate was synthesized according to the method of Fieser (1). ^{35}S-labeled bile salt sulfates were prepared with [^{35}S]-chlorosulfonic acid (Amersham/Searle Corp., Des Plaines, Ill.). [^{14}C]lithocholate was purchased from California Bionuclear Co., Sun Valley, Calif. These substances were further purified by paper electrophoresis in a 0.025 M sodium citrate buffer, pH 5.8, at 50 V/cm for 1.5 h to remove traces of sulfate ion. The labeled bile salt sulfate was then eluted from the paper with water.

P. aeruginosa was isolated from human feces. After isolation from human fecal material, the microorganism was specifically identified by the Microbiology Laboratory at the University of California, Davis, as *P. aeruginosa*. The following criteria were utilized for identification. It was identified as a gram-negative rod that was oxidase positive and grew optimally at 42°C as a non-lactose fermenter on MacConkey agar. It utilized glucose by oxidative fermentation, but not maltose. It gave a positive arginine decarboxylase reaction, but negative lysine and ornithine decarboxylase reactions. Using these criteria, the organism was identified as *P. aeruginosa*. The organism was cultivated on a minimal support medium, substituting the sulfated lithocholate for the sulfur source. The cultures were maintained aerobically at 37°C in a salt medium consisting (per 100 ml) of: sodium phosphate (pH 6.8), 3 mmol; NH_4Cl, 0.1 g; $FeCl_3$, 0.85 g; $CaCl_2$, 0.005 g; sodium benzoate, 1.1 g; and ^{35}S-labeled lithocholate sulfate (specific activity, 5.8×10^5 cpm/mg), 0.25 mg, as the only sulfur source. In the buffered medium the final pH was 6.8. Stock cultures were maintained at 25°C on tryptic soy agar (Difco Laboratories, Detroit, Mich.) with 5% sheep blood and minimal support salt medium agar (1.5% to 100 ml of minimal support medium). On nutrient agar, *P. aeruginosa* form their characteristic smooth round colonies with a fluorescent greenish-blue color, whereas on the minimal support agar the bacteria exhibit a light-green color and form smaller, grainy-looking colonies. Cultures from the minimal support agar were streaked periodically on blood agar to check for contamination.

Cell growth in the liquid culture was followed by measuring the absorbancy at 540 nm on a Varian Techtron model 635. Inocula for the liquid cultures were taken from the minimal support agar plate.

The amount of ^{35}S-labeled sulfated lithocholate remaining in the culture medium after incubation was measured by removing 50 μl of the solution and applying this sample to a thin-layer plate (Adsorbosil-5, Applied Science Laboratories, Inc., State College, Pa.). The plate was developed in a solvent system containing n-butanol–acetic acid–water (10:1:1, vol/vol) for 6 h. In this system, inorganic sulfate stays at the origin and lithocholate moves with the solvent front, lithosulfate having an R_f value of 0.81. Visualization of the standard was achieved by spraying with 10% phosphomo-

FIG. 2. Cell growth curve and extent of sulfated lithocholate hydrolysis. The incubation medium contained 15 ml of minimal support salt medium with 38 μg of ^{35}S-labeled sulfated lithocholate (1.8 × 10^6 cpm). A sample (0.05 ml) was removed from the incubation medium at the specified time interval and applied to a thin-layer plate. The plate was developed, and the area corresponding to the sulfated lithocholate was scraped and counted in a liquid scintillation counter as described in the text. Cell growth was measured by the absorbancy at 540 nm.

TABLE 1. Effect of MgSO$_4$ on the incubation of bile salt sulfatase[a]

MgSO$_4$ (M × 10^3)	Optical density (540 nm)	Sulfated lithocholate hydrolyzed (%)
0.0	1.12	92
1.0	0.98	83
2.5	1.46	23
5.0	0.97	5
10.0	1.01	2
20.0	1.16	2

[a] P. aeruginosa was incubated in 1 ml of minimal support salt medium containing 27 μg of ^{35}S-labeled sulfated lithocholate (total count, 63,000 cpm) with various amounts of MgSO$_4$. After 17 h of incubation, 0.1 ml of the incubation medium was removed to determine the extent of sulfated lithocholate hydrolyzed, as described in the text.
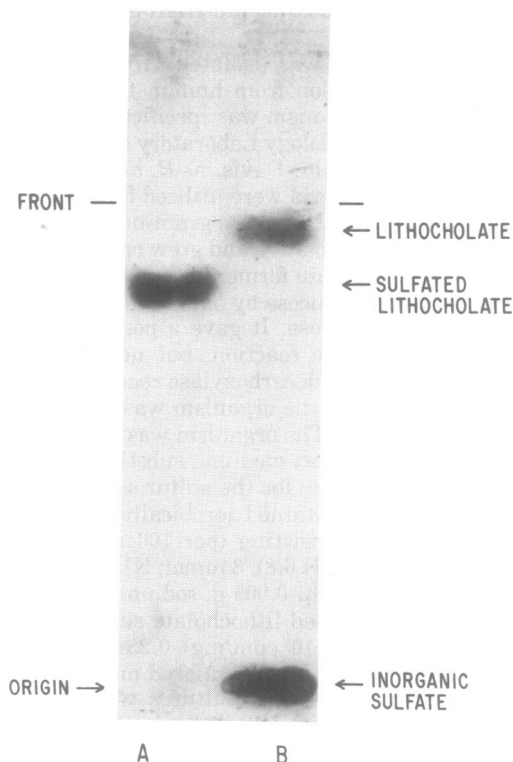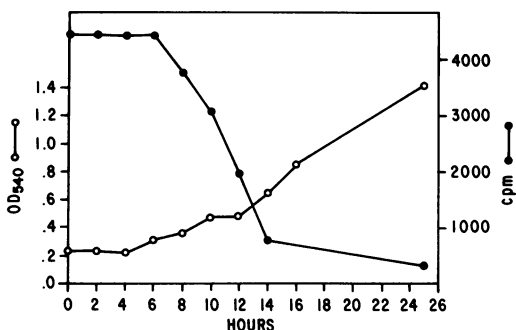

FIG. 1. Radioautogram of the incubation mixture after thin-layer chromatography. The incubation medium contained 0.5 ml of minimal support salt medium (as described in the text) with 2 μg of doubly tagged (^{35}S, ^{14}C) sulfated lithocholate (75,000 cpm) and P. aeruginosa. A sample (0.05 ml) was removed at zero time (lane A) and at 15 h of incubation (lane B). The solution was applied to a thin-layer plate, and the plate was developed in a solvent system containing n-butanol–acetic acid–water (10:1:1, vol/vol) for 6 h.

lybdic acid. Radioactive sulfated lithocholate was scraped from the plate, added to 10 ml of Bray's scintillation fluid (1), and counted in a Beckman LS-230 scintillation counter.

Since Palmer (5) demonstrated that lithocholate was further metabolized to other compounds when sulfated lithocholate was incubated with mixed fecal flora, doubly tagged [^{14}C]lithocholate-[^{35}S]sulfate was used to ascertain the desulfation of the substrate. Figure 1 shows the result of using doubly tagged sulfated lithocholate incubated in the bacterial culture. After an incubation period of 15 h, a sample was placed on a thin-layer plate and a radioautogram was developed. Two radioactive

spots were found, one corresponding to lithocholate and the second to the inorganic sulfate (Fig. 1B).

Subsequent assays were carried out using ^{35}S-labeled sulfated lithocholate as the only sulfate source. The time course of cell growth and the extent of sulfated lithocholate hydrolysis indicated no hydrolysis of sulfated bile salt in the first 6 h (Fig. 2). By the 12th hour, 50% of the substrate had been hydrolyzed, and by the 24th hour, almost 100% breakdown was achieved. The optimal time for enzyme induction is at the 13th hour, and the optimal pH is 7.5.

Periodically, cell growth occurred without a corresponding hydrolysis of sulfated lithocholate. This could be due to contamination by sulfate ion in the medium. Inhibition study was initiated to determine the level of sulfate ion necessary to influence enzyme induction (Table 1). As the concentration of $MgSO_4$ was increased, bacterial cell growth increased, with a corresponding decrease in the amount of sulfated lithocholate hydrolyzed.

Additional experiments with selected aerobic and anaerobic bacteria, i.e., *Escherichia coli*, *Bacteroides fragilis*, *Bacteroides cadaveris*, *Streptococcus faecalis*, and bifidobacteria, as well as proteolytic enzymes contained in snake venom and human pancreatic juice, showed no bile salt sulfatase activity.

Isolation and characterization of this enzyme is currently under way. We hope that utilization of this enzyme in bile salt sulfate analysis will significantly improve existing methodology. In this way, the biological significance of sulfated bile salts in man can be more clearly and accurately defined.

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