

[Chem. Pharm. Bull.]  
[29(7)1975—1980(1981)]

## Studies on the Comparative Ability of $\beta$ -Glucuronidase Preparations to Hydrolyze Bile Acid Glucuronides<sup>1)</sup>

JUNICHI GOTO, AKIKO SATO, KAZUHIKO SUZAKI, and TOSHIO NAMBARA\*

*Pharmaceutical Institute, Tohoku University, Aobayama, Sendai, 980, Japan*

(Received December 11, 1980)

The abilities of four  $\beta$ -glucuronidase preparations, *Helix pomatia*, *Patella vulgata*, *Escherichia coli*, and beef liver, to hydrolyze bile acid glucuronides were determined as a function of pH and time. The substrates included 3-glucuronides of free, glycine- and taurine-conjugated lithocholate, chenodeoxycholate, deoxycholate, and cholate. In general, the optimal pH for  $\beta$ -glucuronidase-catalyzed hydrolysis of these substrates was more acidic in the case of nonbacterial  $\beta$ -glucuronidase preparations than in the case of the *E. coli* enzyme. It was also dependent upon the number of hydroxyl groups on the steroid nucleus, but not upon the side chain structure. No evidence was obtained of any overall superiority of any one enzyme preparation in hydrolyzing bile acid glucuronides.

**Keywords**—bile acid glucuronide; glycine-conjugate; taurine-conjugate; phenolphthalein monoglucuronide;  $\beta$ -glucuronidase preparation; enzymatic hydrolysis; pH-activity curve; optimal pH; high-performance liquid chromatography

In recent years, considerable attention has been focused on the metabolism of bile acids in patients with hepatobiliary diseases.<sup>2,3)</sup> The bile acid glucuronides have previously been shown to be present in the urine and plasma of patients with extra- and intrahepatic cholestasis and in trace amounts in the urine of healthy subjects.<sup>4,5)</sup> The methods commonly used for the quantitation of glucuronides of unconjugated and conjugated bile acids involve prior hydrolysis with  $\beta$ -glucuronidase and then with alkali, followed by chromatographic separation of the deconjugated bile acids.<sup>6)</sup> However, a suitable procedure for enzymatic hydrolysis of these glucuronides has not been definitely established. It would seem desirable, therefore, to ascertain the susceptibility of bile acid glucuronides to hydrolysis by  $\beta$ -glucuronidases in order to develop a reliable method for the analysis of these glucuronides in biological fluids.

For the measurement of  $\beta$ -glucuronidase activity, the amount of glucuronic acid liberated from glucuronides is usually determined.<sup>7,8)</sup> However, the individual hydrolysis rates of various substrates used for incubation cannot be estimated separately by this method. In the previous study of this series we synthesized several 3-glucuronides of unconjugated and conjugated bile acids by unequivocal routes for use as authentic specimens.<sup>9)</sup> The present paper deals with a study on the comparative ability of four kinds of  $\beta$ -glucuronidase preparations to hydrolyze bile acid glucuronides by means of high-performance liquid chromatography (HPLC).

### Materials and Methods

**Instruments**—The apparatus used was a JASCO Tri Rotar high-performance liquid chromatograph (Japan Spectroscopic Co., Tokyo) equipped with a Uvidec-100 II ultraviolet (UV) detector (Japan Spectroscopic Co.) monitoring the absorbance at 205 nm, a RCM-100 cartridge holder, and a comparison chamber (Waters Assoc., Milford, Mass.). A Radial-Pak A column (Waters Assoc.) was used under ambient conditions.

**Materials**—The bile acid glucuronides were synthesized in these laboratories by the methods reported previously.<sup>9)</sup> All the chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. Sephadex LH-20 was supplied by Pharmacia Fine Chemicals (Uppsala). Piperidino-hydroxypropyl Sephadex LH-20 (PHP-LH-20) and eluents used for ion-exchange chromatography were prepared as described in the previous paper.<sup>10)</sup> A Sep-pak C<sub>18</sub> cartridge from Waters Assoc. was washed successively with methanol (5 ml), acetonitrile (5 ml), and water (10 ml). Estriol and sodium phenolphthalein

monoglucuronide were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo) and Chugai Pharmaceutical Co. (Tokyo), respectively.

$\beta$ -Glucuronidase preparations derived from *Escherichia coli*, *Helix pomatia*, and *Patella vulgata* were supplied by Sigma Chemical Co. (St. Louis, Mo.) and that from beef liver by Tokyo Zokikagaku Co. (Tokyo). The enzymatic activities were measured at the optimal pH with sodium phenolphthalein monoglucuronide as a substrate.<sup>7)</sup> For the incubation experiments 0.1 M acetate buffer (pH 3.5–5.5) and 0.1 M phosphate buffer (pH 6.0–8.5) were used.

**Hydrolysis of Bile Acid Glucuronides with  $\beta$ -Glucuronidase Preparations**—Bile acid glucuronides (90–150  $\mu$ g) were incubated with an enzyme preparation (60–100 Fishman units) in buffer solution (1 ml) at 37°C for 4 h. The incubation mixture was immediately cooled to 0°C, diluted with ice-cooled 0.5 M phosphate buffer (pH 7.0, 3 ml), and then passed through a Sep-pak C<sub>18</sub> cartridge. The cartridge was washed with water (2 ml) and 1.5% ethanol (4 ml), and the bile acid fraction was eluted with 90% ethanol (4 ml). The eluate was then applied to a column packed with PHP-LH-20 acetate form (100 mg, 18 mm  $\times$  6 mm i.d.). After removal of neutral compounds with 90% ethanol (2 ml), unconjugated, glycine- and taurine-conjugated bile acids were stepwisely eluted with 0.1 M acetic acid in 90% ethanol (4 ml), 0.2 M formic acid in 90% ethanol (4 ml), and 0.3 M acetic acid-potassium acetate in 90% ethanol (pH 6.5, 4 ml), respectively. The taurine-conjugated fraction was evaporated to dryness *in vacuo* below 40°C, and the residue was redissolved in water (1 ml) and passed through a Sep-pak C<sub>18</sub> cartridge. The cartridge was washed with water (2 ml) to remove salts, then taurine-conjugated bile acids were eluted with 90% ethanol (4 ml). Next, 0.2 or 1.0  $\mu$ g of estriol was added to the unconjugated and conjugated fractions as an internal standard, respectively, and each solution was evaporated to dryness *in vacuo* below 40°C. The residue was redissolved in ethanol (100–200  $\mu$ l) and a 5–30  $\mu$ l aliquot was subjected to HPLC according to the procedure described in the previous paper.<sup>11)</sup>

**Recovery Test for Bile Acids added to the Incubation Mixture**—The test samples were prepared by dissolving 5 or 50  $\mu$ g each of chenodeoxycholate, glyco- and taurochenodeoxycholate, glycocholate, glycodeoxycholate, and glycolithocholate in ice-cooled 0.1 M acetate buffer (pH 5.0, 0.9 ml). The test solution was mixed with the *Helix pomatia* enzyme preparation (100 Fishman units) in water (0.1 ml). The mixture was immediately diluted with 0.5 M phosphate buffer (pH 7.0, 3 ml), cleaned up using a Sep-pak C<sub>18</sub> cartridge and PHP-LH-20, and then subjected to HPLC.

## Results and Discussion

An important initial step in the analysis of steroid glucuronides is the hydrolytic cleavage of the  $\beta$ -glucuronoside linkage. Several kinds of readily obtainable  $\beta$ -glucuronidase preparations are currently used for this purpose. However, precise information on the comparative ability of  $\beta$ -glucuronidase preparations to hydrolyze bile acid glucuronides has not hitherto been available. It seemed desirable, therefore, to ascertain the susceptibility of individual pure bile acid glucuronides to hydrolysis by  $\beta$ -glucuronidases, at the optimal pH in each case.

In this study four kinds of  $\beta$ -glucuronidase preparations (beef liver, *E. coli*, *Patella vulgata*, and *Helix pomatia*) were used, adjusted to the same potency at their respective optimal pH values with respect to hydrolysis of a given amount of the standard substrate, phenolphthalein monoglucuronide.

The initial effort was directed to the development of a method for the determination of deconjugated bile acids liberated when bile acid glucuronides are incubated with a  $\beta$ -glucuronidase preparation. A novel method for the simultaneous determination of bile acids in human bile was previously developed by means of HPLC using Radial-Pak A as a column and 0.3% ammonium phosphate (pH 7.7)/acetonitrile (19:8 and 15:8) as mobile phases.<sup>11)</sup> Therefore, an efficient procedure for clean-up of the test samples prior to HPLC was investigated. An enzyme preparation (100 Fishman units) was preincubated at 37°C for 4 h in acetate buffer. The incubation mixture was fortified with known amounts of representative free and conjugated bile acids, diluted with phosphate buffer (pH 7.0), and passed through a Sep-pak C<sub>18</sub> cartridge. The cartridge was washed with water and 1.5% ethanol to remove inorganic salts and other polar substances, then the desired bile acid fraction was eluted with 90% ethanol. The eluate was applied to a PHP-LH-20 column, and unconjugated, glycine- and taurine-conjugated bile acids were stepwisely eluted in the manner described in the previous paper.<sup>11)</sup> The unchanged bile acid glucuronides were recovered by subsequent elution with 1% ammonium carbonate in 70% ethanol. As listed in Table I, all bile acids were recovered almost quantitatively. On the basis of these data, a standard procedure

TABLE I. Recovery of Representative Free and Conjugated Bile Acids added to the Incubation Mixture

Bile acid	Added ( $\mu\text{g}$ )	Found	Recovery $\pm$ S.D. (%)
Chenodeoxycholate	5.0	5.0	100.1 $\pm$ 9.3
	50.1	48.5	96.8 $\pm$ 5.1
Glycocholate	5.0	4.6	91.4 $\pm$ 8.2
	50.6	46.4	91.7 $\pm$ 3.6
Glycochenodeoxycholate	5.0	4.6	92.4 $\pm$ 6.2
	50.2	48.3	96.2 $\pm$ 1.7
Glycodeoxycholate	5.0	4.8	95.4 $\pm$ 4.8
	50.2	47.8	95.2 $\pm$ 1.5
Glycolithocholate	5.0	4.6	91.3 $\pm$ 5.5
	50.1	49.2	98.2 $\pm$ 3.0
Taurochenodeoxycholate	5.0	4.5	90.7 $\pm$ 8.5
	50.5	49.3	97.7 $\pm$ 3.0

n=6.

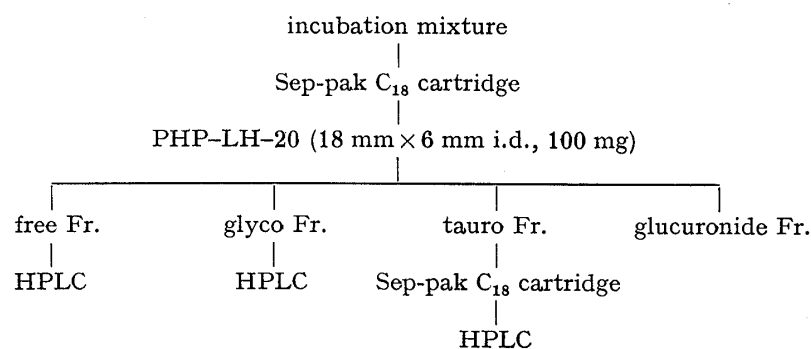


Chart 1. General Procedure for the Determination of Bile Acids

for the determination of deconjugated bile acids liberated in the incubation medium was established as shown in Chart 1.

It has been demonstrated that some enzyme preparations contain bile acids.<sup>6)</sup> Accordingly, bile acids contamination in the four enzyme preparations was checked prior to use. The stability of bile acid glucuronides at various pHs was also examined. The glucuronides were incubated without addition of the enzyme preparation, and deconjugated bile acids were determined by the standard method. The absence of nonenzymatic hydrolysis during the present assay procedure was thus confirmed.

The effect of pH on the enzymatic hydrolysis of bile acid glucuronides was then investigated. Glycochenodeoxycholate 3-glucuronide was incubated with the  $\beta$ -glucuronidase preparations at various pHs. As illustrated in Fig. 1, the optimal pHs were

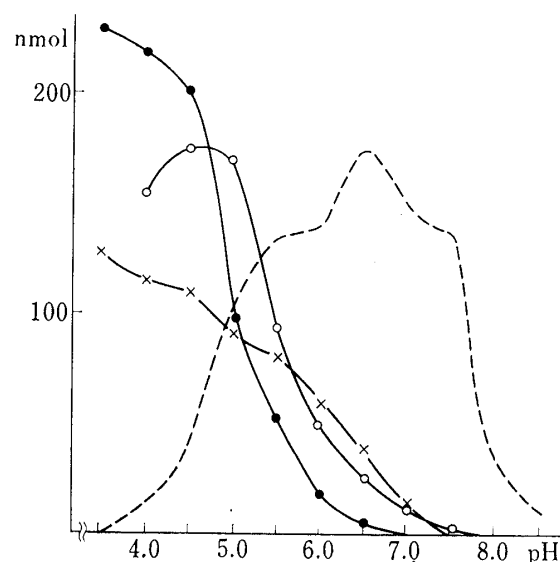


Fig. 1. Effect of pH on the Enzymatic Hydrolysis of Glycochenodeoxycholate 3-Glucuronide

—○—: *Helix pomatia*, —●—: *Patella vulgata*, ---: *E. coli*, —×—: beef liver.

4.5 and 6.5 for the enzyme preparations from *Helix pomatia* and *E. coli*, respectively. On the other hand, *Patella vulgata* and beef liver exhibited the highest activity at pH 3.5. Further investigation in the pH region below 3.5 was not carried out because artifacts may arise. These results, however, are substantially the same as those for steroid hormone glucuronides reported previously.<sup>7)</sup>

The influence of the side chain structure on the hydrolysis rate of bile acid glucuronides was then investigated. A mixture of chenodeoxycholate, glyco- and taurochenodeoxycholate 3-glucuronides was incubated under the conditions described above. The results obtained are shown in Fig. 2. The effect of the conjugate form on the hydrolysis rate was dependent upon the  $\beta$ -glucuronidase preparation. However, no significant difference in the pH-activity curve was recognized among the three glucuronides. As for glycochenodeoxycholate 3-glucuronide in the presence of chenodeoxycholate 3-glucuronide and its taurine-conjugate, *Patella vulgata* and beef liver exhibited an optimal pH, respectively. The hydrolytic properties were somewhat different from those observed when each substrate was incubated alone with the  $\beta$ -glucuronidase preparations (see Fig. 1). A plausible explanation for this phenomenon is not at present available and must await further investigation.

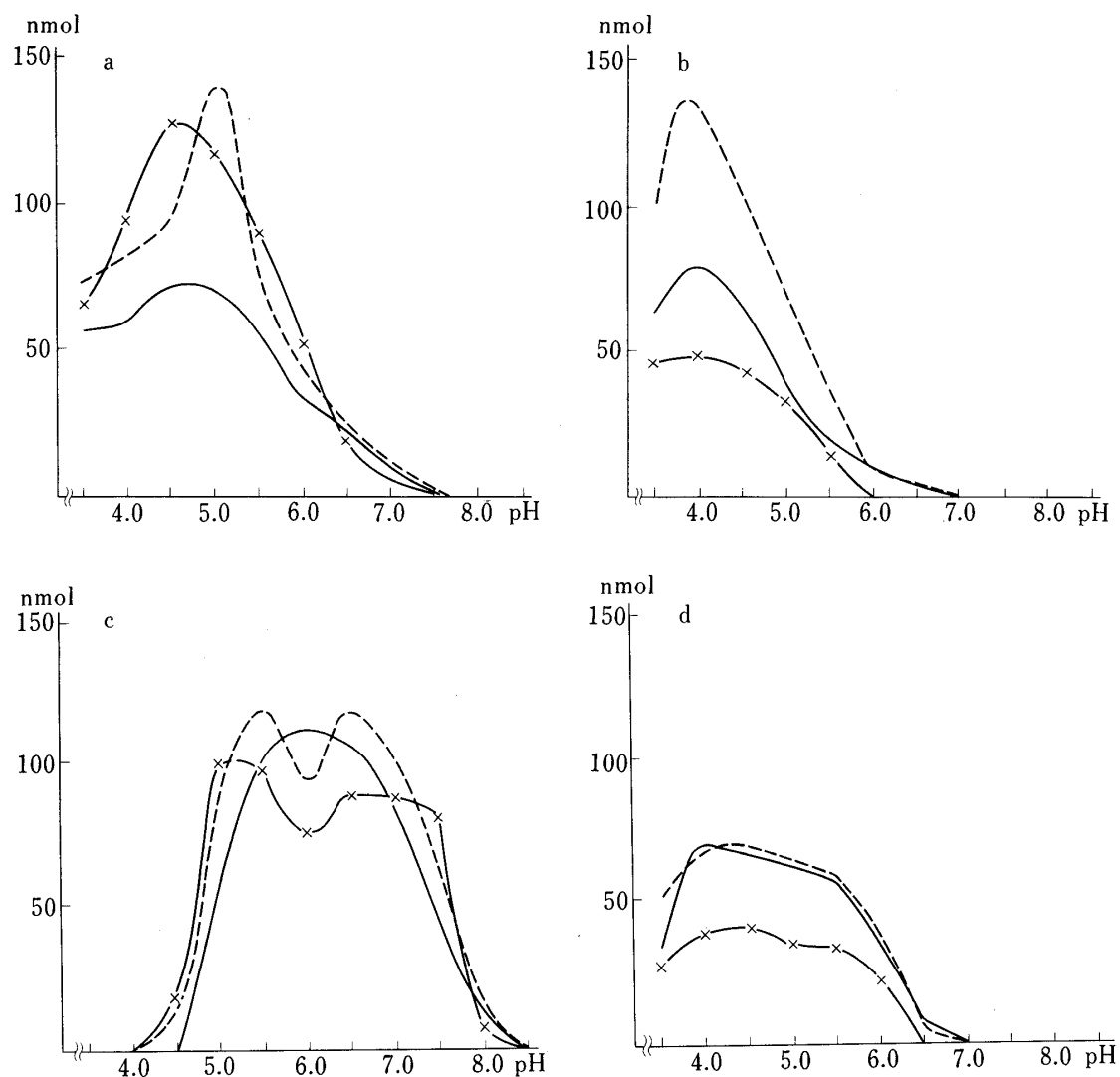


Fig. 2. Effect of pH on the Enzymatic Hydrolysis of Unconjugated, Glycine-, and Taurine-conjugated Chenodeoxycholate 3-Glucuronides

a) *Helix pomatia*, b) *Patella vulgata*, c) *E. coli*, d) beef liver.

—x—: chenodeoxycholate, —: glycochenodeoxycholate, ----: taurochenodeoxycholate.

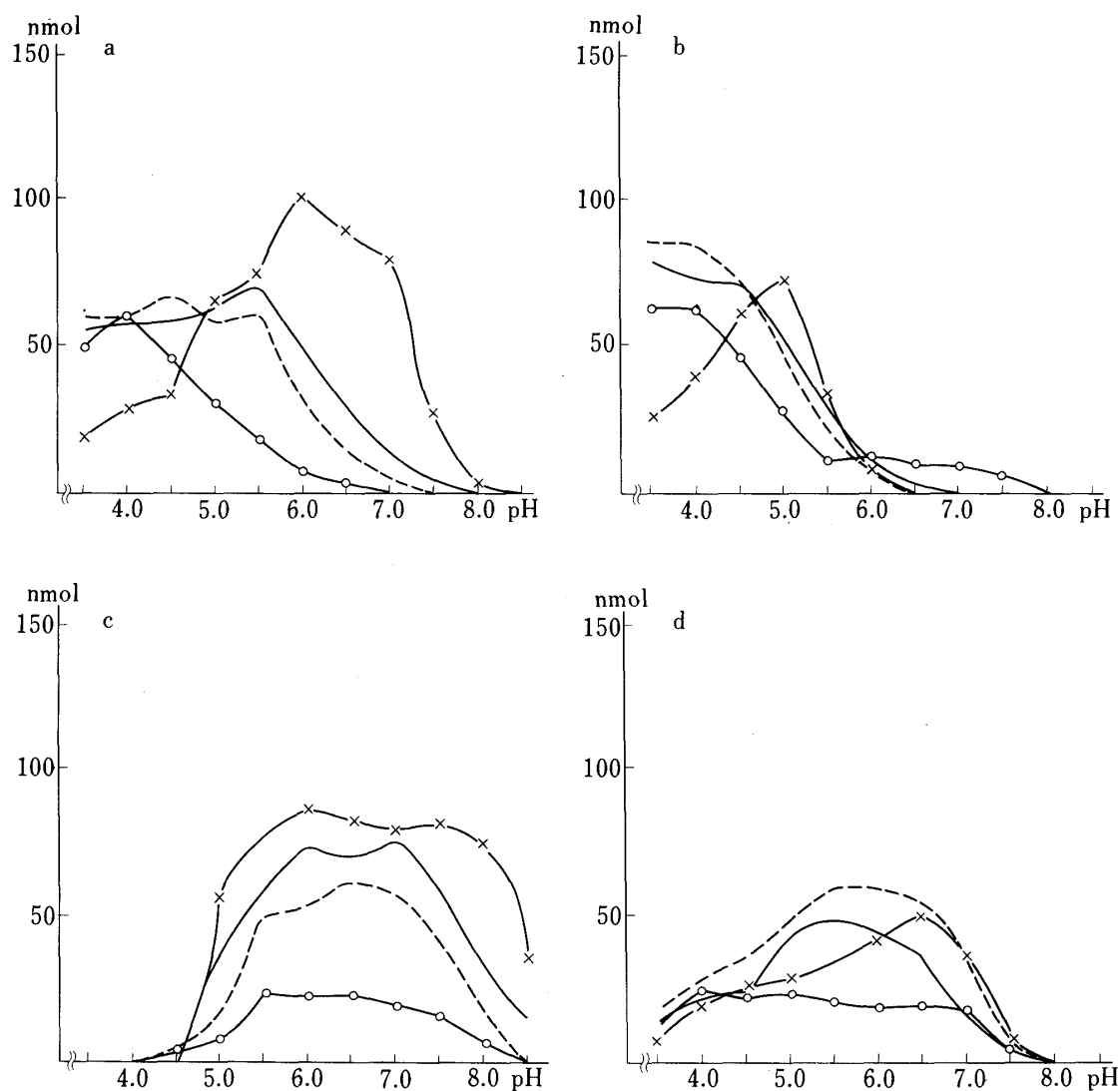


Fig. 3. Effect of pH on the Enzymatic Hydrolysis of Glycine-conjugated Bile Acid 3-Glucuronides

a) *Helix pomatia*, b) *Patella vulgata*, c) *E. coli*, d) beef liver.

—x—: glycolithocholate, —: glycochenodeoxycholate, ---: glycodeoxycholate, —○—: glycocholate.

Interesting results were obtained when a mixture of glycine-conjugated cholate, chenodeoxycholate, deoxycholate, and lithocholate 3-glucuronides was incubated with the  $\beta$ -glucuronidase preparations (see Fig. 3). In all cases, the hydrolysis rate of glycocholate 3-glucuronide was lower than those of other glucuronides. In addition, hydrolysis of lithocholate 3-glucuronide exhibited an optimum at a somewhat higher pH region while that of glycocholate 3-glucuronide showed the lowest optimal pH except in the case of the *E. coli* enzyme. The dihydroxy bile acid glucuronides exhibited similar pH-activity curves. It should be noted that the optimal pH for enzymatic hydrolysis of bile acid glucuronides is dependent upon the number of hydroxyl group on the steroid nucleus but not upon the side chain structure.

Recently, it was demonstrated that biliary  $\beta$ -glucuronidase activity, which was measured by using phenolphthalein monoglucuronide as a substrate, was significantly inhibited by the presence of conjugated chenodeoxycholate and deoxycholate; conjugated cholate showed the least inhibitory effect among bile acids tested.<sup>12)</sup> The effect of lithocholate was not investigated by these workers. In the present study the pH-activity curve with glycolithocholate 3-glucuronide was somewhat different from those with other substrates, and enzymatic hydrolysis of the former glucuronide proceeded more readily in the higher pH region. When

glycine-conjugated cholate, chenodeoxycholate, and lithocholate 3-glucuronides were incubated independently with  $\beta$ -glucuronidase derived from *Helix pomatia*, the results obtained were almost identical with those in Fig. 3a. Therefore, it seems unlikely that deconjugated tri- and dihydroxy bile acids inhibit the enzymatic hydrolysis of glycolithocholate 3-glucuronide in the lower pH region. On the other hand, it is possible that the affinity of mono- and dihydroxy bile acid glucuronides for the enzyme is much higher than that of cholate 3-glucuronide and hence, the hydrolysis rates of the former two are higher than that of the latter. On the basis of these data, suitable pHs for enzymatic hydrolysis of bile acid glucuronides in biological fluids are 4.5 for the *Helix pomatia*, *Patella vulgata*, and beef liver enzymes, and 6.5 for the *E. coli* enzyme.

Finally, a mixture of unconjugated, glycine- and taurine-conjugated deoxycholate 3-glucuronides was incubated with the four enzyme preparations at the selected pHs (6.5 for the *E. coli* enzyme and 4.5 for others) and the time courses of enzymatic hydrolysis were determined. On incubation for 6 h at 37 °C, these substrates underwent hydrolysis almost quantitatively with the *Helix pomatia* and *Patella vulgata* enzymes, but remained partially intact with the *E. coli* and beef liver enzymes.

The basis of the comparative data presented here is the quantitation of deconjugated bile acids by means of HPLC. In this procedure, free, glycine- and taurine-conjugated bile acids formed from various substrates can be simultaneously determined without interference by unchanged glucuronides. This convenient and reliable method is also applicable to the assay of bile acid uridinediphosphoglucuronyl transferase activity. In conclusion, there is no evidence for a distinct overall superiority of any one enzyme preparation for the hydrolysis of the bile acid glucuronides studied here. However, the data do permit a choice in the case of several glucuronides. A judicious selection of optimal conditions should result in a good performance with any one of the four enzyme preparations.

**Acknowledgement** This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, which is gratefully acknowledged.

#### References and Notes

- 1) Part CLXVII of "Studies on Steroids" by T. Nambara; Part CLXVI: H. Hosoda, N. Kawamura, and T. Nambara, *Chem. Pharm. Bull.*, **29**, 1969 (1981). The following trivial names are used in this paper: lithocholate = 3 $\alpha$ -hydroxy-5 $\beta$ -cholan-24-oic acid; chenodeoxycholate = 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholan-24-oic acid; deoxycholate = 3 $\alpha$ ,12 $\alpha$ -dihydroxy-5 $\beta$ -cholan-24-oic acid; cholate = 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholan-24-oic acid.
- 2) R.H. Palmer and M.G. Bolt, *J. Lipid Res.*, **12**, 671 (1971).
- 3) I. Makino, H. Hashimoto, K. Shinozaki, K. Yoshino, and S. Nakagawa, *Gastroenterology*, **68**, 545 (1975).
- 4) P. Back, *Hoppe-Seyler's Z. Physiol. Chem.*, **357**, 213 (1976).
- 5) W. Fröhling and A. Stiehl, *Eur. J. Clin. Invest.*, **6**, 67 (1976).
- 6) B. Alme, A. Nordén, and J. Sjövall, *Clin. Chim. Acta*, **86**, 251 (1978).
- 7) M. Wakabayashi and W.H. Fishman, *J. Biol. Chem.*, **236**, 996 (1961).
- 8) M. Wakabayashi, H.H. Wotiz, and W.H. Fishman, *Biochim. Biophys. Acta*, **48**, 198 (1961).
- 9) J. Goto, K. Suzuki, and T. Nambara, *Chem. Pharm. Bull.*, **28**, 1258 (1980).
- 10) J. Goto, M. Hasegawa, H. Kato, and T. Nambara, *Clin. Chim. Acta*, **87**, 141 (1978).
- 11) J. Goto, H. Kato, Y. Saruta, and T. Nambara, *J. Liquid Chromatogr.*, **3**, 991 (1980).
- 12) K.-J. Ho, L.-H.C. Ho, and O.R. Kruger, *J. Lab. Clin. Med.*, **93**, 916 (1979).