Heparin cofactor II (HCII) is a glycoprotein in human plasma that inhibits thrombin rapidly in the presence of heparin or dermatan sulfate.\textsuperscript{1,4} Inhibition occurs by formation of a stable equimolar complex between HCII and thrombin. Unlike antithrombin III (ATIII), which inhibits all of the serine proteases of the intrinsic coagulation system,\textsuperscript{5} HCII inhibits thrombin but not other coagulation factors.\textsuperscript{2,4,6-8} HCII requires approximately a tenfold higher concentration of heparin than does ATIII for maximum activity.\textsuperscript{5} In contrast, dermatan sulfate increases the rate of inhibition of thrombin by HCII \textemdash 1,300-fold but has no appreciable effect on the rate of inhibition of thrombin by ATIII.\textsuperscript{2,10}\n
We have reported that incubation of \textsuperscript{125}I-thrombin with plasma in the presence of 100 \textmu{g}/mL of dermatan sulfate yields a single radioactive complex with an apparent mol wt \textup{29,000} that comigrates with the thrombin-HCII complex during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE);\textsuperscript{9} activity detected by the assay is decreased in plasma absorbed with monospecific antibodies against HCII; and (c) purified antithrombin III (ATIII) is unreactive in the assay system. Addition of Polybrene to the assay permits determination of HCII activity in samples containing \textless 12 \textmu{U}/mL of heparin. The range of HCII concentrations in normal individuals is \(1.2 \pm 0.4 \mu\text{mol}/L (\text{mean} \pm 2 \text{SD}, n = 34)\). HCII activity was determined in 54 consecutive patients undergoing evaluation for the possibility of disseminated intravascular coagulation (DIC). Ten of the 11 patients with documented DIC had decreased HCII activity as compared with only 7 of the 43 patients without DIC (\(X^2 = 19.3, P < .0001\)). The concentrations of HCII and ATIII varied in parallel in most of the patients tested. A significant correlation between decreased HCII activity and decreased serum albumin concentration was also observed in these patients and in eight additional patients with hepatic failure in the absence of DIC. We conclude that HCII activity is decreased in many patients with DIC and hepatic failure.\textsuperscript{\textcopyright 1985 by Grune \& Stratton, Inc.}

#### MATERIALS AND METHODS

**Materials.** Tosyl-Gly-Pro-Arg-p-nitroanilide (Chromozym TH) was obtained from Boehringer Mannheim, Indianapolis; benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide (Kabi 5-2222) from Helena Laboratories, Beaumont, Tex; Polybrene from Aldrich Chemical Co., Milwaukee; polyethylene glycol (PEG) (Carbowax-6000) from Union Carbide, New York; \textsuperscript{125}I-sodium iodide (\textasciitilde 17 Ci/mg) from Amersham, Washington Heights, III; and protein A-Sepharose from Pharmacia, Piscataway, N.J. Heparin (Panheparin, 150 USP U/mL) was obtained from Abbott Laboratories, North Chicago, Ill; and porcine skin dermatan sulfate (chondroitin sulfate B) was obtained from Sigma Chemical Co., St. Louis. The dermatan sulfate was treated with 0.24 mol/L NaNO\textsubscript{2} in 1.8 mol/L acetic acid for 80 minutes at 22 °C to degrade contaminating heparin\textsuperscript{9} and dialyzed against water prior to use.

**Proteins.** Human thrombin, HCII, and ATIII were purified and assayed as previously described.\textsuperscript{4} The concentration of HCII was determined spectrophotometrically using the extinction coefficient \(E_{280} = 11.7.\textsuperscript{4} \) The mol wt of HCII was assumed to be 65,600.\textsuperscript{4} Thrombin was labeled with \textsuperscript{125}I to yield a final specific activity of 6 to 12 \times 10\textsuperscript{3} cpm/\mu g protein as previously described;\textsuperscript{4} the labeled preparation had a specific fibrinogen-clotting activity \textasciitilde 90% that of unlabeled thrombin. Purified human coagulation factor Xa was provided by Drs George Broze and Joseph Miletich. Bovine serum albumin (crystallized and lyophilized) was obtained from Sigma.

**Electrophoresis.** SDS-PAGE was performed in the presence of 2-mercaptoethanol in 7.5% polyacrylamide gels with the Laemmli buffer system.\textsuperscript{11} Autoradiography was performed as previously described.\textsuperscript{4}

**Collection of plasma.** Venous blood (4.5 mL) was collected into evacuated tubes containing 0.5 mL of 0.129 mol/L of buffered sodium citrate (Vacutainer \#6418, Becton Dickinson, Rutherford, N.J.), and the cells were removed by centrifugation. HCII activity was stable for at least one year when plasma was stored at \textasciitilde 20 °C. Samples of plasma could be thawed and refrozen at least six times without loss of HCII activity.

**HCII functional assay.** Reactions were carried out in 1.5-mL conical polypropylene tubes (Eppendorf) at 22 °C. Forty microliters of a test sample diluted in 0.15 mol/L of NaCl, 0.02 mol/L of Tris-HCII, pH 7.4 (Tris/NaCl buffer), containing 0.1% PEG was mixed with 60 \mu{L} of Tris/NaCl buffer containing 4 \mu{g} of Polybrene, 133 \mu{g}/mL of dermatan sulfate (nitrous acid-treated), 8 mmol/L of EDTA, and 1 mg/mL of bovine serum albumin. Twenty microliters of thrombin (8 to 12 NIH U/mL in Tris/NaCl/0.1%...
PEG) was then added with rapid mixing. Exactly 60 seconds after the addition of thrombin, 60 μL of 0.75 mmol/L Chromozym TH in water was added with rapid mixing. After an additional 60-second incubation, hydrolysis of the substrate was stopped by addition of 60 μL of glacial acetic acid. Precipitated glycosaminoglycans were removed by centrifugation for ten minutes in an Eppendorf microcentrifuge, and the absorbance at 405 nm of the supernatant solution was determined. The absorbance of a blank solution containing all of the reagents except thrombin was subtracted from each data point. The blank varied with individual plasma samples and ranged from 0.01 to 0.1 absorbance unit. The assay was standardized with duplicate 40-μL samples containing 0, 1, 2, 3, or 4 μL of normal pooled plasma. Standard curves of residual thrombin activity (A_405) vs the quantity of plasma present are shown in this range. Diluted test samples (40 μL) containing 1 to 4 μL of plasma were assayed in duplicate and the average absorbance was calculated after subtraction of the blank. The percentage of activity compared with that found in the normal pooled plasma was then determined from the standard curve. Because the mean activity of samples obtained from 34 normal individuals was 118% of the activity of the normal pooled plasma routinely used to standardize the assay, the activity of each sample was divided by 1.18 to normalize the value.

ATIII functional assay. ATIII was assayed by a modification of the factor Xa inhibition assay described by Ödegård et al. Eighty microliters of test plasma diluted in Tris/NaCl buffer containing 1.25 U/mL heparin and 1 mg/mL of bovine serum albumin was incubated with 10 μL of purified human factor Xa (7.5 μg/mL) for 60 seconds at 22°C. Sixty microliters of a solution containing 83 μg/mL of Polybrene and 2.5 mmol/L of S-2222 in water was then added, and the incubation was allowed to continue for ten minutes. Hydrolysis of the substrate was stopped by addition of 60 μL of glacial acetic acid, and the absorbance at 405 nm was determined. The absorbance of a blank solution containing all of the reagents except factor Xa was subtracted from each data point. The standard curve was linear in the range 0.00 to 0.08 μL of plasma assayed.

Immunoabsorption of HCII. Antiserum against HCII was prepared from rabbits immunized subcutaneously with 100 μg of purified HCII in complete Freund's adjuvant and boosted at three-week intervals until suitable titers of antibody were obtained. The antiserum was judged monospecific for HCII by electrophoretic immunoblotting against plasma as well as against purified HCII (E. A. Jaffe and D. M. Tollefsen, unpublished observations). Antiserum (100 μL) was added to 1 mL of a 10% suspension (packed bead volume) of protein A-sepharose in 0.15 mol/L of NaCl, 0.02 mol/L of sodium phosphate buffer, pH 7.6, and mixed continuously for one hour at 22°C. The beads were then washed by centrifugation three times with Tris/NaCl buffer. The beads were resuspended in 1 mL of a 1:10 dilution of pooled human plasma in Tris/NaCl/0.1% PEG and were mixed continuously for two hours at 22°C. The beads were then removed by centrifugation and the absorbed plasma was stored at −20°C.

Other assays. Determinations of the platelet count, partial thromboplastin time, fibrinogen, fibrinogen/fibrin degradation products (FDPs), and albumin were performed by standard techniques in the clinical laboratories of Barnes Hospital, St Louis.

RESULTS

Specificity of the assay for HCII in heparinized plasma. The HCII activity in plasma was determined by measuring the extent of inhibition of thrombin after addition of the protease to a diluted sample premixed with dermatan sulfate. In preliminary experiments in which 125I-thrombin was used, the products of the reaction were analyzed by SDS-PAGE. A single radioactive complex with a mol wt of 96,000 was formed, representing thrombin covalently bound to HCII. However, if a low concentration of heparin was also present, additional thrombin inhibition occurred due to formation of the thrombin-ATIII complex (mol wt 85,000).

As shown in Fig 1 (lane a), Polybrene prevented formation of the thrombin-ATIII complex in heparinized plasma but had no effect on formation of the thrombin-HCII complex under standard assay conditions. No detectable complexes were formed when heparin and Polybrene were present without dermatan sulfate (lane b). Both thrombin-HCII and thrombin-ATIII complexes were formed, however, in the presence of dermatan sulfate and heparin when Polybrene was omitted (lane c). Experiments in which the 2-μL plasma sample was replaced by 2 μL of 198 μg/mL ATIII (lanes d and e) indicate that activation of the purified inhibitor by heparin was also prevented by Polybrene. In contrast, experiments with 2 μL of 78 μg/mL HCII (lanes f and g) indicate that activation of HCII by dermatan sulfate was qualitatively unaffected by Polybrene under standard assay conditions, although higher concentrations of Polybrene diminished formation of the thrombin-HCII complex (not shown). The concentrations of ATIII and HCII used in these incubations were approximately equivalent to their mean plasma concentrations.

The effects of heparin and Polybrene on the assay system were assessed quantitatively in the experiment shown in Fig 2. In this experiment, the extent of inhibition of thrombin by 2 μL of plasma under standard assay conditions was ~28% as measured with a chromogenic substrate. In incubations with or without dermatan sulfate and in the absence of Polybrene (Fig 2, closed symbols), inhibition of thrombin by ATIII became apparent when heparin was present in the assay at final concentrations between 1 × 10⁻³ and 1 × 10⁻² U/mL. Addition of 2 μg/mL of Polybrene prevented activation of ATIII at final heparin concentrations in the reaction mixture.
significant. Furthermore, Fig 2 confirms that Polybrene had exceeded 1.2 U/mL in order for activation of ATIII to become final vol of 120 μL under the standard conditions described. Heparin was included in the incubations at the final concentrations indicated. Incubations were performed without dermatan sulfate (O, Polybrene present; ●, Polybrene absent) or with 66 μg/mL of dermatan sulfate (□, Polybrene present; ■, Polybrene absent). The final concentration of Polybrene was 2 μg/mL.

≤0.2 U/mL (Fig 2, open symbols). The concentration of heparin in the original plasma sample must, therefore, exceed 12 U/mL in order for activation of ATIII to become significant. Furthermore, Fig 2 confirms that Polybrene had no effect on the ability of dermatan sulfate to activate HCII in the assay system (cf open and closed squares at heparin no effect on the ability of dermatan sulfate (0). The concentration of heparin in the original plasma sample must, therefore, exceed 12 U/mL under the standard conditions described. Heparin was included in the incubations at the final concentrations indicated. Incubations were performed without dermatan sulfate (O, Polybrene present; ●, Polybrene absent) or with 66 μg/mL of dermatan sulfate (□, Polybrene present; ■, Polybrene absent). The final concentration of Polybrene was 2 μg/mL.

Standardization of the HCII assay. Figure 3 indicates the results of the HCII assay standardized with normal pooled plasma (closed circles). Inhibition of thrombin was linear in the range 0 to 4 μL of plasma added. SD for five determinations at each quantity of plasma added ranged from 1% to 3% of the mean values. No detectable inhibition occurred with 4 μL of plasma in the absence of dermatan sulfate (not shown). Results obtained with 0.075 to 0.225 μg of purified HCII, when superimposed on the standard curve (open circles), indicate that the pooled plasma sample contained 68 μg/mL of HCII. Mixtures of plasma and purified HCII in various proportions gave strictly additive results (eg, 2 μL of plasma plus 0.14 μg of HCII gave a result equivalent to 4 μL of plasma), indicating that plasma does not contain other molecules that significantly interfere with the assay.

An amount of purified ATIII (0.8 μg) approximately equivalent to that found in 4 μL of plasma was unreactive in the HCII assay (Fig 3, open squares). Furthermore, a sample of pooled plasma absorbed with anti-HCII antibodies linked to protein A-sepharose contained only 6% of the HCII activity present in an equal volume of unabsorbed plasma, while plasma absorbed with control antibodies contained 95% of the original activity (not shown).

HCII activity in normal individuals. The HCII activity in plasma samples from 34 normal donors was 118 ± 45% (mean ± 2 SD) of the activity in the pooled plasma standard shown in Fig 3. The range of HCII concentrations in the normal population was, therefore, 80 ± 30 μg/mL or 1.2 ± 0.4 μmol/L (mean ± 2 SD). The individual data points for the normal individuals are plotted in Fig 4 (controls) as percentages of the normal mean value.

HCII activity in patients with DIC. HCII activity was determined in 145 consecutive plasma samples submitted to the clinical hematology laboratory from 54 patients in whom the diagnosis of disseminated intravascular coagulation (DIC) was suspected (Fig 4). Fourteen of the patients were receiving heparin at the time the samples were collected. No inhibition of thrombin occurred in control assays in which the dermatan sulfate was omitted, confirming that the amount of Polybrene added was sufficient to neutralize the heparin present in the samples. Patients 1 through 22 each had two or more samples tested for HCII activity, whereas patients 23 through 54 had only one sample tested. In general, multiple samples collected from a single patient at different times gave similar results in the HCII assay, indicating that the levels of HCII were fairly stable in these patients during the period of sampling (2 to 20 days). Nineteen of the 54 patients had at least one HCII determina-

![Fig 2. Effect of Polybrene and heparin on inhibition of thrombin by plasma. HCII assays were performed with 2 μL of plasma in a final vol of 120 μL under the standard conditions described.](image)

![Fig 3. Standardization of the HCII assay. Assays were performed with the indicated amounts of normal pooled plasma (●, mean values), purified HCII (O), or purified ATIII (□). The error bars indicate the range of values obtained for five determinations performed with each quantity of plasma.](image)

![Fig 4. HCII activities in normal individuals and patients. HCII activities are plotted as percentages of the mean value obtained for 34 normal individuals (Controls). The dashed lines indicate the normal range for HCII (62% to 138%, mean ± 2 SD) determined from the control population. Each symbol represents the average of duplicate determinations performed on a single plasma sample. Patients with DIC as defined in Table 1. ●, patients without DIC.](image)
Table 1. Patients With Abnormal HCII Activity

<table>
<thead>
<tr>
<th>Patient No.*</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnoses</th>
<th>Mean Percentage of HCII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>F</td>
<td>Pre-eclampsia</td>
<td>+ 19</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>M</td>
<td>Hepatic cirrhosis, bacterial peritonitis</td>
<td>+ 19</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>F</td>
<td>Hepatic cirrhosis, pancreatitis, pneumonia</td>
<td>+ 29</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>M</td>
<td>Alcoholic hepatitis, subdural hematoma</td>
<td>+ 35</td>
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<tr>
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<td>50</td>
<td>F</td>
<td>Metastatic ovarian carcinoma, septicemia</td>
<td>+ 36</td>
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<td>59</td>
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<td>Acute nonlymphocytic leukemia, septicemia</td>
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<td>F</td>
<td>Pre-eclampsia</td>
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<td>M</td>
<td>Chronic renal failure, myo-cardiac infarction</td>
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<tr>
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<td>Chronic myelogenous leukemia, S/P marrow transplant</td>
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<td>28</td>
<td>M</td>
<td>Abdominal gunshot wound, S/P colectomy</td>
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<td>F</td>
<td>S/P coronary artery bypass</td>
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<td>+ 51</td>
</tr>
<tr>
<td>54</td>
<td>65</td>
<td>F</td>
<td>Narcotic overdose, respiratory distress syndrome</td>
<td>+ 28</td>
</tr>
</tbody>
</table>

Decreased HCII activity

Increased HCII activity

*Patients from Fig 4 who had at least one abnormal HCII determination, ie, HCII <62% or >138% of the mean value for normal individuals.

†Patients were considered to have DIC (+) if they met at least three of the following criteria: FDPs >8 μg/mL; platelet count <150,000/μL; fibrinogen <150 mg/dL; and activated partial thromboplastin time >38 seconds.

‡Died.

Figure 5. Relationship between HCII and ATIII activities. HCII activities are plotted as in Fig 4. ATIII activities are plotted as percentages of the mean value obtained for 24 normal individuals (not shown). The range for ATIII activity was 62% to 138% (mean ± 2 SD) in the normal population. Patients with DIC, ◆; patients without DIC, O.

Figure 6. Relationship between HCII and serum albumin concentrations. Each symbol represents a corresponding HCII activity and serum albumin concentration in 49 of the patients in Fig 4 as well as eight additional patients with known hepatic failure. The normal range for serum albumin concentration was 3.5 to 5.0 g/dL. Patients from Fig 4 with DIC, ◆; patients from Fig 4 without DIC, O; patients with hepatic failure who did not have DIC, X.
HCII activity in DIC and hepatic failure

HCII activity is directly related to the coagulopathy is unknown. A single patient with DIC in whom the HCII activity was consistently evaluated suggests that the presence of HCII is not sufficient to prevent DIC. In the future, a test for the thrombin-HCII complex or measurement of the half-life of circulating HCII may indicate whether HCII is consumed during DIC. In the present study, there was also a significant correlation between decreased HCII activity and a serum albumin concentration ≤ 3.5 g/dL (Fig 6), including patients who had no evidence of DIC. These results indicate that HCII is decreased in some patients with hepatic failure independent of the presence of DIC and suggest that HCII synthesis occurs in the liver. Recent experiments demonstrating that HCII is synthesized by cultured human hepatoma cells support this conclusion.

The relationships between the activities of HCII and ATIII in 22 patients are shown in Fig 5. In general, patients with decreased HCII activity also had decreased ATIII, although there were two exceptions. Similarly, two patients with normal amounts of HCII had ATIII concentrations that were decreased. Thus, it appears that independent processes may determine the concentrations of HCII and ATIII in various disease states. We have studied three patients with hereditary ATIII deficiency (40% to 50% of normal activity), all of whom had HCII activities within the normal range as previously reported.

The function of HCII in vivo may eventually be elucidated by the discovery of patients with primary HCII deficiency. In a preliminary study of 105 patients with documented venous thrombosis, we have found no one in whom the concentration of HCII was less than normal in the absence of hepatic disease. (D.M. Tollefsen and J. Hirsh, unpublished observations. The assays for HCII were performed by the technique described herein in the laboratory of D.M.T.) Therefore, if HCII deficiency occurs, it is a rare cause of venous thrombosis. Furthermore, preliminary experiments of MacIntosh et al have failed to demonstrate inhibition of thrombin by HCII in a perfused rabbit heart preparation. Thus, the normal function of HCII may not be to inhibit intravascular thrombin and thereby to prevent thrombosis. Alternatively, HCII may inhibit thrombin in extravascular sites where dermatan sulfate is prevalent and thereby regulate activities of the protease such as mitogenesis or chemotaxis.

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Heparin cofactor II activity in patients with disseminated intravascular coagulation and hepatic failure

DM Tollefsen and CA Pestka