Pharmacokinetics of Activated Protein C in Guinea Pigs

By H. Berger Jr, C.G. Kirstein, and C.L. Orthner

Protein C is a vitamin K-dependent zymogen of the serine protease, activated protein C (APC), an important regulatory enzyme in hemostasis. In view of the potential of human APC as an anticoagulant and profibrinolytic agent, the pharmacokinetics and tissue distribution of APC were studied in guinea pigs. The plasma elimination of a trace dose of $^{125}$I-APC was biphasic following an initial rapid elimination of approximately 15% of the injected dose within 1 to 2 minutes. This rapid removal of $^{125}$I-APC from the circulation was found to be a result of an association with the liver regardless of the route of injection. Essentially identical results were obtained with active site-blocked forms of APC generated with either diisopropylfluorophosphate or D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, which indicates that the active site was not essential for the liver association. Accumulation of all three forms of APC in the liver peaked at 30 minutes and then declined as increasing amounts of degraded radiolabeled material appeared in the gastrointestinal tract and urine. Removal of the $\gamma$-carboxyglutamic acid (gla) domain of diisoproplyphosphoryl-APC resulted in a 50% reduction in the association with liver and an accumulation in the kidneys. Protein C and protein S were cleared from the circulation at rates approximately one-half and one-fourth, respectively, that of APC. Both in vitro and in vivo, APC was found to form complexes with protease inhibitors present in guinea pig plasma. Complex formation resulted in a more rapid disappearance of the enzymatic activity of APC than elimination of the protein moiety. These findings indicate two distinct mechanisms for the elimination of APC. One mechanism involves reaction with plasma protease inhibitors and subsequent elimination by specific hepatic receptors. The other mechanism involves the direct catabolism of APC by the liver via a pathway that is nonsaturable over a substantial dose range and independent of the active site. This pattern of elimination is distinctly different from that observed with the homologous coagulation enzymes thrombin, factor IXa, and factor Xa.

HUMAN PROTEIN C is a two-chain glycoprotein (Mr, 62,000) that is structurally homologous to the vitamin K-dependent coagulation proteins, such as factors IX and X.$^{1,2}$ Like these proteins, it circulates in plasma as a zymogen. As a result of activation of the coagulation pathway, however, protein C undergoes thrombin-catalyzed proteolysis of a dodecapeptide from the N-terminus of its heavy chain, which results in activated protein C (APC), a serine protease with relatively narrow specificity. APC acts as a natural anticoagulant and has been shown to inactivate the coagulation factors VIIIa and Va proteolytically.$^{1}$ In addition, APC enhances clot lysis,$^{1,7}$ which has been attributed to its ability to neutralize type 1 plasminogen activator inhibitor.$^{1,2}$ Recent studies have shown that APC infusion in animals has an antithrombotic effect and is able to prevent platelet deposition and fibrin accretion to thrombi.$^{14,16}$ In light of the potential usefulness of APC as an antithrombotic and profibrinolytic agent, we have examined the pharmacokinetics of human plasma-derived APC in guinea pigs. This study was performed in connection with a separate study demonstrating the antithrombotic efficacy of human APC in the guinea pig that is the subject of a manuscript in preparation.

MATERIALS AND METHODS

Materials. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PFACK), human $\omega_1$-macroglobulin, human $\alpha$-antitrypsin, and both human and bovine antithrombin III were obtained from Calbiochem Corp (San Diego, CA). Bovine serum albumin (BSA), Triton X-100, and diisopropyl fluorophosphate (DFP) were from Sigma Chemical Co (St Louis, MO). All electrophoresis reagents and Hz hydrazide gel were from Bio-Rad Laboratories (Rockville Centre, NY). Mouse monoclonal antibody (MoAb) ESC 3 to human protein C, goat antihuman protein S antibody, and rabbit thrombomodulin were from American Diagnostica Inc (Greenwich, CT). Iodo-Gen, Iodo-Beads, and dextran sulfate-agarose were from Pierce Chemical Co (Rockford, IL). Kabir chromogenic substrate S-2266 and factor Xa were obtained from Helena Laboratories (Beaumont, TX). Dade reagents for clotting assays were from Baxter Healthcare Corporation (Charlotte, NC). Nembu- tal was from Abbott Laboratories (North Chicago, IL). Heparin sodium was from Eli Lilly and Co (Indianapolis, IN). Sodium $^{131}$I-iodide and sodium $^{125}$I-iodide were from Amersham Corp (Arlington Heights, IL). Male Dunken Hartley guinea pigs were from Charles River Breeding Laboratories (Wilmington, MA).

Protein preparations. Protein C and protein S were purified from human plasma as described previously.$^{17}$ The purity of both preparations was greater than 95% as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein C was then converted to APC by incubation with 0.1% to 0.5% (wt/wt) protein C activator purified from Agkistrodon contortrix contortrix venom.$^{18}$ The snake venom activator was removed from the preparation by adsorption onto SP-Sephadex.$^{19}$ APC was inactivated by either incubation at 37°C for 1 hour with 5 mmol/L DFP, which generated what will be referred to as diisopropolphosphoryl-APC (DIP-APC), or incubation at 37°C for 30 minutes with 10 μmol/L PPACK. Excess reagent was removed by chromatography on Sephadex PD-10 columns. Loss of amidolytic activity on the chromogenic substrate S-2266 was used as an index of inactivation. Removal of the N-terminal $\gamma$-carboxyglutamyl acid (gla)-containing domain of APC was achieved by limited digestion with chymotrypsin as described by Esmen et al.$^{19}$ The period of digestion...
was chosen by previously monitoring the cleavage over time on SDS-PAGE under reducing conditions. The reaction was terminated by the addition of DFP to a final concentration of 5 mmol/L and incubation at 37°C for 30 minutes. Gla-domainless DIP-APC was separated from the cleaved N-terminal light chain peptide by chromatography on QAE-Sephadex as described by Eason et al.9

Human thrombin was purified as previously described26 and was generously supplied by Dr H.E. Fuchs, Duke University. DIP-thrombin was prepared by incubation with 5 mmol/L DFP at 37°C for 30 minutes followed by removal of excess reagent as described above.

Radioiodination of proteins. All proteins were labeled with 125I by the Iodo-Gen method.27 Briefly, a film containing 0.6 µg of Iodo-Gen was dried on the bottom of cryovials. Aliquots of 30 µL of the desired protein at a concentration of 1 mg/mL (in 0.02 mol/L Tris-HCl, pH 7.4; 0.3 mol/L NaCl; 0.1% Triton X-100) were labeled by incubation with 0.3 mCi of 125I for 5 minutes on ice. 125I-labeled protein C, APC, DIP-APC, PPACK-APC, and protein S were freed of unincorporated label by capturing them on MoAb columns, prepared by coupling the antibodies to 11z hydrazide gel according to the manufacturer's instructions. In the case of APC, this procedure fortuitously also served to remove a small amount of 40,000-d material, which was identified as a degraded form of APC by immunoblotting studies (C.L. Orthner, unpublished results). These proteins were eluted in 0.1 mol/L glycine, pH 3, containing 2 mol/L guanidine HCl, and immediately neutralized by the addition of an appropriate amount of 2.5 mol/L Tris-HCl, pH 9.0. The preparations were then dialyzed against 0.05 mol/L Tris-HCl, pH 7.4, containing 0.1 mol/L NaCl and 0.002 mol/L CaCl₂. Alternatively, unincorporated label was removed by gel filtration on Sephadex PD-10 columns equilibrated in this latter buffer. Specific activities were routinely 5 µCi/µg. Preliminary studies indicated that the pharmacokinetics of the 125I-labeled proteins prepared by either method were the same. It has been the experience of one of the authors (H.B.) that cleaner preparations result from the use of the antibody columns, and that loss of enzymatic or cofactor activity has not been a problem. For example, 1 µg of purified APC before labeling generated 0.11 optical density unit/min at 405 nm were taken with a Titertek Multiscan plate reader (Flow Laboratories, Inc, McLean, VA). Standard curves were generated by spiking aliquots of plasma on ice with known amounts of APC, immediately adding barium citrate, and processing as above. If the detection limit is defined as the amount of APC that produces twice the background reading, then the assay was sensitive enough to detect 0.25 µg of APC per milliliter of plasma.

Pretreatment of plasma with methylamine was carried out as described by Svenson and Howard.29 The treated plasma was then dialyzed overnight against phosphate-buffered saline (PBS) containing 20 mmol/L trisodium citrate.

The anticoagulant activity of APC was monitored by the activated partial thromboplastin time determined on a fibrometer. The cofactor effect of protein S was determined by a factor Xa one-stage clotting assay as described by D'Angelo et al.24

Pharmacokinetic studies. Guinea pigs weighing 360 ± 40 g were anesthetized with 35 mg/kg Nembutal intraperitoneally. Anesthesia was maintained for the duration of the experiment by supplementing with subcutaneous injections of Nembutal at 10 mg/kg as needed. Body temperatures were maintained by placing the animals on a heating pad thermostatted to 37°C by a circulating water bath. Cannulas were inserted into the right carotid artery and/or the left jugular vein and filled with a heparin lock. Preliminary experiments performed in the absence of heparin verified that the presence of heparin in the cannulas did not influence the results. Nevertheless, when blood samples were taken, the first 0.5 mL was discarded; the blood remaining in the cannula was then flushed back into the animal using saline, and the cannula refilled with a heparin lock. Blood was sampled from the arterial cannula; radiolabeled proteins were injected through either cannula.

Bolus injections of approximately 2 µCi and 0.1 µg to 0.4 µg of a given 125I-labeled protein and 1 µCi of 125I-BSA were given in 1 mL of saline and followed by a flush of 0.5 mL of saline. Blood samples were withdrawn at desired intervals by a two-syringe technique whereby the first 0.5 mL of blood was discarded and the next 0.45 mL of blood was collected into 0.05 mL of 3.8% trisodium citrate. No more than seven such samplings were performed on an individual animal, which limited blood loss to 15% to 20% of the total blood volume. Plasma was prepared by centrifugation at 2,500g for 10 minutes at 4°C, or by immediate centrifugation for 2 minutes in an Eppendorf microfuge when the amidolytic activity of APC was to be determined. To compensate for potential losses of activity during centrifugation, guinea pig plasma at 37°C was spiked with known amounts of APC and cocentrifuged with the blood samples. Then, barium citrate fractions were prepared immediately from aliquots of the recovered plasma. The losses in amidolytic activity encountered in handling the samples in this manner were approximately 10%. Alternatively, blood samples were collected in 1/10 volume of 3.8% trisodium citrate containing 0.11 mol/L p-aminobenzamidine, which preserved all of the amidolytic activity. Both procedures yielded the same results. In preliminary experiments the radioactivity recovered in the plasma from a given amount of blood was found to contain essentially all of the radioactivity, indicating that insignificant amounts of radiolabel were associated with the cellular elements. Radioactivity precipitable in 10% trichloroacetic acid (TCA) was taken as an index of intact protein and that soluble in 10% TCA as degraded protein. A Packard Autogamma 5000 multichannel system coupled to the CompuSphere computer matrix analysis program was used to determine the individual contents of 125I and 111In.

At selected times after injection of 125I-labeled proteins, major organs were removed, washed briefly in saline, and counted in toto. In preliminary experiments, it was found that little radioactivity was associated with brain. Therefore, brain was not routinely monitored. Samples of liver were taken at various times and homogenized in 10% TCA to determine the relative contents of TCA-soluble and TCA-precipitable radioactivities. The fraction of
The disappearance curves for all of the proteins studied were described by Davis or in the presence of a slow beta phase with a t½ of 133 minutes. From 60 minutes to 180 minutes, identical results on the elimination of 125I-APC were obtained if the guinea pigs were first administered, which indicates that this compartment was nonsaturable over this dose range. The subsequent elimination of 125I-APC was biphasic, characterized by a rapid alpha phase with a ½ of 13 minutes followed by a slower beta phase with a ½ of 133 minutes. From 60 minutes through 180 minutes, ≥ 85% of the total radioactivity in the plasma was TCA-precipitable, which indicates that the bulk of the radioactivity remained as intact APC over this time period. Identical results on the elimination of 125I-APC were obtained if the guinea pigs were first administered a dose of human protein S sufficient to achieve an initial plasma level of 25 μg/mL (results not shown).

Similar studies were performed using radiolabeled DIP-APC and PPACK-APC, inactive forms of the enzyme in which the active site serine or histidine, respectively, was blocked. Surprisingly, the elimination of these active-site

The disappearance curves for all of the proteins studied were analyzed by graphic curve peeling, and all curves were fitted to the following equation: C(t) = R exp (-at) + S exp (-ßt) where a and ß are the slopes of the initial and terminal phases, respectively, and R and S are the y-intercepts on a semilogarithmic plot of the fractional amount of protein circulating as a function of time. Pharmacokinetic parameters were calculated from these disposition parameters using standard equations after normalization of the sum of the intercept terms, R and S, to 1. This is the same treatment of the data as would have resulted had the disappearance curves been plotted as a percentage of the radioactivity in the initial plasma sample.

The data presented on the tissue distribution of radioactivity were corrected for the amount of plasma entrapped in each organ. The volume of plasma entrapped was calculated from the cpm 131I in each organ divided by the concurrent cpm 131I/ml plasma corrected for dilution by anticoagulant. Then the cpm 131I to be subtracted from each organ was determined by multiplying the volume of plasma entrapped by the concurrent cpm 131I/ml plasma corrected for dilution by anticoagulant.

PAGE: Nondenaturing PAGE was performed in 6% gels as described by Davis or in the presence of SDS as described by Laemmli. Molecular weight markers used on SDS gels were myosin (200,000), phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000). For autoradiography, gels were dried and exposed to Kodak X-Omat x-ray film at −70°C.

RESULTS

Plasma elimination of APC and related proteins. Figure 1 shows the plasma elimination of radiolabeled APC after intraarterial injection into guinea pigs, determined by monitoring TCA-precipitable radioactivity. A plot of the radioactivity precipitable by barium citrate was indistinguishable over the time frame indicated. At prolonged circulation times (eg, ≥ 6 hours) a decrease in barium citrate precipitability was noticed that amounted to a 30% decrease at 6 hours. This same phenomenon was observed upon incubation of 125I-APC in guinea pig plasma in vitro.

An explanation for this alteration of APC was not sought, but the experiments were confined to a duration of 3 hours or less. The precipitable radioactivity could be totally accounted for on SDS-PAGE as species with M, values ≥ 62,000 (data not shown), which indicates that the curve depicted represents the clearance of intact protein. At the earliest observable time (1 to 2 minutes), approximately 15% of the 125I-APC was no longer in the circulation but was associated with the liver (see below). The same percentage of radioactivity was found associated with the liver whether only a trace amount (0.1 μg) of 125I-APC was injected or a 1,000-fold excess of unlabeled APC was simultaneously administered, which indicates that this compartment was nonsaturable over this dose range. The subsequent elimination of 125I-APC was biphasic, characterized by a rapid alpha phase with a ½ of 13 minutes followed by a slower beta phase with a ½ of 133 minutes. From 60 minutes through 180 minutes, ≥ 85% of the total radioactivity in the plasma was TCA-precipitable, which indicates that the bulk of the radioactivity remained as intact APC over this time period. Identical results on the elimination of 125I-APC were obtained if the guinea pigs were first administered a dose of human protein S sufficient to achieve an initial plasma level of 25 μg/mL (results not shown).

Similar studies were performed using radiolabeled DIP-APC and PPACK-APC, inactive forms of the enzyme in which the active site serine or histidine, respectively, was blocked. Surprisingly, the elimination of these active-site
blocked forms of APC was almost indistinguishable from that of APC (only the disappearance curve for DIP-APC is shown in Fig 1). Control studies in which radiolabeled DIP-APC or PPACK-APC was spiked into guinea pig plasma in vitro and incubated at 37°C for 3 hours demonstrated that the adducts were stable under these conditions, as evidenced by their inability to form SDS-stable high molecular weight complexes. This result is in contrast to the situation with APC, where such complexes were observed when analyzed by SDS-PAGE (results not shown). As was the case with APC, approximately 15% of the injected 125I-DIP-APC rapidly associated with liver. The plasma elimination of γ-carboxyglutamic acid (gla)-domainless DIP-APC was only slightly different from that of DIP-APC (Fig 2). However, removal of the gla domain did have a significant effect on the tissue distribution of the molecule (see below).

As is seen in Fig 2, essentially quantitative recoveries of protein C and protein S were observed at the earliest sampling time, followed by biphasic elimination curves for both of these proteins. The elimination of protein C was slower than that of APC and its modified forms, while the elimination of protein S was decidedly the slowest of the proteins examined.

As seen in Fig 1, the decline of APC amidolytic activity in vivo was markedly faster than the elimination of radiolabeled APC protein, which suggested the formation of APC-inhibitor complexes. Subsequent to a 50% loss of enzymatic activity by the first sampling time (1 minute), the decline in APC activity followed essentially first order decay kinetics with a ½ of approximately 8 minutes. The discrepancy between protein and activity levels at the initial sampling time was accounted for by the formation of protease-inhibitor complexes even within this brief time frame (see below).

The disposition parameters for all of the proteins studied are listed in Table 1. In contrast to the plasma elimination of APC and its modified forms, the elimination of protein C and protein S fit well to a two-compartment model and showed no evidence for fast association with a noncirculating compartment. This is seen in the fact that the sums of the extrapolated γ-intercepts for the α and β phases (R plus S) were 0.93 and 1.00 for protein C and protein S, respectively, while only 0.78 and 0.80 for APC and DIP-APC, respectively. This appears to be an inherent difference in the pharmacokinetics of protein C versus APC, rather than an artifact of protein preparation, because both proteins were iodinated and desalted by immuno-capture using the same procedure. Furthermore, identical plasma elimination curves were obtained when the proteins were desalted by gel filtration (data not shown).

Because the association of APC and its modified forms with liver was too fast to be studied pharmacokinetically, the sum of the disposition parameters, R and S, were normalized to unity as described in Materials and Methods. This normalization results in the distribution of these forms to the liver appearing in the volume of distribution term, Vd. Pharmacokinetic parameters were calculated by using standard equations for a two-compartment model and are presented in Table 2. The clearance of protein C, 0.35 mL/min/kg, was approximately half that of APC and its modified forms, as was its fractional catabolic rate (k0) of 0.006 inverse minutes. The elimination of protein S, whose N-terminal half is a structural homologue of protein C and the vitamin K-dependent coagulation proteins,23 was clearly the slowest, characterized by a clearance of 0.15 mL/min/kg and a k0 of 0.003 inverse minutes.

**Tissue distribution studies.** The distribution of radioactivity in selected tissues at several times following injection of the various radiolabeled proteins is shown in Fig 3. At 10 minutes postinjection, 60.0% of the 125I-APC was found in the plasma while 27.9% was found in the liver (Fig 3A). The radioactivity in the liver increased to 32.1% at 30 minutes and then declined as increasing amounts were found in the gastrointestinal tract and urine. The percentage of the radioactivity associated with the liver that was soluble in TCA increased from 1.4% at 10 minutes to 13.9% at 120 minutes (Table 3). From 50% to 70% of the TCA-soluble protein C; (▲) protein C; (△) gla-domainless DIP-APC; (◇) protein S.

![Graph](image-url)

**Table 1. Disposition Parameters for Proteins of the Protein C System in Guinea Pigs**

<table>
<thead>
<tr>
<th>Protein</th>
<th>R (min⁻¹)</th>
<th>S (min⁻¹)</th>
<th>β (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated protein C</td>
<td>0.42</td>
<td>0.062</td>
<td>0.38</td>
</tr>
<tr>
<td>DIP-APC</td>
<td>0.30</td>
<td>0.069</td>
<td>0.48</td>
</tr>
<tr>
<td>Gla-domainless DIP-APC</td>
<td>0.41</td>
<td>0.122</td>
<td>0.48</td>
</tr>
<tr>
<td>Protein C</td>
<td>0.22</td>
<td>0.233</td>
<td>0.71</td>
</tr>
<tr>
<td>Protein S</td>
<td>0.17</td>
<td>0.154</td>
<td>0.83</td>
</tr>
</tbody>
</table>

The disposition parameters were derived from graphic curve peeling of the biphasic exponential clearance curves shown in Figs 1 and 2 as described by Gibaldi and Perrier. The intercept terms, R and S, are presented as fractions of the injected dose.
Pharmacokinetic parameters were calculated from the disposition parameters listed in Table 1. Vc is the central volume of distribution, Vd is the total volume of distribution, kₚ is the elimination rate constant, kᵣ is the reflux rate constant from the peripheral compartment to the central compartment, Cl is the clearance, and AUC is the extrapolated area under the curve for doses of each protein of 250 μg/kg.

The distribution of radiolabeled DIP-APC in the various organs followed the same general pattern, except that approximately 25% to 30% less label was found in the liver at the earlier sampling times (Fig 3B). Figure 3C shows that the organ distribution of radiolabeled gla-domainless DIP-APC was dramatically altered in comparison with DIP-APC; a 50% reduction in the amount of radioactivity in liver and a 200% increase in kidneys were noted 30 minutes after injection (P < .01, Student's t-test). Figure 3D shows the tissue distribution of protein C, where approximately 10% was associated with the liver at 15 to 30 minutes following injection, the remainder being in the circulation. Less than 1% of any radiolabeled protein studied was found in the bile, heart, lungs, or spleen at any sampling time.

The distribution of radioactivity in selected tissues immediately after injection was also studied as a function of the route of administration. Table 4 shows that 14% to 18% of ¹²⁵I-APC or ¹²⁵I-DIP-APC was associated with the liver at 1 to 2 minutes after injection, regardless of the route of administration. This finding was in sharp contrast to the results obtained with ¹²⁵I-DIP-thrombin, where the tissue distribution in the guinea pig was clearly influenced by the route of administration. The localization of ¹²⁵I-DIP-thrombin was primarily to the vascular bed first encountered. These results are similar to those found for ¹²⁵I-DIP-thrombin in the rabbit.³²

**Studies in vitro.** The time course of inactivation of APC in vitro was studied in freshly prepared guinea pig or human plasma. As shown in Fig 4, the time course in citrated guinea pig plasma was biphasic and unaffected by the presence of heparin. The initial rate of inactivation of 5 μg

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**Table 2. Pharmacokinetic Parameters for Proteins of the Protein C System in Guinea Pigs**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vc (mL/kg)</th>
<th>Vd (mL/kg)</th>
<th>kₚ (min⁻¹)</th>
<th>kᵣ (min⁻¹)</th>
<th>Cl (mL/min/kg)</th>
<th>AUC (min⁻¹μg·min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>65</td>
<td>125</td>
<td>0.010</td>
<td>0.020</td>
<td>0.27</td>
<td>0.65</td>
</tr>
<tr>
<td>DIP-APC</td>
<td>66</td>
<td>101</td>
<td>0.010</td>
<td>0.017</td>
<td>0.038</td>
<td>0.67</td>
</tr>
<tr>
<td>Gla-DIP-APC</td>
<td>58</td>
<td>101</td>
<td>0.013</td>
<td>0.048</td>
<td>0.069</td>
<td>0.77</td>
</tr>
<tr>
<td>Protein C</td>
<td>56</td>
<td>71</td>
<td>0.006</td>
<td>0.052</td>
<td>0.179</td>
<td>0.35</td>
</tr>
<tr>
<td>Protein S</td>
<td>52</td>
<td>63</td>
<td>0.003</td>
<td>0.025</td>
<td>0.129</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**Table 3. Proportion of Tissue-Associated Radioactivity That Is Soluble in TCA and the Fraction of TCA-Soluble Material as Iodide**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>10 min</th>
<th>30 min</th>
<th>120 min</th>
<th>10 min</th>
<th>30 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>&lt;1</td>
<td>1.0</td>
<td>4.8</td>
<td>ND</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Liver</td>
<td>1.4</td>
<td>9.3</td>
<td>13.9</td>
<td>0.5</td>
<td>0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

At the indicated times after injection of ¹²⁵I-DIP-APC, the percentages of the total radioactivity present in plasma and liver that were soluble in 10% TCA are shown. The right hand portion of the table lists the fraction of the TCA-soluble radioactivity attributable to free iodide. The data presented represent the means of duplicate experiments at each time point.

Abbreviation: ND, not determined.
Table 4. Tissue Distribution of Radiolabeled Proteins After Various Routes of Injection

<table>
<thead>
<tr>
<th>Route of Injection</th>
<th>Plasma</th>
<th>Heart</th>
<th>Liver</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-APC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraarterial</td>
<td>82.3</td>
<td>0.5</td>
<td>14.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Intravenous</td>
<td>70.3</td>
<td>0.5</td>
<td>15.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Intraportal</td>
<td>83.7</td>
<td>0.5</td>
<td>16.0</td>
<td>1.3</td>
</tr>
<tr>
<td>125I-DIP-APC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>82.9</td>
<td>0.5</td>
<td>18.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Intraportal</td>
<td>85.0</td>
<td>0.4</td>
<td>13.5</td>
<td>1.2</td>
</tr>
<tr>
<td>125I-DIP-Thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraarterial</td>
<td>31.7</td>
<td>0.3</td>
<td>15.7</td>
<td>8.5</td>
</tr>
<tr>
<td>Intravenous</td>
<td>25.2</td>
<td>0.9</td>
<td>14.2</td>
<td>36.3</td>
</tr>
<tr>
<td>Intraportal</td>
<td>27.5</td>
<td>0.3</td>
<td>53.9</td>
<td>3.3</td>
</tr>
</tbody>
</table>

At 1 to 2 minutes after injection of the radiolabeled protein, the indicated tissues were counted for their contents of total radioactivity. No corrections for the plasma entrapped in the organ were applied. Values represent the average of duplicate determinations.

APC/mL of guinea pig plasma in vitro proceeded at a linear rate of 7.5% inhibition per minute over the first 5 minutes (inset, Fig 4). This rate was slower than the initial decline of enzymatic activity in vivo starting from the same concentration of APC. In contrast to the results obtained in guinea pig plasma, inactivation in human citrated plasma was essentially monophasic characterized by a t½ of approximately 30 minutes. As reported previously by others, heparin clearly enhanced the first-order rate of inactivation in human plasma, reducing the t½ to approximately 10 minutes.

Figure 5 shows complex formation between 125I-APC and inhibitors in guinea pig plasma as analyzed by nondenaturing PAGE. In citrated plasma (lanes 4 and 5), a time-dependent decrease in 125I-APC (band d) was accompanied by an increase in two or more 125I-APC inhibitor complexes. The faster migrating complex (band b) was diffuse and appeared to consist of more than one component. Band b migrated similarly, albeit somewhat more slowly, than the complex of 125I-APC with human α1-antitrypsin (lane 3, band c). The slower migrating complex (band a) barely

Fig 4. Effect of heparin on APC inactivation in plasma in vitro. Aliquots of citrated human or guinea pig plasma were spiked with 5 µg/mL APC in the absence of heparin (open symbols) or the presence of 10 U/mL heparin (closed symbols) and incubated at 37°C. The amidolytic activity remaining as a function of time of incubation was determined. (C, •) Human plasma; (C, ■) guinea pig plasma. The inset shows the inhibition in guinea pig plasma over the first 5 minutes. Notice that the ordinate is linear in contrast to logarithmic in the main graph.

Fig 5. Autoradiographic demonstration of complex formation between 125I-APC and protease inhibitors in guinea pig plasma in vitro. 125I-APC was incubated at 37°C under the following conditions: lane 1, APC alone; lane 2, human α1-macroglobulin, 4 hours; lane 3, human α1-antitrypsin, 2 hours; lane 4, plasma, 15 minutes; lane 5, plasma, 90 minutes; lane 6, heparinized plasma, 15 minutes; lane 7, heparinized plasma, 90 minutes; lane 8, methylamine-treated plasma, 15 minutes; lane 9, methylamine-treated plasma, 90 minutes. Reactions were terminated by the addition of PPACK to a final concentration of 100 µmol/L. Aliquots were then subjected to nondenaturing PAGE on a 6% gel. The top of the photograph represents the top of the separating gel.
entered the gel and comigrated with the complex of $^{125}\text{I}}$-APC with human $\alpha_\text{1}$-macroglobulin (lane 2). Methylamine treatment of guinea pig plasma completely abolished its ability to form this slowly migrating complex with $^{125}\text{I}}$-APC while not affecting formation of the faster migrating complex(es) (Fig 5, lanes 8 and 9). In concordance with these results, the methylamine-treated plasma lost approximately half of its capacity to inhibit APC (data not shown). A similar time-dependent disappearance of $^{125}\text{I}}$-APC and appearance of inhibitor complexes occurred in either citrated or heparinized guinea pig plasma in vitro (compare lanes 4 and 5 with lanes 6 and 7). This result was consistent with the lack of effect of heparin on the inactivation of APC in guinea pig plasma noted above. In vitro, the amount of complex formation correlated directly with the inhibition of enzymatic activity of APC (data not shown).

Inhibitor complexes with identical electrophoretic mobilities to those seen in vitro were also found in samples obtained following injection of $^{125}\text{I}}$-APC into guinea pigs (Fig 6). Complex formation was evident at the earliest sampling time, 1 minute. These samples were collected into an anticoagulant containing a concentration of PPACK that was sufficient to block complex formation during processing of the blood (results not shown). The slowly migrating inhibitor complex comigrated with the complex of $^{125}\text{I}}$-APC with guinea pig $\alpha_\text{1}$-macroglobulin, which was purified by the method of Lonberg-Holm et al. Incubation of the guinea pig $\alpha_\text{1}$-macroglobulin with APC for 15 minutes at 37°C resulted in an 80% reduction of the amidolytic activity of APC against the substrate, S-2266. The amount of $^{125}\text{I}}$-APC associated with the slowly migrating complex peaked at 10 minutes and then declined, while the amount associated with the faster migrating complex(es) peaked at 20 minutes.

The complexes formed upon incubation of $^{125}\text{I}}$-APC in human plasma in vitro were studied using the same nondenaturing gel system (Fig 7). Two complexes (bands c and d), one of which comigrated with the complex of $^{125}\text{I}}$-APC with $\alpha_\text{1}$-antitrypsin (band d), were evident at all times of incubation. The formation of the complex labeled band c, which appeared as a doublet, was clearly accelerated in the presence of heparin (compare lanes 4 and 6, noting the difference in time of incubation) and presumably reflected complex formation with protein C inhibitor, the only APC-inhibitor complex whose formation is known to be accelerated by heparin. On prolonged incubation, a trace of a third complex (band b) became apparent, particularly in the presence of heparin. This complex was consistent with the poor reactivity that was observed between $^{125}\text{I}}$-APC and the purified human protein (lane 2). The amount of total complex formation observed with the nondenaturing gel system (assessed by counting the individual radioactive regions of the gel) was consistent with the degree of inhibition of amidolytic activity (results not shown).

**DISCUSSION**

This study provides evidence that there are at least two elimination mechanisms for APC in the guinea pig. One mechanism involves the formation of APC inhibitor complexes that are subsequently cleared by the liver, presumably by specific receptor systems that recognize the inhibitor portion of the complex as seen in other systems. The other mechanism is the direct interaction of APC itself with liver. This conclusion is based on the results of studies in which active site-blocked forms of APC were used. Control studies confirmed that, in contrast to APC, DIP-APC and
PPACK-APC remained inactive and unable to form inhibitor complexes in vitro and in vivo. Yet the plasma elimination of APC and both of these modified forms was very similar. As was found for APC, only 85% of the total dose of $^{125}$I-DIP-APC was present in the circulation at 1 minute following injection; the other 15% could be almost completely accounted for in the liver. This fast association with the liver was unique for APC and its active site-blocked analogs.

Over half (50% to 70%) of the degraded, radiolabeled material present in the liver at any time was in the form of free iodide, presumably due to the deiodinase activity of liver acting on $^{125}$I-iodotyrosine resulting from degradation of the protein. The radiolabeled material that appeared in the gastrointestinal tract was not associated with tissue but found exclusively in the luminal contents. It was present as greater than 90% free iodide, probably resulting from liver catabolism and subsequent secretion by the parietal cells in the stomach. The excretion of radiolabel via the gastrointestinal tract has been seen in other studies conducted in rodents using $^{125}$I-labeled proteins.

Though the plasma elimination curve of gla-domainless DIP-APC was only slightly different from that of DIP-APC, there was a large difference between the two forms in tissue distribution. A marked reduction in the association with liver and an increased association with kidneys was noted with $^{125}$I-labeled gla-domainless DIP-APC. This had the net effect of accelerating the rate of the $\alpha$ phase of clearance approximately twofold while barely altering the $\beta$ phase.

It is important to note the differences in the elimination of APC and homologous coagulation enzymes. Lollar and Owen found that in rabbits the plasma elimination of human DIP-thrombin was dramatically faster than that of thrombin. They also found that the tissue distribution of DIP-thrombin was dependent on the route of administration; most of the dose was recovered in the lungs soon after intravenous injection and in the liver after intraportal injection. The distribution was widespread after intraarterial injection. In addition, they found that the binding of $^{125}$I-DIP-thrombin to the nonplasma compartment was saturable and reversible. These results provided evidence for high-affinity, active site-independent binding sites for thrombin on the endothelium. Similar studies of radiolabeled DIP-factor Xa and factor IXa in the mouse demonstrated the presence of binding sites for these enzymes on the endothelium. These in vivo results have been confirmed and extended in studies of the binding of thrombin to cultured endothelial cells. In the case of thrombin, one of the major receptors has been shown to be thrombomodulin, which shares sequence homology with the low-density lipoprotein (LDL) receptor.

The results of the present study on the elimination of
125I-APC are in sharp contrast to the elimination of the coagulation enzymes described above. We found little difference in the plasma elimination of 125I-APC and its active site-blocked forms. Furthermore, while the present study confirmed the dependency of tissue distribution of DIP-thrombin on its routes of injection, the tissue distributions of 125I-APC and 125I-DIP-APC were found to be independent of the route of administration. In addition, essentially quantitative recoveries of 125I-APC and 125I-DIP-APC were obtained at the earliest sampling times by summation of the radiolabel present in plasma and liver. However, a minor endothelial compartment would not have been detectable. Thus, in contrast to the situation with the coagulation enzymes, the results of the present investigation indicate that the extrahepatic endothelium does not play a major role in the elimination of APC. Consistent with the present results are studies of others of 125I-APC binding to cultured bovine aortic endothelial cells, in which it was found that APC binding was protein S-dependent, but even in its presence very few APC binding sites per cell were found. Because protein S is thought to exhibit species specificity in its interaction with APC, we also examined the elimination of trace 125I-APC in animals preinjected with human protein S to achieve an initial plasma concentration of 25 μg/mL. The presence of autologous protein S had no effect on radiolabeled human APC elimination in the guinea pig. Therefore, under these conditions as well, there was no evidence for a measurable endothelial compartment. However, one cannot rule out the possibility of species specificity in the interaction of human protein S with guinea pig endothelium.

The other mechanism of APC catabolism involves the formation of inhibitor complexes and their subsequent clearance by specific hepatic receptors as has been reported for other protease-inhibitor complexes. Human APC has been shown to be inactivated by two members of the serpin family of protease inhibitors in human plasma in vitro. It has been reported that the reaction of APC with protein C inhibitor is enhanced by heparin, while APC inactivation by α1-antitrypsin is unaffected by heparin. Our results of spiking 125I-APC into human plasma were consistent with these reports. In addition, we observed the minor formation of two additional inhibitor complexes on prolonged incubation, particularly in the presence of heparin, with lower electrophoretic mobilities. One of these comigrated with the complex of 125I-APC with purified α1-macroglobulin, and the other had a mobility intermediate between the top of the gel and the region containing the complexes of 125I-APC with protein C inhibitor and α1-antitrypsin.

Addition of 125I-APC to guinea pig plasma resulted in the formation of two major inhibitor complexes as analyzed by the same nondenaturing PAGE system that was used for the human samples. Complexes indistinguishable by PAGE formed either in vitro following addition of 125I-APC to plasma or in vivo following bolus administration of 125I-APC to the animals. The very slowly migrating species has been identified as a complex of 125I-APC with an α1-macroglobulin, tentatively designated α1-macroglobulin based on the isolation procedure. This conclusion is based on the following data: (1) the complex had a very low mobility on PAGE similar to that seen with purified human α1-macroglobulin and identical to that seen with purified guinea pig α1-macroglobulin; (2) the formation of this complex was completely and selectively blocked by pretreatment of the plasma with methylamine, followed by its removal; and (3) the inhibitor has been purified, shown to be sensitive to methylamine, and shown to be capable of inhibiting the amidolytic activity of APC on the substrate, S-2266 (H. Berger, manuscript in preparation). The degree of inhibition of APC on this low molecular weight substrate was somewhat surprising in view of the commonly held belief that proteases in complex with α1-macroglobulins retain nearly full activity on such substrates. However, Gyzander and Teger-Nilsson have reported that plasmin in complex with human α1-macroglobulin exhibited only 50% of the activity compared with free plasmin on the substrate, S-2251. The inhibition of the amidolytic activity of APC by guinea pig α1-macroglobulin agreed with the finding that methylamine-treated guinea pig plasma lost its ability to form this complex along with much of its ability to inhibit the amidolytic activity of APC. The second APC-inhibitor complex(es) with the faster mobility migrated similarly, but not identically, to the complex of APC with purified, human α1-antitrypsin. It is tempting to speculate that this may be a complex with guinea pig α1-antitrypsin, because the formation of this complex was unaffected by heparin. However, the identity of the inhibitor, or inhibitors, remains to be determined.

Complexes of APC with both protein C inhibitor and α1-antitrypsin have been identified in the plasma of patients with disseminated intravascular coagulation. Interestingly, in a recent study in the chimpanzee whereby APC was formed in vivo by infusion of factor Xa and phospholipid, a predominant APC-inhibitor complex of low mobility was observed on nondenaturing PAGE, similar to that seen in the guinea pig. While attempts to identify this complex were unsuccessful by sandwich enzyme-linked immunosorbent assay (ELISA), this technique may be rather insensitive for APC complexed with α1-macroglobulin because of its trapping mechanism. One possible explanation for the results seen in the guinea pig is that human APC exhibits a different reactivity towards protease inhibitors in guinea pig plasma than guinea pig APC would. Alternatively, APC of either species may exhibit a different reactivity in vivo versus in vitro similar to what was seen previously with factor Xa or thrombin. Whether α1-macroglobulin is a relevant inhibitor of APC in humans under either pathologic or pharmacologic conditions remains to be elucidated.

One final point, the clearance of the enzymatic activity of APC in vivo was faster than that of the protein moiety. Three different processes were identified that affected the circulation of enzymatic activity: (1) association of APC with the liver; (2) reaction with protease inhibitors; and (3) direct catabolism of APC by liver. These results predict that in the pharmacologic use of APC, greater attention should be directed towards the pharmacokinetics of enzymatic activity than towards the protein itself.
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Pharmacokinetics of activated protein C in guinea pigs
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