that anti-jelly serum inhibits cleavage of fertilized eggs.

As shown in this experiment, the agar diffusion technic serves as a convenient method for demonstrating the conversion of a precipitating, multivalent antibody to a non-precipitating, univalent form by papain digestion. These univalent non-precipitating antibody fragments do inhibit the fertilizability of eggs presumably by blocking the receptor molecules. The exact site of the inhibitory action has not been determined. It is hoped that studies employing fluorescein-labeled antibody will give a visual means of localizing the specific receptor sites.

Summary. Papain digested (univalent) anti-jelly serum inhibits the fertilizability of eggs of *R. pipiens* just as does the undigested anti-jelly serum. This observation, combined with the precipitation inhibiting action, strongly suggests a direct blocking of receptor sites in the egg jelly that perform a significant role(s) in fertilization.

1. Shaver, J. R., Barch, S. H., *Acta Embryol. et Morphol. Exp.*, 1960, v3, 180.

2. Shivers, C. A., *Am. Zool.*, 1961, v1, 118.

3. Metz, C. B., *Intern. Rev. Cytol.*, 1961, v11, 219.

4. Tyler, A., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 197.

5. Metz, C. B., Schuel, H., *Am. Zool.*, 1961, v1, 463.

6. Porter, R. R., *Nature*, 1958, v182, 670.

7. Kekwick, R. A., *Biochem. J.*, 1940, v34, 1248.

8. Nisonoff, A., *Biochem. Biophys. Research Comm.*, 1960, v3, 466.

Received April 5, 1962. P.S.E.B.M., 1962, v110.

Comparison of Bile Acids in Intestinal Contents of Germfree and Conventional Rats.*† (27526)

BENGT E. GUSTAFSSON AND ARNE NORMAN

Department of Germfree Research, Karolinska Institutet, Stockholm, and Department of Clinical Chemistry, Stockholms Läns Centrallasarett, Danderyd, Sweden.

After administration of cholic acid-24-C^{14} to conventional rats the isotope is excreted *via* feces and the half-life time of cholic acid estimated to 2-3 days(1,2). The main labeled excretory products have been identified as 3,12-dihydroxy-7-ketocholanic, deoxycholic and 3-hydroxy-12-ketocholanic acids(3,4). The microbial transformation mainly occurs in cecum, where almost all conjugated bile acids are hydrolyzed. In the small intestine the bile acid composition is mainly the same as in the bile.

Germfree rats and rats treated with chemotherapeutics have much longer half-life time of cholic acid-24-C^{14} than conventional rats(5,6). The labeled excretory products consist of taurocholic acid and a small amount of glycocholic acid. The amount of bile acids excreted by germfree rats is less than half of that excreted by conventional rats (1.9 and 5.1 mg per day respectively)(7). The intestinal flora thus cause an increased elimination of bile acids from the enterohepatic circulation. The rate of cholate excretion in feces by rats is also influenced by the composition of the diet(2). The possibility exists that these dietary influences are also related to changes in the intestinal flora.

The aim of the present investigation was to further elucidate the difference of bile acid turnover in germfree and conventional rats by comparing the physical state of bile acids in intestinal content present in small intestine, cecum and colon.

Experimental. The material comprised 2 germfree rats reared according to Gustafsson (8,9) on his semi-synthetic diet D 7, 4 conventional rats of the same strain and on diet D 7 and 4 conventional rats of a strain of

* Bile acids and steroids 121.

† This work is part of investigations supported by grants from Statens medicinska forskningsrad, Wallenbergsstiftelsen and Nat. Inst. Health, Bethesda, Md.

Sprague-Dawley origin reared on a pelleted practical type diet (P). All rats were male and weighed 200-300 g. The animals were kept in metabolism cages and given diets and water *ad libitum*.

Cholic acid-24-C^{14} was given as sodium salt dissolved in saline intraperitoneally. During the experiments the rats were given the usual diet *ad libitum*. Forty-eight hours after dosing the rats were killed, and the intestinal tract removed. The contents of the small intestine, cecum and colon were transferred separately to different precooled flasks. After addition of about 10 parts of saline the contents were homogenized with a magnetic stirrer for 5 minutes. This and subsequent operations were carried out at 0-5°C. The suspensions were transferred to tubes and centrifuged for 60 minutes at 25,000 × *g*. The supernatant was decanted and the sediment suspended in 1/15 M phosphate buffer, pH 7.4, and centrifuged as above. The sediment was extracted 3 hours in a Soxhlet extractor using acetone.

The isotope was determined in the combined supernatant and phosphate washing and in the acetone extract. After evaporation hydrolysis was carried out with 1 M sodium hydroxide in a closed steel tube for 6 hours at 120°C. The final ether extract was subjected to reversed phase partition chromatography using Phase system F1.(10). Moving phase: methanol/water 55:45; Stationary phase: chloroform/heptane 9:1. Four ml of stationary phase were supported on 4.5 g hydrophobic Supercel. The fractions collected from the columns were titrated and isotope content determined on aliquots after plating.

Results. Distribution of labeled bile acids between supernatant and sediment after centrifugation of intestinal content. By centrifugation of intestinal content suspended in saline at 25,000 × *g* for 60 minutes a clear supernatant could be obtained. Fig. 1 gives per cent of the total amount of labeled bile acids in the intestinal content which was recovered in the supernatant and sediment respectively. In the small intestine of conventional rats more than 95% of the labeled bile acids occurred in the supernatant, *i.e.*, in a

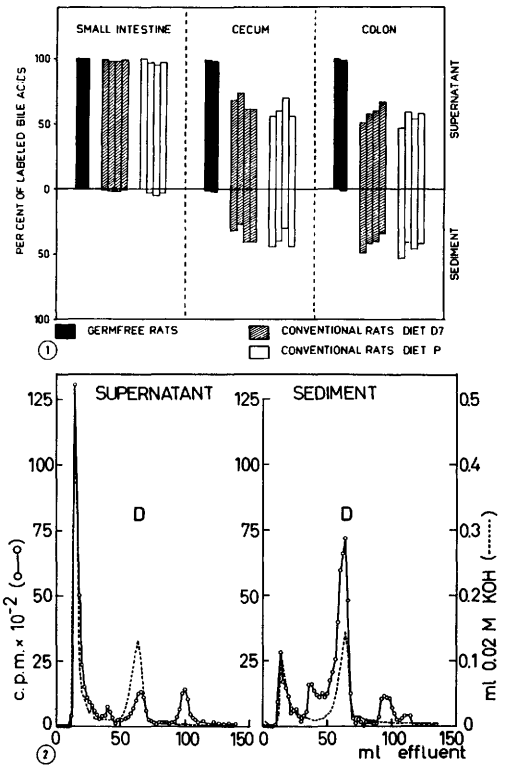

FIG. 1. % of labeled bile acids recovered from supernatant and sediment after centrifugation of intestinal contents at 25,000 x *g* for 60 minutes.

FIG. 2. Chromatographic separations of hydrolyzed labeled bile acids present in supernatant and sediment after centrifugation of cecal content isolated from a conventional rat 2 days after administration of cholic acid-24-C^{14}. Reference substance: deoxycholic acid (D). O——O, radioactivity; ····· titration values.

water soluble form. In the cecum of rats on diet D 7 a mean of 35% (range: 27-41%) and on diet P a mean of 40% (range: 30-44%) was obtained in the sediment and thus present in the water insoluble fraction. Somewhat greater fractions of isotope were recovered from the sediments after centrifugation of the contents of colon. No significant differences were observed between the two groups of conventional rats reared on diets D 7 and P respectively. In the germfree rats the conditions were quite different as the labeled bile acids were present in the supernatants in all segments of the intestinal tract.

Composition of labeled bile acids present in supernatant and sediment. Column chromatographic analysis using phase system FI separates metabolites of cholic acid with hy-

TABLE I. Labeled Metabolites of Cholic Acid without Hydroxyl- or Ketogroup at C-7 in Per Cent of Total Amount of Labeled Material in Sediment and Supernatant. Cecal Contents of Conventional Rats on Diet P.

Rat No.	Supernatant	Sediment
M 11	74	95
M 12	52	91
M 13	50	85
M 14	65	94
M 15	45	84
M 16	55	80

droxyl- or ketogroup at C-7 from metabolites without corresponding groups. Cholic and 3, 12-dihydroxy-7-ketocholanic acid (in effluent fraction 10-20 ml) appear prior to deoxycholic (40-60 ml) and 3-hydroxy-12-ketocholanic acid (80-110 ml). Representative chromatograms of hydrolyzed labeled bile acids in supernatant and sediment of centrifuged cecal content are shown in Fig. 2. Metabolites of the same chromatographic behavior were present in supernatant and sediment. However, the relative composition of the metabolites was different. The main metabolites in the sediment were deoxycholic and 3 - hydroxy - 12 - ketocholanic acid. Similar analysis of cecal contents of conventional rats reared on diet P are summarized in Table I. The labeled bile acids present in the sediment always had a higher amount of metabolites without hydroxyl- or ketogroup at C-7 than those present in the supernatant.

The fact that a high amount of cecal bile acids in conventional rats contrary to germfree rats is recovered from the water-insoluble fraction after centrifugation can certainly be ascribed to the action of the intestinal flora. Different mechanisms for this action of the intestinal flora are possible and are subjected to further investigations.

Summary. Two days after administration of cholic acid-24-C^{14} to germfree and conventional rats the intestinal contents were separated by centrifugation at 25,000 $\times$ *g*. 1. The labeled bile acids throughout the intestinal tract of germfree rats were recovered in the supernatant. 2. In the conventional rats almost all labeled bile acids in the small intestine were recovered in the supernatant whereas 27-44% of the labeled bile acids in cecum were found in the sediment. 3. The labeled bile acids in the sediment after centrifugation of cecal content had a higher per cent of deoxycholic and 3-hydroxy-12-ketocholanic acid than those present in the supernatant.

The authors are indebted to Dr. G. Widström, Dept. of Clinical Chemistry, for valuable help.

1. Lindstedt, S., Norman, A., *Acta Physiol. Scand.*, 1956, v38, 121.

2. Portman, O. W., Murphy, P., *Arch. Biochem. Biophys.*, 1958, v78, 367.

3. Norman, A., Sjövall, J., *J. Biol. Chem.*, 1958, v233, 872.

4. Portman, O. W., *Arch. Biochem. Biophys.*, 1958, v78, 125.

5. Gustafsson, B. E., Bergström, S., Lindstedt, S., Norman, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 467.

6. Lindstedt, S., Norman, A., *Acta Physiol. Scand.*, 1956, v38, 129.

7. Gustafsson, B. E., Norman, A., Sjövall, J., *Arch. Biochem. Biophys.*, 1960, v91, 93.

8. Gustafsson, B. E., *Acta Pathol. et Microbiol. Scand.*, 1948, Suppl. 73.

9. ———, *Ann. N. Y. Acad. Sci.*, 1959, v78, 1.

10. Sjövall, J., *Acta Physiol. Scand.*, 1953, v29, 232.

Received April 9, 1962. P.S.E.B.M., 1962, v110.