

## Bile Acid Inhibition of *Clostridium Botulinum*

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Bile acids and their glycine and taurine conjugates were tested *in vitro* for inhibition of *Clostridium botulinum* types A and B. Cholic acid inhibited most strains at 2 mg/ml, whereas chenodeoxycholic acid inhibited all strains at 0.4 mg/ml. Deoxycholic acid inhibited one strain at 0.08 mg/ml and other strains at 0.4 and 2 mg/ml. Lithocholic acid inhibited all strains at 0.016 mg/ml. Glycine conjugates also showed considerable inhibition of some strains, whereas taurine conjugates were inactive.

Bile acids are known to inhibit various intestinal bacteria (6, 7, 18, 19). Floch et al. (5) found that lactobacilli were inhibited *in vitro* by cholic and deoxycholic acids, whereas enterobacteria and enterococci were not. Others reported on the alteration of intestinal bacteria in various pathological conditions of the intestinal tract. Shimada et al. (16) found *Clostridium perfringens* in 10 bile specimens from elderly patients who had undergone biliary tract surgery; none was found in controls. Lykkegaard and Justesen (8) showed that diverse bacterial populations developed in the gall bladder, common bile ducts, and bile of patients with strictures, stones, or diseases of the gall bladder. In such patients, several isolates of clostridia were made; normal subjects had no culturable bacteria in the gall bladder or its secretions.

The ability of various primary, secondary, and conjugated bile acids to inhibit a number of strains of *C. botulinum* types A and B is the subject of this report.

### MATERIALS AND METHODS

A total of 21 cultures of *C. botulinum* types A and B were obtained from the following sources: Center for Disease Control, Atlanta, Ga. (C. Hatheway); Food and Drug Administration, Washington, D. C. (R. Lynt); U. S. Department of Agriculture Food Safety and Quality Service, Beltsville, Md. (R. Johnston); Northern Regional Research Center, Peoria, Ill.; The American Type Culture Collection, Rockville, Md.; and U. S. Army Natick Research and Development Command, Natick, Mass. (A. Anellis). Stock cultures were maintained on cooked meat medium and were transferred to fluid thioglycolate medium and incubated for 48 h at 30°C in a controlled environment incubator (National Appliance Co.) with an N<sub>2</sub> atmosphere. These were diluted by adding 0.2 ml of culture to 5 ml of assay medium, which consisted of 0.5% yeast extract, 0.5% tryptone, 0.2% glucose, 0.1% sodium thi-

oglycolate, and 0.12% K<sub>2</sub>HPO<sub>4</sub> at pH 6.6. All cultures grew within 18 h in this medium without bile acids. Two drops of the suspension was added to 5 ml of assay medium in tubes (15 by 125 mm).

Bile acids were added as sodium salts; 1% solutions were made up in water and heated to boiling, if necessary, to effect solution (lithocholic and glycolithocholic acids are insoluble in cold water). One-milliliter amounts of the 1% solutions and appropriate dilutions were added to 4 ml of assay medium made up to 1.25× normal concentration. These were autoclaved at 15 lb/in<sup>2</sup> for 15 min. After inoculation, the assay tubes were incubated at 30°C under N<sub>2</sub> in the controlled atmosphere incubator. Observations were made at 1, 2, and 5 days. (There was no change after 2 days, however.) Assessment of growth was by observing turbidity development or, with insoluble bile acids, by plunging a hot loop into the tubes; the evolution of gas in any quantity indicated growth.

### RESULTS

The effects of bile acids on the growth of 21 strains of *C. botulinum* types A and B are shown in Table 1. Of the four unconjugated acids, cholic acid was least inhibitory; the minimal inhibitory concentration was 2 mg/ml or greater for nearly all strains. The glycine and taurine conjugates of cholic acid also were not inhibitory. Chenodeoxycholic acid inhibited all cultures at 0.4 mg/ml; glycochenodeoxycholic acid was less active and the taurine conjugate showed no inhibition at 2 mg/ml. Deoxycholic acid, a secondary bile acid formed by bacterial action on cholic acid, also inhibited nearly all strains at 0.4 mg/ml; in a second experiment, this compound inhibited cultures B1218 and 169 at 0.3 mg/ml, although it allowed growth at 0.2 mg/ml. Its glycine conjugate was less active than the free acid, and its taurine derivative showed no inhibition. Table 1 shows that the most inhibitory bile acid was lithocholic acid, also a secondary acid derived

TABLE 1. Inhibition of *C. botulinum* by bile acids

Culture no.	Type	Source <sup>b</sup>	Minimal inhibitory concn (mg/ml) of the following acids: <sup>a</sup>											
			Cholic	Cheno- deoxy- cholic	Deoxy- cholic	Litho- cholic	Glyco- cholic	Glyco- cheno- deoxy- cholic	Glyco- deoxy- cholic	Glyco- litho- cholic	Tauro- cholic	Tauro- cheno- deoxy- cholic	Tauro- deoxy- cholic	Tauro- litho- cholic
20PLALCA	A	CDC	2	0.4	0.4	0.016	>2	2	2 <sup>c</sup>	2	>2	>2	>2	>2
174091A	A	CDC	>2	0.4	2	0.016	>2	2 <sup>c</sup>	2 <sup>c</sup>	0.4	>2	>2	>2	>2
1	A	FSQS	2	0.4	0.4	0.016	>2	2 <sup>c</sup>	2	0.4	>2	>2	>2	>2
2	A	FSQS	2	0.4	0.08	0.016	>2	2 <sup>c</sup>	2	0.4	>2	>2	>2	>2
33A	A	Natick	2	0.4	0.4	0.016	>2	2 <sup>c</sup>	2	0.4	>2	>2	0.4	>2
B1218	A	NRRC	2	0.4	0.4	0.016	2	0.4	2	0.08	>2	>2	>2	>2
25763	A	ATCC	0.4	0.4	0.4	0.016	2	2 <sup>c</sup>	2	0.08	>2	>2	>2	>2
3	B	FSQS	2 <sup>c</sup>	0.4	2	0.016	>2	0.4	2	0.08	>2	>2	>2	>2
4	B	FSQS	2	0.4	2	0.016	2	2 <sup>c</sup>	2 <sup>c</sup>	0.4	>2	>2	>2	>2
53B	B	Natick	2	0.4	0.4	0.016	2	2 <sup>c</sup>	2 <sup>c</sup>	0.08	>2	>2	>2	>2
7949	B	ATCC	2	0.4	0.4	0.016	>2	2 <sup>c</sup>	2 <sup>c</sup>	0.08	>2	>2	>2	>2
62	A	FDA	>2	0.4	0.4	0.016	2 <sup>c</sup>	2 <sup>c</sup>	ND <sup>d</sup>	ND	ND	ND	ND	ND
69	A	FDA	>2	0.4	0.4	0.016	0.4	2 <sup>c</sup>	ND	ND	ND	ND	ND	ND
78	A	FDA	>2	0.4	0.4	0.016	0.4	2	ND	ND	ND	ND	ND	ND
426	A	FDA	>2	0.4	0.4	0.016	2	2	ND	ND	ND	ND	ND	ND
429	A	FDA	>2	0.4	0.4	0.016	0.4	2 <sup>c</sup>	ND	ND	ND	ND	ND	ND
169	B	FDA	>2	0.4	0.4	0.016	0.4	2 <sup>c</sup>	ND	ND	ND	ND	ND	ND
383	B	FDA	>2	0.4	0.4	0.016	0.4	2	ND	ND	ND	ND	ND	ND
642	B	FDA	>2	0.4	0.4	0.016	0.4	2	ND	ND	ND	ND	ND	ND
999	B	FDA	>2	0.4	0.4	0.016	2	2	ND	ND	ND	ND	ND	ND
8688R	B	FDA	>2	0.4	0.4	0.016	0.4	2 <sup>c</sup>	ND	ND	ND	ND	ND	ND

<sup>a</sup> Log<sub>5</sub> dilutions giving concentrations of 2, 0.4, 0.08, and 0.016 mg/ml.

<sup>b</sup> CDC, Center for Disease Control; FSQS, U.S. Department of Agriculture Food Safety and Quality Service; Natick, U.S. Army Natick Research and Development Command; NRRC, Northern Regional Research Center; ATCC, American Type Culture Collection; FDA, Food and Drug Administration.

<sup>c</sup> Very slight growth at 0.4 mg/ml.

<sup>d</sup> ND, Not done.

from chenodeoxycholic acid. Lithocholic acid inhibited all strains at the lowest level tested, 0.016 mg/ml. In another test, it inhibited the first 10 strains in Table 1 at 0.008 mg/ml and permitted growth at 0.004 mg/ml. Glycolithocholic acid was less inhibitory, showing a minimal inhibitory concentration of 0.4 mg/ml for most strains. The taurine conjugate was not active at 2 mg/ml. There were no apparent differences in bile acid inhibition of A and B types of *C. botulinum*.

### DISCUSSION

The striking differences in the ability of the various bile acids to inhibit *C. botulinum* suggest that these may be involved in the inability of the organism to proliferate in normal adult intestinal tracts. *C. botulinum* does grow in some infants, however, producing typical botulism symptoms. No case of such intestinal proliferation in an adult has been reported.

Sharp et al. (14) found fetal bile to contain only chenodeoxycholic and cholic acids, the former in greater quantity. Meconium contained deoxycholic acid, but this was not present in the

stools of newborn infants. Norman and Strandvik (11) found only small amounts of cholic and chenodeoxycholic acids in the duodenal contents of infants fed breast milk and later reported (12) that orally administered lithocholic acid was excreted as the glycine and taurine conjugates. Bongiovanni (2) did not detect lithocholic acid in infant bile, whereas Mower et al. (10) found the lithocholic acid content of adult feces to be directly correlated with age ( $P < 0.005$ ).

Alteration of bile salts by intestinal bacteria, resulting in the production of deoxycholic and lithocholic acids and the deconjugation of glycine and taurine conjugates, has been reported by several workers (3, 4, 15, 17, 18).

In the light of these reports, it is interesting to speculate that the growth of *C. botulinum* in the intestinal tracts of some infants may be the result of a lack of inhibitory bile acids, such as lithocholic or deoxycholic acids. The validity of this hypothesis must await further studies on the bile acid content of infants with botulism.

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