The antithrombin activity of glucuronic esters of bilirubin

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SYNOPSIS The plasma of patients with pronounced jaundice as well as that of cats with experimental obstructive jaundice inhibits the clotting of normal plasma with thrombin. The influence of different bile constituents—bilirubin, bilirubin glucuronides, sodium salts of bile acid—on the clotting of plasma or fibrinogen with thrombin was examined. Bilirubin glucuronides in concentrations above 8 mg. % showed antithrombic activity but other substances were inactive. Glucuronide acid and phenolphthalein glucuronide in concentrations up to 100 mg. % did not have any influence on the thrombin time.

It is well known that a prolongation of the plasma thrombin time can be observed in the course of liver disease associated with jaundice. According to Ratnoff (1953) and Alagille and Soulier (1957), the prolonged thrombin time in liver disorders is due to a deficiency of some factor in plasma rather than to the appearance of inhibitory substances. Ratnoff’s (1953) view is that in plasma there is a co-factor of thrombin-fibrinogen interaction which is defined as ‘thrombin accelerator’, and its amount may be decreased in liver disease. Other authors suggested an increase of antithrombin activity in the plasma of these patients (Schwarz, Wanner, and Koller, 1951; Sokal, Schmid, and Hörder, 1955). Masure (1956) postulated qualitative modifications in fibrinogen molecules resulting in an increased resistance of fibrinogen to the action of thrombin.

We have found that the plasma of patients with pronounced jaundice, as well as that of cats with experimental obstructive jaundice, inhibits the clotting of normal plasma with thrombin, and the purpose of this paper is to examine the influence of bile constituents on the thrombin-fibrinogen interaction in vitro because it has been found that bilirubin glucuronides exhibit antithrombic activity.

METHODS AND MATERIALS

Blood from patients and from normal subjects was collected into 0.1 M ammonium oxalate in the proportion of one part of anticoagulant to nine parts of blood. Plasma was obtained by centrifuging for 10 min. at 1,500 r.p.m.

Cats’ plasma was obtained in the same manner from the animals with experimental obstructive jaundice produced by ligating the choledochus duct and from control cats. Fibrinogen was prepared from bovine plasma by the method of Blomback and Blomback (1957). Thrombin was purchased from Hoffman la Roche and Co.

Bilirubin, glucuronic acid, phenolphthalein glucuronide, sodium salts of choletic and desoxycholetic and glycocholic acid were commercial preparations. As a solvent for bilirubin, 0.1 M Na₂CO₃ was used.

Bilirubin glucuronides were prepared by the method of Cole, Lathe, and Billing (1954) from urine, blood, and bile from human patients with liver disorders. The identity of ester preparations obtained was confirmed by paper chromatography (Schmid, 1957), absorption spectra (Cole et al., 1954), and the direct van den Bergh reaction. The preparations were protein free and freely soluble in water.

Paalsch buffer was made up of solutions A and B.

A Sodium borate 0.05 M 19-108 g. Na₂B₄O₇ 10 H₂O. 1,000 ml.

B Boric acid solution in saline, 12-404 g. H₃BO₃, 2-925 g. NaCl, distilled water to 1,000 ml.

and 1:2 vol. of A was mixed with 8:8 vol. of B. The final pH should be 7-6.

This buffer was used as a diluent for all reagents unless otherwise stated.

METHODS

The thrombin time was determined as follows:—

Plasma, 0.2 ml., or 0.2% fibrinogen was mixed with 0.2 ml. of the substance to be tested or the solvent used.
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to prepare the solution of the above substance, and added
to 0·1 ml. thrombin (diluted in order to obtain a clotting
time of 10 to 15 sec. in the control system). Prothrombin
time was determined by the method of Quick (1935) using
rabbit brain thromboplastin.

The recalcification time was tested after adding 0·2 ml.
plasma to the same volume of 0·025 M CaCl₂.

The inhibitory influence of bile constituents on the
prothrombin and recalcification times was determined
after adding one volume of the substance to be tested to
one volume of plasma.

The concentration of bilirubin and bilirubin glucu-
ronides in plasma samples and in the preparations
of bilirubin esters was determined by the method of Malloy
and Evelyn (1937) using a Coleman D 206 B spectrophotometer.

RESULTS

ANTITHROMBIC ACTIVITY OF ICTERIC PLASMAS We
have observed a markedly prolonged thrombin time
in some patients with pronounced jaundice. Patients’
and normal plasmas were mixed in various propor-
tions and the thrombin time was determined in each
mixture. A significant inhibitory influence of icteric
plasma can be seen from the curve shown in Fig. 1.
The same phenomenon was observed when the
mixtures of normal and icteric plasma of cats were
examined (Fig. 2).

INFLUENCE OF BILIRUBIN, BILIRUBIN GLUCURONIDES,
GLUCURONIC ACID, PHENOLPHTHALEIN GLUCURONIDES,
AND SODIUM SALTS OF BILE ACIDS ON THROMBIN TIME
The influence of all these substances on clotting of

FIG. 1. Influence of icteric plasma on the thrombin time
of normal plasma. Patient was suffering from obstructive
jaundice due to cholelithiasis. Level of total bilirubin in
serum is 51 mg. % and of bilirubin glucuronides 40·3 mg. %.

normal plasma and on fibrinogen with thrombin was
examined (Figs. 3 and 4). It was found that bilirubin
up to a concentration of 200 mg. % did not influence the thrombin time. On the
contrary, bilirubin glucuronides showed a significant
inhibitory influence in concentrations above 8 mg.
%1. Therefore we examined glucuronic acid and
phenolphthalein glucuronide and found that these
substances in concentrations up to 100 mg. % did
not have any influence on thrombin time.

THE INFLUENCE OF BILIRUBIN GLUCURONIDES ON
PROTHROMBIN AND RECALCIFICATION TIMES It can be
seen from Table I that the thrombin time of plasma
and of fibrinogen as well as of the prothrombin
and recalcification times of plasma are prolonged in
the presence of bilirubin glucuronides.

1The values refer to the initial concentrations of solutions which were
examined.

DISCUSSION

It appears that bilirubin glucuronides inhibit
thrombin-fibrinogen interaction, while other tested
bile constituents are not active. We were not able
to confirm the finding quoted by Scheraga and
Laskowski (1957) concerning the inhibition of
fibrinogen-fibrin conversion by bile acids.

The possibility cannot be excluded that the dif-
ferent reactivity of bilirubin and its esters is related

FIG. 2. Influence of icteric cat plasma on the thrombin
time of normal cat plasma. Level of total bilirubin in serum
21·7 mg. % and of bilirubin glucuronides 17 mg. %.
FIG. 3. Influence of free bilirubin, bilirubin glucuronides, glucuronic acids, phenolphthalein glucuronides and bile acid salts on the clotting time of normal plasma with thrombin. Initial concentrations: Bilirubin esters, 17·5 mg. %; glyccholeic acid, 20 mg. %; free bilirubin, 200 mg. %; glucuronic acid, phenolphthalein glucuronide, sodium glycocholate, sodium deoxycholate, sodium salt of choleic acid, 100 mg. %; and cholic acid, 25 mg. %.

FIG. 4. Influence of bilirubin esters, free bilirubin, sodium salt of cholic acid, glycocholic acid, deoxycholic acid, and glucuronic acid on clotting time of 0·2% fibrinogen with thrombin. Initial concentrations: Bilirubin esters, 19 mg. %; free bilirubin, 200 mg. %; sodium glycocholate, sodium deoxycholate, glucuronic acid 100 mg. %; and sodium salt of cholic acid, 100 mg. %.
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A raised level of bilirubin glucuronides could be one of the most specific factors influencing the thrombin time in the course of hepatic jaundice.

Measuring the prothrombin time is considered to be one of the most sensitive liver tests, for it permits the evaluation of the ability of liver parenchyma to synthesize plasma clotting factors. (It should be noted that the high levels of bilirubin glucuronides may interfere with the prothrombin time.) It is possible that the high level of bilirubin glucuronides in the plasma of icteric patients may be a contributory factor in the development of a haemorrhagic tendency.

### TABLE I

<table>
<thead>
<tr>
<th>Concentration of Bilirubin-glucuronides Solution (mg.%)</th>
<th>Thrombin Time (sec.)</th>
<th>Prothrombin Time (sec.)</th>
<th>Recalcification Time (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>40</td>
<td>70</td>
<td>30</td>
</tr>
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<td>12</td>
<td>11</td>
<td>21</td>
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<td>3.12</td>
<td>11</td>
<td>10</td>
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<td>Buffer</td>
<td>11</td>
<td>11</td>
<td>18</td>
</tr>
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</table>

To differences in solubility of these substances in plasma protein solutions, or to a decrease in the level of free bilirubin in the presence of plasma protein.

Further studies are necessary to elucidate the detailed mechanism of the inhibitory action of bilirubin glucuronides.

The prolongation of the thrombin time in liver disorders may well be of a complex nature and due to several factors. An increase in fibrinolytic activity of blood in hepatic patients may result in the appearance of the fibrinogen digestion product antithrombin VI (Niewiarowski and Kowalski, 1958; Kowalski, 1960). The dysproteinaemia, particularly low albumin level and high y globulin level favour a prolongation of the thrombin time (Jim, 1957).

### REFERENCES