Inhibitory Effect of Bile Constituents upon Bacterial \(\beta\)-Glucuronidase Activity

I. Effect of Normal Bile

By

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Maki et al.\(^1\) found in 1962 that the bile of patients with calcium bilirubinate stones, which are common gallstones in the Japanese, generally had higher \(\beta\)-glucuronidase activity than the control bile and the optimal pH for the activity was around 7.0, similarly to that for bacterial preparations of \(\beta\)-glucuronidase. These observations have led to the hypothesis on the mechanism of gallstone formation that \(\beta\)-glucuronidase derived from bacteria, *Escherichia coli* in most instances, catalyzes hydrolysis of bilirubin glucuronide into free bilirubin and glucuronic acid, and the former combines with calcium to form an unsoluble salt which is the main constituent of calcium bilirubinate stones.

However, it was also revealed by the same authors that the infected biles was not always associated with an elevated \(\beta\)-glucuronidase activity. This fact may be interpreted to suggest the presence of bile constituents which inhibit the enzyme activity. The present study has been undertaken to evaluate the above assumption on the experimental basis. The present communication, as the first report of the series, deals with the inhibitory effect of the normal bile upon the bacterial \(\beta\)-glucuronidase activity.

MATERIALS AND METHODS

The test materials were obtained by puncture of the gallbladder or the common bile duct on laparotomy of surgical cases excluding those with liver or biliary tract diseases. The puncture was performed under aseptic precautions.

*Estimation of the inhibitory effect.* The assay system for the inhibitory effect of the bile on the bacterial \(\beta\)-glucuronidase activity was as outlined in Table I. One-tenth ml. each of a 20 mg/ml aqueous solution of a bacterial \(\beta\)-glucuronidase preparation (Sigma Chemical Co.) was taken into test tubes A and B, and 0.1 ml.
TABLE I. Incubation System for Assaying Inhibitory Effect on Bacterial $\beta$-Glucuronidase Activity*

<table>
<thead>
<tr>
<th>Tube</th>
<th>Enzyme</th>
<th>Sample</th>
<th>Substrate</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>B</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>C</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* ml. of ingredients

each of a 1:10 test bile into tubes B and C. To each tube was added 0.1 ml. of a 0.01 M phenolphthalein glucuronide solution and then the final volume of each mixture was made 1.0 ml., with 1/5 M phosphate buffer at pH 6.8. The mixtures were then kept at 38°C for an hour; the amount of phenolphthalein liberated during the incubation period was estimated for each reaction mixture, according to Sato's modification, as a measure of the $\beta$-glucuronidase activity. The enzyme activities of the mixtures A and B were compared with each other to evaluate the inhibitory effect of the bile sample, while the mixture C served as the control. The duration of the assay reaction was fixed at one hour as mentioned above. This was based on the result of a preliminary experiment which is shown later in the appropriate section.

The test was performed with 10 bladder bile and five choledochus bile samples. In three cases in which both bladder bile and choledochus bile samples were obtained, the total bilirubin contents of the samples were measured by the Evelyn-Malloy method to estimate the concentrations of the biles; the relation between the inhibitory effect and the concentration of bile was investigated.

Dilution experiment. The effect of dilution on the inhibitory effect of the bile upon bacterial $\beta$-glucuronidase was studied using three sets of bladder and choledochus bile samples. In this experiment the bladder bile samples were diluted 1:10 to 1:80 and the choledochus bile samples 1:1 (undiluted) to 1:20; subsequent procedures were the same as described above.

RESULTS

In a preliminary experiment in which the reaction mixtures A and B of a sample were incubated for varying periods, the quantity of phenolphthalein liberated by the incubation was found to be approximately proportional to the duration of incubation in a range up to 3 hours (Fig. 1), justifying the assay time of one hour.

In all the samples studied, the mixtures A and B revealed definite $\beta$-glucuronidase activities, while the control mixture C, containing the normal bile but no
Inhibitory Effect of Bile Constituents upon Bacterial $\beta$-Glucuronidase Activity

Fig. 1. Reaction curves for hydrolysis of phenolphthalein glucuronide by bacterial $\beta$-glucuronidase with (B) or without (A) a 1:10 bile sample. The reaction systems were as described in Table I. The ordinate indicates the amount of phenolphthalein produced by the hydrolysis.

bacterial $\beta$-glucuronidase, did not show any $\beta$-glucuronidase activity at all. Moreover, $\beta$-glucuronidase activity of the mixture B, with a test bile sample, was always lower than that of the corresponding mixture A. This apparently indicates an inhibitory effect of the bile upon the bacterial $\beta$-glucuronidase activity. The grade of inhibition has been expressed with the inhibition ratio as defined by the following formula:

$$\text{Inhibition ratio} = \frac{\beta\text{-glucuronidase activity of "A"} - \beta\text{-glucuronidase activity of "B"}}{\beta\text{-glucuronidase activity of "A"}} \times 100$$

Distribution of the inhibition ratio. All the bile samples examined, both bladder samples and choledochus samples, exhibited perceptible inhibitory effects at the dilution of 1:10. Table II shows the distribution of the inhibition ratio in 10 samples of the 1:10 bladder bile. The ratio showed a comparatively narrow range, from 29.9% to 41.2%, and the average was 37.4%. The individual variation of the inhibition ratio in five samples of the 1:10 choledochus bile was somewhat larger than in the case of the bladder bile; the ratio ranged between 5.2% and 34.0%, the average being 18.1% (Table III).
### Table II. Inhibitory Effect of 1:10 Bladder Bile on Bacterial β-Glucuronidase Activity

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Inhibition ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stomach cancer</td>
<td>39.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>40.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>41.2</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>39.6</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>39.5</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>40.3</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>39.0</td>
</tr>
<tr>
<td>8</td>
<td>Peptic ulcer</td>
<td>32.4</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>38.0</td>
</tr>
<tr>
<td>10</td>
<td>Gastric polyp</td>
<td>34.4</td>
</tr>
</tbody>
</table>

Average: 37.4%

### Table III. Inhibitory Effect of 1:10 Choledochus Bile on Bacterial β-Glucuronidase Activity

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Inhibition ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stomach cancer</td>
<td>22.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>34.0</td>
</tr>
<tr>
<td>4</td>
<td>Peptic ulcer</td>
<td>16.7</td>
</tr>
<tr>
<td>5</td>
<td>Gastric polyp</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Average: 18.1%

Fig. 2. The relation between the inhibition ratio and the total bilirubin content in bladder bile samples (dotted lines) and choledochus bile samples (solid lines). Dilution 1:10. The marks identify patients.
In the three cases subjected to estimation of bilirubin content of the bile, the inhibition ratio of the bladder sample as well as that of the choledochus sample was found to be higher in cases with higher bilirubin content (Fig. 2), suggesting a parallelism between the concentration of the bile and the inhibitory effect upon bacterial β-glucuronidase.

**Effect of dilution upon inhibition ratio.** Although dilution of the sample generally resulted in a decrease in the inhibitory effect on the β-glucuronidase activity, the effect of dilution was much more remarkable in the choledochus bile than in the bladder bile; in the former a sharp fall of the inhibition ratio took place at comparatively low dilutions, whereas the inhibition ratio of the latter was considerably lowered only at high dilutions (Fig. 3). It was also observed that the inhibition ratio of the choledochus bile was always lower than that of the bladder bile of the same individual, when compared at the same dilution.

![Figure 3](image)

**Fig. 3.** Effect of dilution on the inhibitory effect of bladder bile samples (dotted lines) and choledochus bile samples (solid lines). The marks identify patients.

**DISCUSSION**

According to Maki et al., the bile of patients of cholelithiasis, especially those with calcium bilirubinate stones, exhibits a considerable β-glucuronidase activity, while the activity is insignificant or completely absent in the bile of normal
subjects or patients with various conditions other than cholelithiasis. Although
this and other facts suggest that β-glucuronidase in the bile is of bacterial origin,
it has been also known that the bile without gallstones is sometimes free of
β-glucuronidase activity even when it is considerably infected with *E. coli*. This
means that the strain and quantity of the bacteria are not the only factors deter-
mining the β-glucuronidase activity of the bile; the inhibitory effect of bile con-
stituents upon the enzyme should be taken into consideration as a possible factor.

Since Oshima\(^3\) reported in 1936 on a strong inhibitory effect of sodium salts
of oxidicarbonic acids upon β-glucuronidase of the bovine spleen, a number
of substances have been known to inhibit the β-glucuronidase activity, including
some saccharic acids,\(^4,5\) glucuronic acid,\(^6\) heavy metals,\(^7\) ascorbic acid\(^8\) and
ammonium chloride.\(^9\) It is probable that some of these substances appear in
the bile under normal or pathologic conditions to influence the β-glucuronidase
activity.

In the present study which was undertaken to evaluate the effect of normal
human bile on the bacterial β-glucuronidase activity, a system consisting of a
bacterial β-glucuronidase preparation and phenolphthalein glucuronide (the
substrate) was used as the assay system. It was confirmed by a preliminary
experiment that the yield of phenolphthalein in this system was approximately
proportional to the duration of incubation for a wide range of the latter, in both
the cases with and without a test bile sample added. The amount of phenolph-
thalein after one hour's incubation was thus taken as a measure of β-glucuronidase
activity.

The results with 15 samples (10 bladder bile and five choledochus bile
specimens) showed that the β-glucuronidase activity of the system with the bile
sample was invariably lower than that without the sample, revealing an
inhibitory effect of the bile on the bacterial β-glucuronidase activity. It was also
shown that the choledochus bile was less remarkable in the inhibition than the
bladder bile of the same case, when compared at the same dilutions. This is
possibly due to the fact that the choledochus bile is less concentrated and, conse-
quently, smaller in quantity of inhibitory substances than the bladder bile.

From these results it may be reasonably concluded that some of bile con-
stituents exert an important effect on the level of β-glucuronidase activity in the
infected bile. Furthermore, that the inhibitory effect was detected not only in
the bladder bile but also in the choledochus bile is interpreted to suggest the
hepatic origin of such inhibitory substances.

**SUMMARY**

Using an assay system consisting of a bacterial β-glucuronidase preparation
and phenolphthalein glucuronide, it was experimentally demonstrated that the
bile, both the bladder bile and the choledochus bile, of patients without liver or
biliary tract diseases had an inhibitory effect on the activity of bacterial β-glucuronidase.

References

2) Sato, T., ibid., 1962, 77, 23.
6) Spencer, B. & Williams, R.T., ibid., 1951, 48, 537.