The Effect of Biliary Bile Acid Concentration and Composition on the Calcium Level in Human Gallbladder Bile

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We analyzed total and ionized calcium concentrations in gallbladder bile of 34 humans in four groups: 8 patients with no gallstone, 11 gallstone patients treated with no gallstone dissolution agents, 8 gallstone patients treated with chenodeoxycholic acid (CDCA) and 7 gallstone patients treated with ursodeoxycholic acid (UDCA). We found that total calcium level ranged from 1.40 to 8.01 mmol/liter, closely related to total bile acid concentration (r=0.759). However, ionized calcium level was maintained in a narrow range of 0.25 to 1.23 mmol/liter and had no relation to total bile acid concentration. UDCA-rich bile showed relatively high level of ionized calcium. We performed ultrafiltration of bile with cut-off molecular weight 1,000 to investigate the interaction between biliary calcium and bile acid aggregates. The proportion of ultrafiltrated bile acid level to that in original bile in the UDCA group was statistically higher than the other groups. Relatively large percentage of smaller bile acid aggregates in UDCA-rich bile may impair its calcium solubility.

It is known that insoluble calcium precipitation in gallbladder bile can act as a nidus for gallstone formation (Been et al. 1979; Wosiewitz 1980) and can form calcified shell of cholesterol gallstones (Bateson et al. 1981; Schoenfield et al. 1981). Such precipitation or calcification of gallstones is clinically important, as it affects successful cholesterol gallstone dissolution. Calcium precipitation occurs theoretically when the product of ionized calcium and some anions (carbonate, phosphate, bilirubinate, palmitate etc.) exceed the solubility product constant of those salts (Moore et al. 1982; Moore 1984; Rege and Moore 1986).
Previous studies demonstrated that calcium precipitation occurred even during treatment with UDCA (Bateson et al. 1981; Raedsch et al. 1981; Ros et al. 1986), however, it has not been observed often during CDCA treatment (Schoenfield et al. 1981).

Bile acids have been proposed as a major calcium binder, and act as buffers which, by lowering ionized calcium concentrations, minimize the risk of calcium precipitation in bile. Recently, several studies were reported which suggested chemical structure of bile acid influences calcium binding capacity and solubility (Sutor et al. 1980; Williamson and Percy-Robb 1980; Rajagopalan and Lindenbaum 1982; Cummings and Hofmann 1984).

We measured biliary calcium by an ion-selective electrode equipped with a newly developed sample container, which made it possible to perform analysis of viscous gallbladder bile samples with small volume. Considering the interaction between biliary calcium and bile acid aggregates, we used ultrafiltration to analyze the distribution of the size or stability of bile acid aggregates in human gallbladder bile. Then, the effect of size distribution of bile acid aggregates on the biliary calcium solubility were investigated.

**MATERIALS AND METHODS**

*Collection of gallbladder bile*

Thirty four gallbladder bile samples were obtained directly by aspiration from gallbladder during operation. All patients gave their informed consent to participate in the study. Eight non-gallstone patients (control group) consisted of 3 gastric cancers, 4 colonic cancers and 1 acute abdomen (peritonitis due to appendicitis) and none of them had hepatobiliary disorders.

Twenty six gallstone patients (GS group) divided into three sub-groups: 11 patients treated with no gallstone dissolution agent (non-treated group), 8 patients with CDCA administration (CDCA-treated group) and 7 patients with UDCA administration (UDCA-treated group). The diagnosis of gallstone before the operation was made on the basis of conventional radiographic and ultrasonographic examinations of the biliary tract. Patients who showed positive cholangiogram by oral or intravenous cholangiography were administered randomly either CDCA or UDCA. Patients of the CDCA-treated group were given CDCA 400 mg/day, and patients of the UDCA-treated group were given 450-600 mg/day for seven days before the operation, and on the 8th day, gallbladder bile samples were obtained directly during the operation.

Bile samples were aspirated from the gallbladder anaerobically and aseptically with 18 gauge needle and put into an anaerobic tube immediately (Nipro Neo Tube, Nipro Ltd. Tokyo). The gallstones extirpated operatively were classified by macroscopic morphology into 8 pure cholesterol stones (1 in non-treated, 3 in CDCA-treated and 4 in UDCA-treated group) and 18 mixed stones (10 in non-treated, 5 in CDCA-treated and 3 in UDCA-treated group). No further investigation of the chemical composition of gallstones was performed in this study.

The pH was measured using an electrode pH meter (Horiba Ltd., Kyoto) immediately after the collection of the gallbladder bile sample.

*Measurement of total and ionized calcium concentrations in gallbladder bile*

An aliquot of original gallbladder bile was diluted with 20 volumes of 1% SrCl₂
solution, and total calcium concentration of duplicate samples were determined by an atomic absorption spectrophotometer (A 855 type, Japan Jarell Ash, Kyoto).

Ionized calcium concentration in gallbladder bile was measured by a calcium selective electrode (Type RW 9415; Philips Ltd. the Netherlands) equipped with a newly developed sample container (Fig. 1). This container was prepared by Towa Denpa Ltd. Tokyo, Japan. It consisted of a glass cylinder (1.2 cm in inside diameter) with a stirrer at the bottom and a ceramic junction on the side wall. One ml of gallbladder bile was put into this container, which was then placed in a saturated KCl solution inside a beaker. A calcium selective ion electrode was inserted into the bile sample and a reference electrode into the KCl saturated solution. Calcium concentration was determined from a digital readout on the display panel within 3 min after an equilibrium was reached. Triplicate measurements of ionized calcium deviated from the mean by less than 1.0%. The accuracy of this equipment was confirmed by measuring standard ionized calcium solution (NOVA biomedical, Waltham, MA, USA) with a known concentration ranged from 0.5 to 2.5 mmol/liter.

Ultrafiltration of human gallbladder bile

Ultrafiltration of gallbladder bile was performed using a disposable ultrafiltration unit of nominal cut-off molecular weight (MW) 1,000 (Japan Millipore Ltd., Tokyo). After 0.5 ml of gallbladder bile was put into the unit, ultrafiltration was started anaerobically with injection of 3 ml of nitrogen gas into the unit by a syringe. This ultrafiltration was continued until 0.2 ml of filtrate was obtained, usually taking 3 hr.

The cut-off MW of this unit was evaluated by using 0.08% of α, β, γ, δ-tetraphenylporphinetrisulfonic acid disulfonic acid tetrahydrate (MW 1123; TPPS, Dojin Chemical Ltd., Kumamoto). The ultrafiltration rate of this compound was calculated from the difference of absorbance at selected wave lengths before and after ultrafiltration: the rate was 3.5% for TPPS. To assess the reproducibility of the ultrafiltration procedure, 7 gallbladder bile samples were filtrated in triplicate, and ultrafiltrated bile acid concentrations were determined. The coefficient of variation was 9.0 ± 2.1%. The possibility of specific absorption of bile acids by the filter membrane was studied using the mixed solution of authentic CDCA, UDCA and cholic acid (CA) in the concentration of 0.5 mM, which was

Fig. 1. A schema of a new type of sample container. A new type of sample container was used for the analysis of a small amount of bile. This container is a glass cylinder with a stirrer at the bottom and a ceramic junction on the side wall. The container containing 1.0 ml of gallbladder bile was then put in a saturated KCl solution inside a beaker. A, calcium ion-selective electrode (type PW 9415, Philips Ltd.); B, reference electrode; C, saturated KCl solution; D, gallbladder bile sample; E, a stirrer; F, a ceramic junction.
under the critical micellarization concentration. There was no significant difference in each bile acid concentration before and after the filtration. Then, specific absorption of bile acid by the filter membrane was excluded.

**Analysis of biliary lipids**

An aliquot of original and ultrafiltrated gallbladder bile sample was diluted with 20 volumes of chloroform-methanol (2:1) solution, and this mixture was agitated in an ultrasonic bath for 15 min. The sample was centrifuged and the extract was analyzed for biliary lipids. Total bile acid (TBA) concentration was determined enzymatically using 3α-hydroxysteroid dehydrogenase (Nyegaard Co., Oslo, Norway) (Iwata and Yamasaki 1964). Bile acid composition was determined by gas-liquid chromatography after enzymatic hydrolysis with chohylglycine hydrolase (Sigma Chemical Co., St. Louis, MO, USA) (Nair and Garcia 1969). Phospholipid was determined by the method of Hoesflmayr and Fried (1966), and cholesterol by the enzymatic method of Allain et al. (1974). The individual composition of biliary lipid was expressed in mole percent.

**Data analysis**

The results were expressed as mean ± standard error (S.E.). The relationship between biliary lipid and calcium fraction (total and ionized calcium) was calculated by linear regression analysis. To compare mean values among the four groups, analysis of variance (ANOVA) followed by the least significant difference (LSD) was used. A p value < 0.05 was considered statistically significant.

**RESULTS**

**Biliary lipid composition**

Total biliary lipid (TBL) concentrations of the 34 bile samples ranged from 1.8 to 12.5 g/100 ml. The average values of TBL and TBA in the control group were 9.7 g/100 ml and 149.8 mmol/liter, respectively, and these values were statistically higher than those in each of GS group (4.6 g/100 ml, 63.4 mmol/liter in non-treated, 5.3 g/100 ml, 83.7 mmol/liter in CDCA-treated and 5.4 g/100 ml, 81.5 mmol/liter in UDCA-treated group, respectively) (p < 0.01). TBL or TBA levels were not significantly different among non-, CDCA-, and UDCA-treated group (Table 1). There was no difference in bile acid composition between control group and non-treated group, as well. In CDCA-treated group, CDCA content was 62.5 ± 7.8 mmol/liter which accounted for 75.8 ± 3.8% of TBA concentration, 83.7 ± 11.0 mmol/liter (CDCA-rich bile); and in UDCA-treated group, UDCA was 44.4 ± 6.0 mmol/liter which accounted for 54.3 ± 2.7% of TBA concentration, 81.8 ± 7.1 mmol/liter (UDCA-rich bile) (Table 2).

**Total and ionized calcium concentrations in gallbladder bile**

Total and ionized calcium concentrations in gallbladder bile from each group are summarized in Table 3. Total calcium concentration ranged from 1.40 to 8.01 mmol/liter (3.55 ± 0.33), which was closely related with TBA (y = 0.034x + 0.450, r = 0.759, p < 0.01) (Fig. 2). Total calcium level was also well correlated with the level of large bile acid aggregates which were calculated by subtracting TBA level in ultrafiltrate from that in original bile (y' = 0.041x + 0.449, r = 0.740, p < 0.01).
On the other hand, ionized calcium concentration ranged from 0.25 to 1.23 mmol/liter (0.69±0.04) and was maintained in a narrow range (y" = 0.002x + 0.514, r = 0.159) (Fig. 2).

Total calcium level in the control group (5.81±0.63 mmol/liter) was statistically higher than that in each of the GS group (2.17±0.27 mmol/liter in non-treated, 3.37±0.65 mmol/liter in CDCA-treated, 3.34±0.28 mmol/liter in UDCA-treated group, respectively) (p <0.01). Total calcium level among the GS group showed no difference. The mean value of ionized calcium concentration in

### Table 1. Comparison of original and ultrafiltrated biliary lipid composition

<table>
<thead>
<tr>
<th>Group</th>
<th>Bile</th>
<th>TBL (g/100 ml)</th>
<th>TBA (mmol/liter)</th>
<th>PL (mmol/liter)</th>
<th>CH (mmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>O</td>
<td>9.7±0.9</td>
<td>149.8±14.9</td>
<td>35.1±2.1</td>
<td>18.9±2.6</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1.2±0.1</td>
<td>27.0±4.1</td>
<td>1.1±0.2</td>
<td>1.2±0.6</td>
</tr>
<tr>
<td>Gallstone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-treated</td>
<td>O</td>
<td>4.6±0.7</td>
<td>63.4±11.5</td>
<td>20.7±2.9</td>
<td>6.3±1.1</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>0.5±0.1</td>
<td>12.2±3.1</td>
<td>0.5±0.1</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>CDCA-treated</td>
<td>O</td>
<td>5.3±0.7</td>
<td>83.7±11.0</td>
<td>19.5±2.4</td>
<td>7.7±1.6</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>0.5±0.1</td>
<td>10.7±2.0</td>
<td>0.7±0.3</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>UDCA-treated</td>
<td>O</td>
<td>5.4±0.7</td>
<td>81.5±7.1</td>
<td>22.2±2.5</td>
<td>6.1±0.9</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>0.8±0.1</td>
<td>18.9±3.1</td>
<td>0.2±0.0</td>
<td>trace</td>
</tr>
</tbody>
</table>

*All values are mean±s.e.

TBL, total biliary lipid; TBA, total bile acid; PL, phospholipid; CH, cholesterol; O, original bile; U, ultrafiltrated bile; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid.

### Table 2. Total and ultrafiltrated bile acid concentrations in original and ultrafiltrated bile

<table>
<thead>
<tr>
<th>Group</th>
<th>TBA in original (mmol/liter)</th>
<th>TBA in ultrafiltrate (mmol/liter)</th>
<th>Filtration rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>149.8±14.9</td>
<td>27.0±4.1</td>
<td>18.1±2.3</td>
</tr>
<tr>
<td>Gallstone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-treated (n=11)</td>
<td>63.4±11.5</td>
<td>12.2±3.2</td>
<td>19.1±1.9</td>
</tr>
<tr>
<td>CDCA-treated (n=8)</td>
<td>83.7±11.0</td>
<td>10.7±2.2</td>
<td>12.7±1.5</td>
</tr>
<tr>
<td>CDCA</td>
<td>62.5±7.8</td>
<td>5.0±1.0</td>
<td>8.4±1.7</td>
</tr>
<tr>
<td>UDCA-treated (n=7)</td>
<td>81.5±7.1</td>
<td>18.9±3.3</td>
<td>22.3±1.8</td>
</tr>
<tr>
<td>UDCA</td>
<td>44.4±6.0</td>
<td>13.6±2.2</td>
<td>30.0±2.6</td>
</tr>
</tbody>
</table>

*All values are mean±s.e.

*p <0.05 and **p <0.01. TBA, total bile acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid.
UDCA-treated group (0.78 ± 0.08 mmol/liter) was relatively high among the three groups, however it was not statistically significant. The relative proportion of ionized to total calcium in the control group (12.7%) was statistically lower than that in each sub-group of the GS group (27.1% in non-treated, 20.4% in CDCA-treated, 23.3% in UDCA-treated group) (Table 3).

The pH ranged from 7.15 to 8.45 without any significant difference among the four groups (Table 3). No correlation was observed between pH and ionized calcium levels.

*Ultrafiltrated bile acid levels (Table 2)*

Ultrafiltration rate of TBA was calculated by dividing TBA concentration in the ultrafiltrate by that in the original bile. The ultrafiltration rate of TBA was not statistically different between the control group (18.1 ± 2.3%) and each of the three GS sub-groups. Among the GS groups, the ultrafiltration rate of TBA in CDCA-treated group (12.7 ± 1.5%) was significantly lower compared with that in UDCA-treated (22.3 ± 1.8%) and non-treated (19.1 ± 1.9%) group.

Regarding the influence of CDCA or UDCA on the size distribution of bile acid aggregates, the amount and bile acid composition of ultrafiltrates were compared between the CDCA- and the UDCA-treated groups. The original TBA levels in both groups showed no statistical difference. The ultrafiltration rate of CDCA itself in CDCA-rich bile was calculated to be 8.4 ± 1.7%, and that of UDCA itself in UDCA-rich bile was 30.0 ± 2.6%, respectively. Therefore, the ultrafiltration rate of UDCA in UDCA-rich bile was about 3.7 times higher than that of CDCA in CDCA-rich bile.

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>Total calcium (mmol/liter)</th>
<th>Ionized calcium (mmol/liter)</th>
<th>I/T Calcium rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.83 ± 0.13</td>
<td>5.81 ± 0.63</td>
<td>0.74 ± 0.09</td>
<td>12.7 ± 1.1</td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallstone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-treated</td>
<td>8.04 ± 0.15</td>
<td>2.17 ± 0.27*</td>
<td>0.59 ± 0.05</td>
<td>27.1 ± 3.0*</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDCA-treated</td>
<td>8.07 ± 0.16</td>
<td>3.37 ± 0.65*</td>
<td>0.69 ± 0.010</td>
<td>20.4 ± 4.5*</td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDCA-treated</td>
<td>7.95 ± 0.07</td>
<td>3.34 ± 0.28*</td>
<td>0.78 ± 0.08</td>
<td>23.3 ± 3.5*</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*aAll values are mean ± s.e.

*p < 0.05 as compared with the control group.

I/T, Ionized/Total.
DISCUSSION

Calcium in the human fluid plays various physiological roles in the ionized form (Moore 1970). Calcium levels in bile might be closely associated with insoluble calcium precipitation, which could cause nucleation and calcification in gallstones (Sutor and Wilkie 1977; Been et al. 1979; Wosiewitz 1980; Bateson et al. 1981; Schoenfield et al. 1981; Abedin et al. 1989).

In 1980, Sutor et al. first measured biliary calcium by atomic absorption and ionized calcium by ion-selective electrode simultaneously and reported that total calcium concentration ranged from 1.40 to 8.01 mmol/liter and increased in proportion to total bile acid concentration. There was a highly significant correlation between these two values and the regression line was $y = 0.034x + 0.450$ ($r = 0.759, p < 0.01$). Ionized calcium, however, was maintained in a narrower range (0.25–1.23 mmol/liter).

Each symbol illustrated in this figure is as follows: Circle: control group, Square: non-treated gallstone group, Triangle: CDCA-treated group, and Pentagram: UDCA-treated group. Closed symbols of each group show total calcium concentrations (mmol/liter) and open symbols show ionized calcium concentrations (mmol/liter).

Fig. 2. Relationship between total or ionized calcium and total bile acid concentrations in 34 gallbladder bile samples. Total biliary calcium concentration ranged widely from 1.40 to 8.01 mmol/liter and increased in proportion to total bile acid concentration. There was a highly significant correlation between these two values and the regression line was $y = 0.034x + 0.450$ ($r = 0.759, p < 0.01$). Ionized calcium, however, was maintained in a narrower range (0.25–1.23 mmol/liter).

We measured total and ionized calcium with reference to biliary lipids in human gallbladder bile. We measured ionized calcium concentration by a calcium ion-selective electrode with a newly developed sample container. Using a conventional container, a considerable amount of bile sample was necessary and viscous bile might prevent biliary calcium from making contact equally with the calcium selective electrode. Considering these problems, we developed a new type of sample container. This container did not require a reference electrode in the bile sample because the reference electrode was bridged to the bile sample through a ceramic junction on the side wall of the container, consequently we could reduce sample volume to 1.0 ml. Equal contact with the electrode was achieved by a stirrer at the bottom of the container.

In our study, total calcium concentration ranged from 1.40 to 8.01 mmol/liter and ionized calcium from 0.25 to 1.23 mmol/liter. Total calcium concentration was closely related to TBA (r=0.759), but ionized calcium remained within a narrow range (Fig. 2). This finding was compatible with the previous reports (Knyrim et al. 1989; Yoneda et al. 1991). Non-ionized calcium may be solubilized by binding mainly to bile acid mixed micelles which may play an important buffer-like role in keeping the constant ionized calcium level to minimize the risk of calcium precipitation in bile (Moore et al. 1982; Moore 1984). Then, higher ionized to total calcium ratio denotes smaller capacity of this buffer action. In the present study, the relative amount of ionized calcium was significantly higher in each of the GS group compared to the control group (Table 3). This is due to the low TBA concentration in the GS group, which implies poor concentrating ability of the gallbladder.

We suppose that bile acid composition also play an important role since compositional changes would influence the size distribution of bile acid aggregates. It is suggested that monomers, dimers, simple and mixed micelles and vesicles coexist in bile, which was predicted by recent studies with quasielastic light scattering (Mazer et al. 1980), nuclear magnetic resonance (Schurtenberger and Lindman 1985) and gel filtration chromatography (Cohen and Carey 1990). Therefore, we introduced an ultrafiltration method to reveal the relation between the size distribution of bile acid aggregates and biliary calcium. Considering the difference of bile acid composition, the ultrafiltration rate of UDCA in UDCA-rich bile was apparently higher than that of CDCA in CDCA-rich bile (30.0% vs. 8.4%). These results may imply that UDCA was filtered more effectively than CDCA, and that in UDCA-rich bile, the small size of aggregates (MW less than 1,000) such as monomer, dimer and simple micelles of bile acids occupied a relatively large percentage compared with those in CDCA-rich bile.

As calcium binding capacity of small size of aggregates is less than that of
mixed micellar bile acids (Moore 1984), UDCA-rich bile may have a high potential to precipitate insoluble calcium salts from the viewpoint of the calcium binding capacity and buffer-like action of bile acid micelles. We hypothesized that the difference in micelle forming properties would influence the stability of solubilized calcium in bile, although ionized calcium levels detected no statistical difference between the CDCA and UDCA group.

Raedsch et al. (1981) reported that calcium solubility decreased in UDCA-rich bile compared to that of CDCA-rich bile in in vitro experiment. Knyrim et al. (1989) demonstrated that UDCA replacement resulted in an increase in the relative proportion of ionized calcium compared to CA and CDCA in human. They proposed that UDCA may bind calcium ions weakly and increase calcium activity in bile compared to other bile acids. These data well correspond with our present results and may be compatible with the recent reports of gallstone calcification during UDCA therapy (Bateson et al. 1981; Raedsch et al. 1981; Ros et al. 1986).

The bicarbonate-carbonate system influences ionized calcium in bile as well. Knyrim et al. (1989) demonstrated that replacement of the bile acid pool with CDCA and UDCA resulted in a significant rise in the concentration of bicarbonate and carbonate in bile. However, we could not investigate biliary bicarbonate concentration in this study. Anaerobic condition was not maintained during measurement of biliary pH, which was ranged 7.15 to 8.45. No correlation was observed between pH and ionized calcium levels in our study, and biliary calcium levels were in agreement with previous reports (Sutor et al. 1980; Knyrim et al. 1989). As all bile acids are already ionized within the range of pH 6.5 to 8.5 (Gleeson et al. 1990), biliary pH are proposed not to have much influence on calcium ion binding of bile acids.

We summarize as follows; (a) biliary ionized calcium level was maintained in a narrow range, and there was no significant change according to the presence or absence of gallstones. (b) As total calcium level depends on TBA concentration, poor bile concentrating ability may induce a high ratio of ionized to total calcium. This would indicate a high risk of insoluble calcium precipitation. (c) Considering the buffer-like action of bile acid mixed micelles on biliary calcium, UDCA-rich bile may possess a high risk to precipitate calcium salts compared with CDCA-rich bile.

In conclusion, disability of gallbladder function and replacement of bile acid pool with UDCA may contribute to unstable calcium solubility in gallbladder bile.

Acknowledgment

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References


