Hydrolysis of Conjugated Bile Acids by Extracellular Enzymes Present in Rat Intestinal Contents.* (29603)

ARNE NORMAN AND OLOF A. WIDSTRÖM (Introduced by B. E. Gustafsson)
Department of Clinical Chemistry, Stockholms Läns Centrallasarett, Danderyd, Sweden

Bile acids are excreted in a conjugated form with more than 90% conjugated with taurine(1). In the small intestine, most of the bile acids are also conjugated, whereas the bile acids in the cecum, colon and feces are almost all unconjugated(2). Since bile acids in the feces of germfree rats and rats treated with antibiotics are almost exclusively conjugated, the hydrolysis of conjugates in untreated or normal rats has been ascribed to the action of microbial enzymes(3,4). The hydrolysis of taurine and glycine conjugates has been demonstrated in pure cultures of several species of Clostridia and enterococci (5).

The present investigation describes the occurrence of enzymes present in rat intestinal contents which have the ability to split conjugated bile acids.

Experimental. 24-C^{14} labeled taurocholate, taurodeoxycholate and glycocholate were synthesized as described earlier(6). *Enzyme preparations.* White male rats of a Sprague-Dawley strain, 3-6 months old, and reared on a standard diet were used. Immediately after killing the rats by a blow on the head, the intestinal tract was removed and divided into the stomach, small intestine, cecum, and colon. The small intestine was divided further into 3 approximately equal parts. The contents of each section were washed out with 10 ml of saline and homogenized with a magnetic stirrer. All operations were carried out at 0-5°C. The saline suspensions were centrifuged for 60 minutes at 25000 × *g*, and the supernatants were then decanted and stored at −17°C.

Enzymatic assay. Each incubation mixture contained 0.2 ml supernatant, 1.6 ml buffer solution and 0.2 ml of a 10 mM water solution of the labeled conjugate. After 10, 20 and 60 minutes incubation at 37°C aliquots were removed and the reaction stopped by immersing the samples in boiling water for 3 minutes. A control, in which saline was substituted for enzyme, was run with each series of assays. Enzyme activity was expressed as μmoles of the conjugate hydrolyzed per minute under the specified assay conditions.

Analytical methods. The hydrolysis of taurocholic and taurodeoxycholic acid was determined by extraction of unconjugated bile acid with ethyl acetate. Each reaction mixture was diluted with hydrochloric acid and water to a final volume of 3 ml. The final concentration of hydrochloric acid was 1 M. After equilibration with 3 ml of ethyl acetate, saturated with water, the isotope content in the ethyl acetate and water phases was determined. The partition coefficient between equal parts of ethyl acetate and M hydrochloric acid for the following bile acids was as follows: cholic acid 97, deoxycholic acid 95, taurocholic 0.02, and taurodeoxycholic acid 0.02. For determination of the hydrolysis of glycocholate, the products of the reaction were separated by paper chromatography according to Sjövall(7) and the ratio of isotope in the conjugated and unconjugated bile acid spots determined.

The possible metabolism of unconjugated bile acids by extracellular enzymes was investigated by column chromatographic separations(8,9).

Results. Hydrolysis of conjugated bile acids. The extracellular enzymes present in the supernatant of cecal contents consistently showed a high ability to split conjugated bile acids. The effect of pH on hydrolysis of taurocholate by these enzymes is shown in Fig. 1. The enzymes show considerable activity over a pH range of 5.0 through 7.0, but activity decreases rapidly

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† Bile acids and steroids 152.

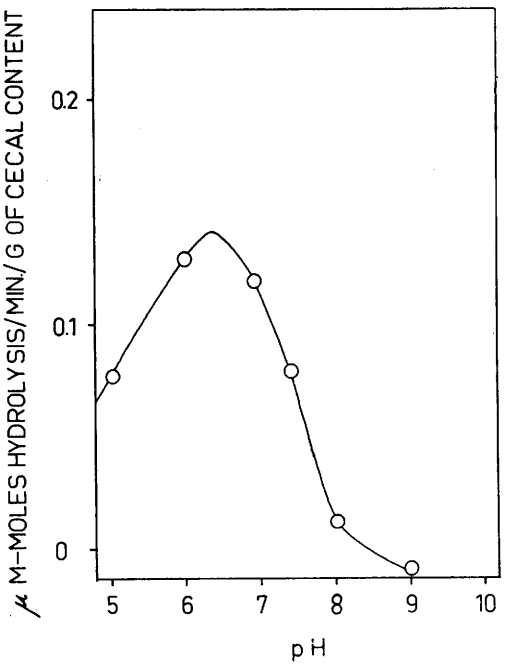

FIG. 1. Effect of pH on enzymatic hydrolysis of taurocholate catalyzed by extracellular enzyme present in the cecum.

above pH 7.5. In the following experiments the reactions were carried out in phosphate buffer at pH 6.5.

The enzymes which split conjugated bile acids are stable to freezing. No significant change in enzyme activity was observed after storing for 14 days at —17°C. After heating 3 minutes at 100°C a complete loss of activity occurred.

The ability of extracellular enzymes in the cecum to split taurocholate, glycocholate and taurodeoxycholate was compared (Table I). No significant difference in rate of hydrolysis of taurocholate, taurodeoxycholate and glycholate was observed.

TABLE I. Hydrolysis of Taurine and Glycine Conjugates by Extracellular Enzymes from the Cecum.*

Conjugate	Hydrolysis at pH 6.5, μMoles/min/g cecal content	
	Mean value	Range
Taurocholate	.245	.20–.27
Taurodeoxycholate	.230	.21–.26
Glycocholate	.215	.20–.24

* Pooled samples from 6 rats.

TABLE II. Distribution of Extracellular Taurocholate Splitting Enzymes in Rat Intestinal Tract.*

Supernatant	Hydrolysis of taurocholate at pH 6.5, μMoles/min/g intestinal content
Stomach	.010
Small intestine	
Upper third	0
Middle "	.007
Lower "	.007
Cecum	.630
Colon	.790

* Pooled samples from 6 rats.

The distribution of taurocholate splitting activity in the intestinal tract was determined and results are shown in Table II. Contrary to the high activity in the cecum and colon, practically no activity was found in the stomach and small intestinal contents.

Transformation of cholic acid. The remaining reaction mixtures from the above experiments with taurocholate -24-C^{14} were pooled and the unconjugated labeled bile acid extracted with ethyl acetate and separated by column chromatography. All the labeled material was eluted in the same fractions as cholic acid. The specific activity on recrystallization with non-labeled cholic acid remained constant. In a few experiments trace amounts of cholic acid -24-C^{14} were added to 2 ml of cecal content supernatant and incubated for 2 hours. No transformation of labeled cholic acid was observed.

Conclusions. Rat cecal content contains extracellular enzymes which catalyze the hydrolysis of taurocholate, taurodeoxycholate and glycocholate. The activity is so high that it is reasonable to assume that the hydrolysis of conjugates entering the cecum is brought about by these extracellular enzymes. During *in vitro* hydrolysis with these enzymes no metabolism of the bile acid molecule occurs.

The distribution of the taurocholate splitting activity in the intestinal tract is in agreement with its presumed microbial origin, *i.e.* little activity was found in the small intestinal contents as compared with the cecal contents.

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Plasma Levels of Various Blood Clotting Factors in Germfree Rats.* (29604)

B. A. WAALER, B. E. GUSTAFSSON, A. HAUGE, D. NILSSON, AND E. AMUNDSEN
Institute of Physiology, University of Oslo, Oslo, Norway and Department of Germfree Research, Karolinska Institutet, Stockholm, Sweden

The blood clotting factors prothrombin (factor I), proconvertin (factor VII), antihemophilic B factor (factor IX) and Stuart-Prower factor (factor X) depend on vit. K for their production and are all produced in the liver. Fibrinogen (factor II), proaccelerin (factor V) and antihemophilic A factor, (factor VIII) may, however, mainly be produced elsewhere in the body. It has been shown that the antihemophilic A factor level in plasma may be considerably raised by injections of adrenalin(1), or after muscular exercise(2,3,4). Fever-producing vaccine injections(5) or unspecific fever induction(6), cause increases in the plasma levels of antihemophilic A factor, proaccelerin and fibrinogen. This increase may indicate that their production is related to the reticulo-endothelial system(5,6). The theory is supported by the observation that patients with diseases of the reticulo-endothelial system, for example Hodgkin's disease, may have very high plasma levels of antihemophilic A factor(7).

These data suggested that it would be of interest to analyse the plasma levels of the blood clotting factors in germfree animals, in which part of the lymphatic system is poorly developed(8,9,10,11,12,13).

Methods and materials. Animals. Seven germfree rats of the Swedish strain from the Department of Germfree Research at Karolinska Institutet were used. These animals were housed in Gustafsson's isolators, and reared according to Gustafsson's methods(9, 14). The 7 control animals, of comparable weight and age, were of the same strain and reared in the same institute as the germfree rats. Each rat received 200 μg vitamin K_1 per day in its food.

Blood collection. Blood was obtained from ether-anesthetized animals by puncture of the right heart ventricle after thoracotomy. 2.7 ml blood was drawn into a plastic syringe containing 0.3 ml of a 3.1% Na-citrate solution ($2H_2O$), transferred to a chilled, paraffinized glass tube and centrifuged for 20 minutes. The plasma was kept deep frozen in a paraffinized glass tube until tested.

Blood clotting tests.

Antihemophilic A factor was measured with the thromboplastin generation test of Pool and Robinson(15).

Fibrinogen was measured spectrophotometrically by the method of Jacobsson(16) with some minor modifications(17).

Prothrombin was measured with the one-stage method using Russel's viper venom in cephalin as reagent(18), modified so as to be insensitive to the plasma content of Stuart-Prower factor(19).

Proaccelerin was measured according to Stormorken(20).

Proconvertin and *antihemophilic B factor* were measured in one-stage systems using

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