Glucuronidase Activity in Intestinal Contents of Rat and Man and Relationship to Bacterial Flora[1] (36510)

THOMAS H. KENT, LAWRENCE J. FISCHER, AND ROSE MARR

Departments of Pathology and Pharmacology, University of Iowa College of Medicine, Iowa City, Iowa 52240

The activity of the hydrolytic enzyme β-glucuronidase is high in the large bowel contents of mammals but low in the small intestinal contents (1–3). The intestinal activity, with an optimum at pH 6, appears to be derived primarily from bacterial sources (3). Intestinal glucuronidase appears to play an important role in the enterohepatic circulation of natural and foreign compounds excreted in the bile as glucuronide conjugates (4, 5). The polar glucuronides are usually not well absorbed from the intestine but after hydrolysis mediated by glucuronidase the more lipid-soluble aglycones are often readily absorbed into the portal circulation. Excretion of substances from the body are considerably delayed by this process.

The newborn animal would be expected to have very little glucuronidase activity derived from bacterial sources. The age at which the young animal attains the adult capacity to hydrolyze glucuronide conjugates is not known. Presumably the increase in glucuronidase activity with age should coincide with establishment of the organisms which produce the enzyme in the intestinal contents. These intestinal organisms have not been well characterized.

The present study was undertaken to determine the development after birth of intestinal glucuronidase activity in rat and man. In addition the development and character of the bacteria responsible for production of intestinal glucuronidase in the rat were investigated. The results provide important correlations between intestinal glucuronidase activity and the establishment of certain anaerobic organisms.

Materials and Methods. Measurement of glucuronidase activity. The microtechnique of Robinson, Blinn and Frank (6) was used because it is simple to perform, accurate, economical, and semiquantitative results were adequate for our purpose. One loop of broth media or one loop of intestinal content, which had been diluted 1:2 with phosphate buffer at pH 6.2 and centrifuged, was mixed with 0.008 ml of 0.001 *M* phenolphthalein glucuronic acid and 1 drop of chloroform. The mixture was drawn into a 1 × 8 mm capillary tube and incubated for 1 hr at 37°. The incubated mixture was then blown into a depression on a white spot plate containing 0.03 ml of 0.15 *M* Na_2CO_3. The resulting pink color was graded 0 to 5+ as an index of glucuronidase activity. By calculation, a strongly positive test (5+) would be roughly equivalent to liberation of 400 μg of free phenolphthalein/g of sample (or 400 Fishman units) of glucuronidase.

Animals and patients. Rats used included newborns delivered at term by cesarean section, 5-day-old weanlings, 25-day-old rats which were 4 days postweaning, and 200–300 g Sprague-Dawley rats. The latter two groups were fed standard rat chow (Wayne Lab Blocks, Allied Mills, Chicago) and water *ad libitum*. Samples of intestinal content were obtained immediately after killing the rats by cervical dislocation or intracardiac administration of Nembutal. Stools from newborn and older infants were obtained from freshly soiled diapers. Feces were obtained by digital removal from patients 15 to 84 years old who were being seen in the medical out patient

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clinic for various disorders such as chest pain, diabetes mellitus, and hypertension. None was receiving antibiotics.

Antibiotics used to suppress rat intestinal flora were similar to those used previously (7). Lincomycin (25 mg) was given intragastrically twice daily and in the drinking water, 500 mg/liter. Neomycin sulfate, 20 mg; polymyxin B sulfate, 2 mg; and bacitracin, 1000 units were given intragastrically twice daily and in the drinking water with 1.3 g/liter neomycin sulfate, 60 mg/liter polymyxin B sulfate and 30,000 units/liter bacitracin.

Microbiological techniques. Methods for quantitative cultures of young rats are the same as reported previously (7–9). Serial 10-fold dilutions were spread on agar plates using a bent glass rod. Blood agar and MacConkey agar (Difco Laboratories) were incubated aerobically at 37°; SF agar (Difco Laboratories), aerobically at 45°; and blood agar, kanamycin–vancomycin blood agar, lactobacillus agar (Baltimore Biological Laboratories), anaerobically at 37°. Small and large intestine could not be separated in newborn and 5-day-old rats, so the entire intestine was ground in sterile saline and counts were determined for the entire intestine. Counts on 25-day-old rats were based on 1 g of cecal content and 4 cm lengths of mid-small intestine (approx 1 g).

For *in vitro* testing of glucuronidase production by bacteria, the following culture conditions were adopted after considerable trial and error. Neomycin sulfate (142 μg/ml) was added to one of two tubes of deep meat broth; the tubes were autoclaved and then stored in Torbal jars at room temperature in an atmosphere of 90% H_2 and 10% CO_2. Just prior to inoculation, menthol glucuronic acid ammonium (Sigma Chemical Company), 0.5 g/ml of broth, which had been sterilized by passage through a Millipore filter, was added. The inoculated media was incubated at 37° anaerobically for 4 days and then tested for glucuronidase activity.

For isolation of individual organisms that liberated glucuronidase into the media, 0.1 ml of broth culture which had been inoculated with cecal content from adult rats and which exhibited 4+ glucuronidase activity after 4 days, was serially diluted in sterile saline, spread on blood agar plates containing 0.5% menthol glucuronic acid ammonium and 142 μg/ml neomycin sulfate, and incubated anaerobically at 37° in 90% H_2 and 10% CO_2. After 4 days incubation, the plates were quickly examined and representative colonies picked for inoculation into deep meat broth containing substrate but not neomycin. Four days after transfer back to deep meat broth the organisms isolated were tested for aerobic and anaerobic growth and gram stained. Organisms from broth with 4+ glucuronidase activity were sent to the State Hygenic Laboratory (courtesy of Dr. Franklin Koontz) for further characterization.

Results. Variation of colonic glucuronidase activity with age and the effect of antibiotics. In rats we found a progressive increase in glucuronidase activity in cecal contents from 5 day, 25 day and adult rats (Table I). Intestinal content was too scant to test newborn (cesarean section) rats. The activity in 25 day and adult rats was suppressed by 4 days'

TABLE I. Cecal Content Glucuronidase Activity in Rats at 1:2 Dilution.

Conditions	No. of rats vs glucuronidase activity					
	Neg	1+	2+	3+	4+	5+
5-day-old rats		1	3	10		
25-day-old rats				3	5	5
Adult rats					2	8
25-day-old rats, lincomycin	7					
Adult rats, lincomycin	9					
Adult rats, neomycin–polymyxin–bacitracin	6					

TABLE II. Fecal Glucuronidase Activity in Man at 1:2 Dilution.

Age	No. of people vs glucuronidase activity					
	Neg	1+	2+	3+	4+	5+
<1 day	4	1				
1–2 days	7	1				
2–3 days	2	2	1		1	
3–4 days	3	1	2			
4–10 days	5	1				
1–3 months	1	2	2			
5–13 months		1	2	1		
Adults	1		2	2	3	7

treatment with lincomycin or a combination of neomycin, polymyxin, and bacitracin (Table I). These antibiotics have previously been demonstrated to suppress anaerobic intestinal flora in rats (6).

Fecal glucuronidase, in man, as determined by the microtechnique at pH 6.2, was essentially absent for the first 10 days after birth and increased only slightly during the first year. Only one adult had negative activity (Table II).

The inhibition of activity by antibiotics suggested that colonic glucuronidase was produced by intestinal flora. Experiments were conducted to determine the types of organisms responsible for the production of glucuronidase activity in rats. The intestines of newborn rats delivered by cesarean section were found to be sterile. The most striking difference between 5-day- and 25-day-old rats was the increased number of bacterioides and micrococci in the cecal content of 25-day-old rats (Table III).

Isolation of organisms producing glucuronidase. Inoculation of 50 colonies of various organisms, including many bacterioides and other representative organisms from the quantitative count plates into various liquid media, failed to result in glucuronidase production. Inoculation of one loop of cecal content, however, into heart infusion broth with or without dextrose resulted in positive glucuronidase activity at 24 hr when incubated anaerobically, but not aerobically. Glucuronidase activity was found with anaerobic media, such as deep meat broth and thioglycolate broth, whether or not it was incubated anaerobically. Because results were most consistent with deep meat broth incubated anaerobically in Torbal jars, these conditions were used for the remaining experiments. The failure to produce glucuronidase activity in transfers of gross cultures to subsequent deep meat broth tubes was corrected by the addition of substrate (0.5% menthol glucuronic acid ammonium) to the media. Without substrate, enzyme activity fell to negligible levels by 3 days, but was maintained for at least 24 days when substrate was present in the media. Further, with substrate in the media, enzyme production was maintained through 8 serial transfers. Lincomycin added to the deep meat broth prevented enzyme production; neomycin did not.

TABLE III. Quantitative Counts on Intestinal Flora in Young Rats.

	Mean[a] $\log_{10} \pm$ SD (no. rats positive/total rats)		
	Intestines 5 days	Cecum 25 days	Small intestine 25 days
Lactobacilli	8.2 ± 0.6 (6/6)	9.2 ± 0.3 (6/6)	8.6 ± 0.7 (6/6)
Bacteroides	4.3 ± 1.1 (6/6)	8.2 ± 0.2 (6/6)	3.5 ± 1.2 (3/6)
α-Hemolytic streptococci	6.0 ± 0.9 (6/6)	6.7 ± 0.6 (6/6)	4.9 ± 0.6 (6/6)
E. coli	6.4 ± 1.0 (4/6)	6.6 ± 0.7 (6/6)	4.9 ± 0.8 (6/6)
Micrococci	(0/6)	5.8 ± 0.6 (6/6)	4.7 ± 0.8 (5/6)
Enterococci	3.3 ± 1.2 (4/6)	4.5 ± 0.7 (6/6)	2.8 ± 0.5 (6/6)

[a] Mean $\log_{10}$ based on animals with positive culture. Numbers represent organisms in small and large intestine at 5 days and in 1 g of cecal content or 4 cm length of mid-small intestine at 25 days.

It was apparent that glucuronidase was being released into the media by anaerobes that required substrate for continued enzyme production. To improve the chances of isolating these organisms while they maintained enzyme production, neomycin and substrate were added to the plates used for isolation and the transfer procedures were performed as quickly as possible to minimize contact with air. In a series of 8 experiments 75 colonies from mixed broth cultures with positive glucuronidase activity were isolated. Ten of the colonies representing a variety of anaerobes (Table IV), resulted in glucuronidase production when inoculated back into deep meat broth.

Discussion. Although glucuronidase is present in mammalian gastrointestinal tissue at all levels (2, 10–12), the very high luminal glucuronidase activity in the colon suggests that fecal glucuronidase content is due mainly to bacterial production and release of the enzyme (1, 2). The glucuronidase in colon is of bacterial type in conventional rats and the much smaller amounts in germ-free rats is of mammalian type (6). Further, glucuronide conjugates are found in cecal content of germ-free but not conventional rats (13). The elimination of glucuronidase activity in colonic content of rats by antibiotic administration, as well as absence of activity in newborn infant feces, as demonstrated in this study, add strong support to this concept. Glucuronidase activity is present in newborn humans (14, 15), but the amounts were small and the pH optimum suggested a tissue origin for the enzyme.

Previous studies have not established the organisms responsible for high levels of glucuronidase activity in colonic contents nor the changes in activity with age. Williams *et al.* (3) found decreased glucuronidase activity in rat cecal content associated with neomycin treatment and decreased numbers of *Escherichia coli*. These workers, however, did not perform anaerobic cultures, nor prove that the enzyme was produced by *E. coli*. In 1949 Buehler, Katzman and Doisy (16) reported isolation of *E. coli* which produced glucuronidase from a patient with cystitis. Enzyme production was greatly enhanced by exposure to air, room temperature and days of incubation (17), conditions not present in the intestine. Smith and Mills (18) found strains of *E. coli* that produced enzyme more rapidly (2–3 days vs 10 days for the Buehler strain). Odell, Priddle and Burt (19) found glucuronidase production by several aerobes isolated from the vaginal tract. Some strains of β-hemolytic streptococci (6, 20, 21), α-hemolytic streptococci (22), coagulase positive and negative staphylococci (23) liberated or contained glucuronidase. Our findings suggest that the organisms previously found to produce glucuronidase are not responsible for fecal glucuronidase activity. Gross fecal cultures only liberated the enzyme under anaerobic conditions and we did not find isolates of the types of organisms described by others to be associated with glucuronidase production.

In this study, the bacteria associated with glucuronidase production were strict anaerobes, which were difficult to isolate. Initial experiments demonstrated that glucuronidase production was associated with high

TABLE IV. Glucuronidase Producing Organisms Isolated From Adult Rat Feces Demonstrated to Produce Glucuronidase.

Organism	No. of isolates	Morphology
Peptostreptococcus	1	Gram + chaining cocci
Anaerobic corynebacteria	3	Gram +/− pleomorphic rods
Propionibacterium	2	Gram + pleomorphic rods
Bacteroides	2	Gram − rods
Clostridium	1	Gram + sporeforming rods
Catenabacterium	1	Gram + filamentous rods

counts of bacterioides and anaerobic conditions. This suggested that intestinal glucuronidase production is associated primarily with bacterioides. Although bacterioides probably make up the majority of anaerobic organisms in the feces, it appears from the results presented here that there are other types of anterobes present, which can produce glucuronidase under certain conditions. Recently, similar types of anaerobes have been isolated from human feces (24, 25).

Further work is needed to determine the significance of intestinal luminal glucuronidase in the biological disposition of endogenous and exogenous compounds which are excreted in the bile as glucuronide conjugates. Alterations in the ability of the body to convert glucuronide conjugates to their potentially more biologically active aglycones have important physiologic and pharmacologic implications. Since the bacterial flora of the intestine is dependent on such factors as age, diet, disease and drug treatment the possibility should be kept in mind that such changes could alter the excretion and biological activity of many natural and foreign compounds.

Summary. Bacterial type β-glucuronidase activity is absent in human feces at birth and increases in human and rat feces with age. In rats there is an association of glucuronidase activity with increases in anaerobic flora. Antibiotics which reduce the anaerobic flora eliminate enzyme activity from rat cecal content *in vivo* and in cultures. Gross cultures of rat cecal content produce glucuronidase only under anaerobic conditions and enzyme production rapidly diminishes in the absence of substrate (glucuronide). Isolation of individual organisms that released glucuronidase into the media was difficult and required maintenance of strict anaerobic conditions and substrate in the media. A variety of anaerobes were found to produce the enzyme.

1. Marsh, C. A., Alexander, F., and Levvy, G. A., Nature (London) **170**, 163 (1952).

2. Conchie, J., and MacDonald, D. C., Nature (London) **184**, 1238 (1959).

3. Williams, J. R., Grantham, P. H., Marsh, H. H., Weisburger, J. H., and Weisburger, E. K., Biochem. Pharmacol. **19**, 173 (1970).

4. Smith, R. L., and Williams, R. T., *in* "Glucuronic Acid Free and Combined" (G. J. Dutton, ed.), p. 457. Academic Press, New York (1966).

5. Jayle, M. F., and Pasqualini, J. R., *in* "Glucuronic Acid Free and Combined" (G. J. Dutton, ed.), p. 507. Academic Press, New York (1966).

6. Robinson, J. J., Blinn, C. W., and Frank, P. F., J. Bacteriol. **64**, 719 (1952).

7. Kent, T. H., Summers, R. W., DenBesten, L., Swaner, J. C., and Hrouda, M., Proc. Soc. Exp. Biol. Med. **132**, 63 (1969).

8. Kent, T. H., Osborne, J. W., and Wende, C. M., Radiat. Res. **35**, 635 (1968).

9. Kent, T. H., Cardelli, R. M., and Stamler, F. W., Amer. J. Pathol. **54**, 237 (1969).

10. Heringová, A., Jirosová, V., and Koldovsky, O., Can. J. Biochem. **43**, 173 (1965).

11. Hänninen, O., Aitio, A., and Hartiala, K., Scand. J. Gastroenterol. **3**, 461 (1968).

12. Jodl, J., Koldovsky, O., Heringová, A., Jirsová, V., and Hofman, J., Biol. Neonatorum **12**, 88 (1968).

13. Grantham, P. H., Horton, R. E., Weisburger, E. K., and Weisburger, J. H., Biochem. Pharmacol. **19**, 163 (1970).

14. Brodersen, R., and Hermann, L. S., Lancet **1**, 1242 (1963).

15. Takimoto, M., and Matsuda, I., Biol. Neonatorum **18**, 66 (1971).

16. Buehler, H. J., Katzman, P. A., and Doisy, E. A., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **8**, 189 (1949).

17. Buehler, H. J., Katzman, P. A., and Doisy, E. A., Proc. Soc. Exp. Biol. Med. **76**, 672 (1951).

18. Smith, E. E. B., and Mills, G. T., Biochem. J. **47**, 49 (1950).

19. Odell, L. D., Priddle, H. D., and Burt, J. C., Amer. J. Clin. Pathol. **20**, 133 (1950).

20. Jacox, R. F., J. Bacteriol. **65**, 700 (1953).

21. Williams, R. E. O., J. Gen. Microbiol. **10**, 337 (1954).

22. Lorina, P. L., Lisanti, V. F., and Chauncey, H. H., Oral Surg. **7**, 998 (1954).

23. Barber, M., Brooksbank, B. W. L., and Kuper, S. W. A., J. Pathol. Bacteriol. **63**, 57 (1951).

24. Sugihara, P. T., Attebery, H. R., Sutter, V. L., and Finegold, S. M., Bacteriol. Proc. **1971**, 108 (1971).

25. Attebery, H. R., Nastro, L. J., and Finegold, S. M., Bacteriol. Proc. **1971**, 108 (1971).

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