Catabolism of hirudin and thrombin–hirudin complexes in the rat

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The metabolic fate of the anticoagulant protein, hirudin, and its complex with thrombin are presently unknown. Therefore we have labelled hirudin and human thrombin–hirudin complex with the residualizing label dilactitol-¹²⁵I-tyramine (*I-DLT) in order to identify their tissue sites of catabolism in the rat. The rapid plasma clearance of hirudin after intravenous injection was unaffected by *I-DLT labelling, and by 2 h 6 % or less of the injected dose remained in the blood. The majority (80.3 ± 4.0 %, $n = 2$) of *I-DLT-hirudin radioactivity recovered in tissues was found in kidney, and kidney was also at least 150 times more active in taking up hirudin, on a weight basis, than any other tissue examined (liver, spleen, skin, muscle, intestine, fat, lung). *I-DLT-hirudin which bound to thrombin was isolated by

chromatography on concanavalin A–Sephacrose; hirudin itself does not bind to concanavalin A. Radioactivity from thrombin–*I-DLT-hirudin was precipitable by anti-thrombin antibody and *I-DLT-thrombin–hirudin was precipitable by anti-hirudin antibody. By 1 h after injection of labelled thrombin–hirudin complexes, the recoveries of radioactivity from hirudin and thrombin in liver were comparable (38.6 ± 3.0 and 36.4 ± 4.1 %, $n = 3$), whereas more radioactivity was recovered in kidney from hirudin than from thrombin (27.6 ± 8.7 compared with 13.6 ± 4.5 %) and less was recovered in lung (0.4 ± 0.2 compared with 17.7 ± 2.9 %). We conclude that hirudin is catabolized predominantly in kidney, whereas the thrombin–hirudin complex is catabolized by both liver and kidney.

INTRODUCTION

The small (7 kDa) polypeptide hirudin is the active anticoagulant protein isolated from the medicinal leech [1]. Hirudin acts by forming a stable non-covalent 1:1 molar complex ($K_d = 20$ fM [2]) with thrombin (EC 3.4.21.5), the key enzyme in the coagulation cascade. Whereas heparin, the most commonly used anticoagulant pharmaceutical, exerts its thrombin-inhibitory activity only when antithrombin III (AT) is present, hirudin does not need a cofactor to inhibit thrombin. Furthermore, the heparin–AT complex reacts with several serine proteases in the coagulation system [3], whereas hirudin reacts selectively and only with thrombin. Thus hirudin has become a promising candidate for antithrombotic treatment [4], and various recombinant hirudin preparations and hirudin analogues are currently under clinical evaluation.

The pharmacokinetics and pharmacodynamics of natural [5,6] and recombinant [7,8] hirudins have been studied previously in healthy volunteers. After intravenous administration, hirudin was cleared from plasma with a half-life of about 1 h, and by 24 h after injection, about 40 % of the administered dose was recovered in urine in an active thrombin-inhibiting form [6]. The cumulative urinary excretion of functionally active hirudin after intravenous injection is species-dependent, being about 40 % for baboons [9], 85 % for dogs [10] and 20 % in pigs (J. Bichler, unpublished work). Of ¹²⁵I-labelled hirudin administered intravenously to rats, about 25 % of the total radioactivity was recovered in urine after 5 h, and functional hirudin accounted for only 15 % of the dose [11].

The findings described above indicate that the mechanism of hirudin clearance is not well understood. Thus the hirudin not recovered in urine might have been eliminated slowly from the body at concentrations below the detection limits of the assays employed or, more likely, it may have been degraded or stored in tissues. Furthermore, of possible clinical relevance, the fate of

hirudin once it is complexed with its target protein thrombin has not been investigated *in vivo*. In this report, we present results from studies in the rat on the clearance of this potential protein pharmaceutical, alone and in complex with human thrombin. Rats were used because the rodent model has consistently shown comparable behaviour for the clearance of both human and murine proteinase inhibitors and proteinase–inhibitor complexes [12].

In this study we have used the residualizing label dilactitol-¹²⁵I-tyramine (*I-DLT) as a radiotracer for monitoring the fate of both hirudin and hirudin–thrombin complex. Residualizing labels permit identification of the tissues and cells active in the uptake and degradation of proteins because the labels become trapped at the site of catabolism, while the carrier protein itself is degraded to diffusible components [13]. As yet these labels have been used primarily as tracers for identifying cells active in the degradation of natural circulating proteins such as albumin [14], lipoprotein [15] or immunoglobulins [16,17]. This work represents the first application of *I-DLT for studies on the clearance of small proteins and enzyme–inhibitor complexes.

MATERIALS AND METHODS

Chemicals and reagents

Human thrombin (T-6759, 3000 National Institute of Health units per mg of protein), BSA, Sephadex G-25, concanavalin A (Con A)–Sephacrose 4B, methyl α -D-mannopyranoside, galactose oxidase and anti-(sheep IgG) antibody were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant desulphato-hirudin (CGP 39393) is co-produced by Ciba-Geigy (Basle, Switzerland) and G.E.N. Therapeutica (Bad Zwischenahn, Germany); its amino acid sequence is identical with the first hirudin variant described [18] except for the absence of the sulphate group on Tyr-63. Rabbit anti-(human thrombin) antibody and human AT were a gift from Dr. Hermann Pelzer,

Abbreviations used: DLT, dilactitol-tyramine; *I-DLT, dilactitol-¹²⁵I or ¹³¹I-tyramine; AT, human antithrombin III; Con A, concanavalin A; buffer A, 10 mM Tris, 1 mM MgCl₂, 0.5 M NaCl, 6 mg/ml poly(ethylene glycol) 6000, 0.2 % NaN₃, pH 8.0.

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Behring Werke (Marburg, Germany). Sheep anti-hirudin antibody was kindly provided by Dr. Reinhart Maschler, G.E.N. Therapeutica. Chromozym-TH (tosylglycylprolylarginine-4-nitranilide acetate) was obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Na¹³¹I was purchased from ICN Pharmaceuticals Inc. (Irvine, CA, U.S.A.) and Na¹²⁵I from Amersham (Arlington Heights, IL, U.S.A.). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), poly(ethylene glycol) 6000 from Fisher Scientific (Pittsburgh, PA, U.S.A.) and NaBH₃CN from Aldrich (Milwaukee, WI, U.S.A.).

Radiolabelling of proteins

Proteins were labelled conventionally using Iodogen as oxidant. Hirudin (50–100 µg) or thrombin (20–40 µg) was dissolved in 25 µl of 0.5 M potassium phosphate buffer, pH 7.7, and placed in an Iodogen-coated tube, followed by addition of 0.3 mCi of Na¹³¹I. After 20 min at room temperature radiolabelled proteins were separated from free ¹³¹I by centrifugal chromatography on a 1 ml Sephadex G-25 column equilibrated in PBS [19]. The specific radioactivity for both protein preparations was 800–1500 c.p.m./ng. Precipitation of ¹³¹I-thrombin by 20 % trichloroacetic acid or of ¹³¹I-hirudin by 90 % acetone, using 1 mg of BSA as carrier protein, was 98 % or more.

Conjugation of *I-DLT to thrombin or hirudin

DLT was radiolabelled and attached to protein as described previously [20]. Briefly, DLT (10 nmol) in Iodogen-coated tubes was labelled with radioactive iodine for 30 min, then transferred to a fresh tube containing 4 units of galactose oxidase and incubated for a further 45 min at 37 °C, to convert the galactose residues into the galactose C-6 aldehyde. Finally, the *I-DLT (0.5–3 mCi) was coupled to amino groups of either hirudin (75–150 µg) or thrombin (50–250 µg) by reductive amination using NaBH₃CN. Labelled protein was separated from unbound *I-DLT as above. Each *I-DLT-protein was 98 % or more precipitable with trichloroacetic acid (thrombin) or acetone (hirudin). Specific activities varied between 300 and 800 c.p.m./ng for thrombin and 1200 and 9000 c.p.m./ng for hirudin.

Preparation of *I-DLT-labelled thrombin–inhibitor complexes

In order to prepare equimolar thrombin–*I-DLT-hirudin or *I-DLT-thrombin–hirudin complexes, a 4–6 molar ratio of inhibitor (60–80 µg) to thrombin (80–100 µg) was incubated in a final volume of 0.2 ml of buffer A [10 mM Tris, 1 mM MgCl₂, 0.5 M NaCl, 6 mg/ml poly(ethylene glycol) 6000, 0.2 %, NaN₃, pH 8.0] for 20 min at room temperature. Unbound *I-DLT-hirudin or hirudin was separated from the complex by affinity chromatography on Con A [21]. The incubation mixture was loaded on to a Con A column (0.5 ml) equilibrated in buffer A. The column was washed with 5 column volumes of buffer A, then bound material was eluted with 5 column volumes of buffer A containing 200 mM methyl α-D-mannopyranoside. Peak fractions (see Figure 3) were pooled and subjected to gel chromatography on a Sephadex G-25 spin column equilibrated in buffer A to remove the methyl α-D-mannopyranoside. Complexes eluted from the Con A column and reisolated by gel filtration on the Sephadex G-25 spin column were checked for the presence of unbound thrombin and hirudin by measuring the enzyme activity of thrombin using the chromogenic substrate Chromozym-TH. No thrombin-inhibitory activity and 2 % or less of free thrombin were detected. For preparation of the *I-DLT-thrombin–AT

complex, Con A-purified *I-DLT-thrombin (0.1 mg) was incubated with an excess of unlabelled AT (0.5 mg) in 0.2 ml (total volume) of buffer A, to ensure complete complexation of *I-DLT-thrombin; no thrombin activity was detected using the chromogenic substrate assay.

Immunoprecipitation of *I-DLT-labelled thrombin–hirudin complexes

Immunoprecipitation studies were carried out according to the procedure of Rush et al. [22]. Briefly, a constant amount (approx. 20 ng) of thrombin–*I-DLT-hirudin complex was incubated with a serial dilution of anti-thrombin antibody in PBS for 1 h at 37 °C, then overnight at 4 °C. Finally, 1 mg of BSA in PBS was added and the incubation mixture was centrifuged for 5 min in an Eppendorf microcentrifuge (16000 g). Radioactivity in the supernatant and pellet was then measured. Alternatively, *I-DLT-thrombin–hirudin complex was incubated with anti-hirudin IgG and the immunoprecipitable fraction of radioactivity was determined.

Studies *in vivo*

Female Sprague–Dawley rats (160–240 g body weight) fed *ad libitum* were maintained on drinking water containing 0.025 % (w/v) NaI for 24 h before use in experiments. *I-DLT-labelled proteins were used within 3 days of preparation for *in vivo* experiments. Injected doses of protein were 20–60 µg/kg for *I-DLT-hirudin, 5–20 µg/kg for *I-DLT-labelled thrombin–hirudin complexes and 5 µg/kg for *I-DLT-thrombin–AT complexes. Radioactive proteins were administered intravenously via the tail vein, blood was sampled and tissues were removed all as described previously [14]; urine was collected by bladder puncture. Plasma samples (typically 25 µl) were precipitated with 250 µl of acetone, and radioactivity in the supernatant and pellet was measured. Hirudin concentration in plasma was determined using the chromogenic substrate assay carried out in the presence of polybrene and urea [23]. Organs were removed *in toto* (liver, kidneys, spleen, lung), or small samples of widely dispersed tissues (skin, skeletal muscle, fat, intestine) were taken and total radioactivity determined as described [14]. To prepare extracts of organs, weighed samples of tissues were homogenized in 4 vol. of ice-cold buffer A, then centrifuged in an Eppendorf microcentrifuge (16000 g) for 5 min to obtain the soluble protein fractions. Thrombin–hirudin complex in Con A fractions of tissue homogenates was measured by an e.l.i.s.a. [24]. Briefly, immobilized anti-thrombin IgG on microtitre plates binds to the thrombin component of the complex; the hirudin moiety of the complex is then recognized by sheep anti-hirudin antibody. Bound complex is detected using anti-(sheep IgG) labelled with peroxidase.

RESULTS

Kinetics of clearance of labelled hirudin preparations

To examine whether the attachment of *I-DLT to hirudin would modify the pharmacokinetic behaviour of the protein, the plasma clearance of radioactivity and functional hirudin activity were measured simultaneously (Figure 1). The recoveries of both radioactivity and thrombin-inhibitory activity were similar. The decline in *I-DLT radioactivity and thrombin-inhibitory activity closely paralleled each other, indicating that the attachment of the residualizing label did not interfere with the normal rapid removal of hirudin from plasma [11].

In separate experiments, ¹²⁵I-DLT-hirudin and conventionally labelled ¹³¹I-hirudin were co-injected into rats (Figure 2a).

Figure 1 Recovery of plasma radioactivity (○) and functional hirudin activity (●), measured by a chromogenic substrate assay, after injection of ^{125}I -DLT-hirudin in rats ($n = 3$, mean $\pm$ S.D.)

Acetone precipitation was used to evaluate the amount of intact protein, compared with radiolabelled degradation products, recovered in plasma. Acetone-precipitable plasma radioactivity was nearly identical for both labelled protein preparations throughout the experiments, and plasma radioactivity from ^{125}I -DLT-hirudin was almost completely acetone precipitable over time. However, the recovery of total and acetone-precipitable radioactivity from the directly labelled hirudin preparation differed significantly after about 20 min, and by 60 min soluble radioactivity represented as much as 50 % of the total. These results are consistent with the expected behaviour of residualizing and conventional tracers, i.e. degradation products from the ^{125}I -DLT-hirudin were retained in tissues at the site of catabolism, whereas a significant fraction of those from ^{131}I -hirudin was released into the circulation. As shown in Figure 2(b), at 2 h after injection, radioactivity from ^{125}I -DLT-hirudin was concentrated in kidney. In contrast, radioactivity from conventionally labelled hirudin was diffusely distributed throughout the body, with a large fraction in skin and muscle.

Purification of thrombin– ^{125}I -DLT-hirudin by chromatography on Con A

Before *in vivo* studies, it was necessary to demonstrate that ^{125}I -DLT-hirudin would form a stable complex with thrombin. Because hirudin does not bind to Con A, whereas the glycoprotein thrombin and the thrombin–hirudin complex do bind to this lectin [25], we could take advantage of the selectivity of Con A to quantify the extent of inhibitor binding, and also to purify complexes. The graph in Figure 3 documents that 1 % or less of applied ^{125}I -DLT-hirudin is bound to Con A. In contrast, about 75 % of ^{125}I -DLT-thrombin was retained on the column, and was eluted with methyl α -D-mannopyranoside; the same percentages of ^{125}I -DLT-thrombin–hirudin complex and ^{125}I -thrombin were also retained by the Con A column (results not shown). All radiolabelled-thrombin preparations not bound to the column did not bind when applied a second time (results not shown), indicating that the capacity of the Con A column had not been exceeded. Thus there was a fraction of the commercial thrombin which could not bind to Con A under the conditions used. When ^{125}I -DLT-hirudin was incubated with thrombin in a $\leq 1:1$ molar ratio, 25 % of the radioactivity bound to Con A. Again, the material that did not bind originally to the column did not bind

Figure 2 Recovery of plasma and tissue radioactivity and hirudin after co-injection of ^{125}I -DLT-hirudin and ^{131}I -hirudin

A mixture of ^{125}I -DLT-hirudin and ^{131}I -hirudin was co-injected into the tail vein of rats ($n = 2$). Blood was taken at the indicated times and the animals were killed at the end of 2 h. (a) Kinetics of plasma clearance of radiolabelled hirudin preparations. Data are shown for total ^{125}I (□) and ^{131}I (■) radioactivity, and acetone-precipitable ^{125}I (○) and ^{131}I (●) radioactivity. Recovery of injected dose in the initial plasma sample (100 s after injection) was $28.0 \pm 0.05\%$ and $30.0 \pm 2.9\%$ for ^{125}I and ^{131}I radioactivity respectively (average $\pm$ range). Absence of range bars indicates that data were within the symbol size. (b) Tissue distribution of radioactivity recovered from radiolabelled-hirudin preparations. Data are expressed for the whole organ as a percentage of the total radioactivity recovered in the body (average $\pm$ range), which was $55.9 \pm 0.8\%$ and $53.0 \pm 2.7\%$, of injected dose for ^{125}I (open bar) and ^{131}I (closed bar) radioactivity. Other = fat, intestine, spleen, lung, plasma.

after reapplication. Only thrombin–hirudin complexes isolated from the Con A column were used for *in vivo* studies.

Immunoprecipitation studies

The stability of the Con A-isolated thrombin– ^{125}I -DLT-hirudin was assessed by immunoprecipitation of the complex with anti-thrombin antibodies. Up to 70 % of the radioactivity from the complex was found to be precipitable, compared with 3 % or less of the radioactivity from ^{125}I -DLT-hirudin alone. To estimate the amount of thrombin immunoprecipitable by the antibody preparation, directly labelled ^{125}I -thrombin and ^{125}I -thrombin–hirudin complex were each incubated with the anti-thrombin antibody and found to be maximally 96 % and 91 % respectively precipitable. Conversely, radioactivity from ^{125}I -DLT-thrombin–hirudin complex was maximally 74 % immunoprecipitable using anti-hirudin antibody, whereas 5 % or less of ^{125}I -DLT-thrombin

Figure 3 Chromatography of ¹²⁵I-DLT-labelled proteins on Con A-Sepharose

Proteins in buffer A, ¹²⁵I-DLT-hirudin (○), ¹²⁵I-DLT-thrombin (●) or a mixture (1:0.8 molar ratio) of thrombin-¹²⁵I-DLT-hirudin complex (■), were loaded on separate Con A columns. Arrow indicates elution with buffer A containing 200 mM methyl α-D-mannopyranoside.

Figure 4 Kinetics of plasma clearance of radiolabelled thrombin-inhibitor complexes and ¹²⁵I-DLT-hirudin

Rats received intravenous injections of ¹²⁵I-DLT-hirudin (○) ($n = 4$), ¹²⁵I-DLT-thrombin-AT (■) ($n = 2$) or a mixture of ¹²⁵I-DLT-thrombin-hirudin (▼) and thrombin-¹²⁵I-DLT-hirudin (▽) complexes ($n = 3$). Blood was removed at the indicated times, and radioactivity measured in plasma is expressed (mean ± S.D.) as a percentage of the injected dose.

alone was immunoprecipitated. Incubation of anti-hirudin antibody with ¹²⁵I-hirudin yielded a maximum of 87% immunoprecipitable radioactivity. These results suggest that the presence of ¹²⁵I-DLT on either thrombin or hirudin may interfere with antibody recognition of the complex, or that there may be a slight dissociation of the complex under the incubation conditions used, i.e. a 1 h incubation at 37 °C, followed by overnight incubation at 4 °C (see below).

Plasma clearance of ¹²⁵I-DLT-labelled enzyme-inhibitor complexes

Figure 4 shows the plasma clearance of radioactivity after intravenous injection of ¹²⁵I-DLT-labelled hirudin and enzyme-inhibitor complexes into rats. We were unable to isolate more than trace amounts of ¹²⁵I-DLT-thrombin-¹²⁵I-DLT-hirudin by

Figure 5 Tissue distribution of radioactivity 1 h after injection of ¹²⁵I-DLT-labelled thrombin-hirudin complexes or ¹²⁵I-DLT-hirudin

Animals described in the legend to Figure 4 were killed after 1 h. Data are expressed (mean ± S.D.) for the whole organ as a percentage of the total radioactivity recovered in the body, which was $72.3 \pm 9.4\%$ and $75.7 \pm 10.4\%$, of injected dose, for ¹²⁵I (hatched bar) and ¹³¹I (closed bar) radioactivity, in the complexes respectively and $81.2 \pm 12.2\%$ of injected dose for ¹²⁵I-DLT-hirudin (open bar). Other = fat, intestine, spleen, lung, plasma, urine.

Con A chromatography. Therefore ¹³¹I-DLT-thrombin-hirudin and thrombin-¹²⁵I-DLT-hirudin were administered simultaneously so that the fate of each component of the complex was followed in the same animal. The plasma clearance of radioactivity from co-injected ¹²⁵I-DLT-labelled thrombin-hirudin complexes was nearly identical, no matter which component of the complex was labelled with ¹²⁵I-DLT, and plasma radioactivity was completely acetone-precipitable at all time points. As previously observed in monkeys [26], the complexes were eliminated from plasma more slowly than hirudin itself. For comparison, the clearance curves for ¹²⁵I-DLT-thrombin-AT and ¹²⁵I-DLT-hirudin are also shown. Despite its larger size the thrombin-AT complex (100 kDa) was eliminated faster than ¹²⁵I-DLT-labelled thrombin-hirudin (42 kDa) or ¹²⁵I-DLT-hirudin (7 kDa).

To evaluate the stability of complexes *in vivo*, plasma was obtained after administration of thrombin-¹²⁵I-DLT-hirudin and immediately applied to a Con A column to estimate the fraction of ¹²⁵I-DLT-hirudin still complexed with thrombin. Con A-bound radioactivity decreased slightly from $84.9 \pm 0.5\%$ after 100 s to $67.6 \pm 8.9\%$ after 60 min ($P = 0.12$; $n = 3$ for each time point, mean ± S.D.). For comparison, $83.5 \pm 1.5\%$ ($n = 4$) of the radioactivity of a portion of the injection solution, kept at room temperature for 1 h, bound when reappplied to the Con A column. Incubation of thrombin-¹²⁵I-DLT-hirudin for 1 h at 37 °C did not result in a significant decrease in the amount of complex recovered, as 80.5% of radioactivity was still retained by Con A, suggesting that the complex should have remained largely intact in the circulation.

Tissue recovery of radioactivity

Figure 5 shows the tissue distribution of radioactivity recovered 1 h after administration of ¹²⁵I-DLT-labelled proteins. At this time, radioactivity remaining in the plasma was below 10% of the injected dose for all administered proteins. Radioactivity from ¹²⁵I-DLT-hirudin injected *per se* was concentrated in kidneys

Table 1 Relative activities of various tissues in the uptake of ¹²⁵I-DLT-labelled hirudin and thrombin–inhibitor complexes

Data are for animals described in the legend to Figure 4, and are expressed as percentage recovered dose/g tissue weight (mean $\pm$ S.D.). Values for all the other organs (fat, intestine, muscle, skin) examined were 0.15 or less.

	Uptake (% dose/g)			
	Liver	Kidney	Lung	Spleen
¹²⁵ I-DLT-hirudin	0.2 $\pm$ 0.1	35.4 $\pm$ 6.3	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0
Thrombin– ¹²⁵ I-DLT-hirudin	5.3 $\pm$ 0.4	19.5 $\pm$ 5.7	0.3 $\pm$ 0.1	1.6 $\pm$ 0.4
¹³¹ I-DLT-thrombin–hirudin	5.1 $\pm$ 1.1	9.6 $\pm$ 3.4	12.9 $\pm$ 0.6	2.7 $\pm$ 0.4

(54.5 $\pm$ 8.8 %, see also Figure 2b). The same pattern of tissue recovery was found (results not shown) for ¹²⁵I-DLT-hirudin which had been regenerated from a thrombin–¹²⁵I-DLT-hirudin complex by trichloroacetic acid precipitation, which destroys thrombin but leaves hirudin intact [27]. The role of the kidney in degradation of hirudin was confirmed, as radioactivity from homogenates of kidneys at 1 h after injection of ¹²⁵I-DLT-hirudin was only 37.9 $\pm$ 9.1 % (n = 5) acetone-precipitable.

Radioactivity from ¹²⁵I-DLT-labelled thrombin–hirudin complexes was found predominantly in liver and kidney (Figure 5). However, whereas the recovery in liver was similar (approx. 35 %) for both proteins, more hirudin was found in kidney than thrombin (28 % compared with 14 %). A significant portion of radioactivity from ¹³¹I-DLT-thrombin–hirudin but not from thrombin–¹²⁵I-DLT-hirudin was also detected in lung (18 % compared with 0.4 %). For comparison, of radioactivity recovered in the body from ¹²⁵I-DLT-thrombin–AT (n = 2), 65 % was found in liver, 11 % in lung and 6 % in kidneys. Table 1 compares the activity of various tissues in the uptake of hirudin and thrombin–hirudin complexes on a weight basis. Kidney was at least 150 times more active in taking up free hirudin than any of the other tissues examined. Besides liver, the spleen also contributed significantly to the uptake of ¹²⁵I-DLT-labelled thrombin–hirudin complexes.

When homogenates from rat tissues obtained 1 h after injection of thrombin–¹²⁵I-DLT-hirudin were analysed by chromatography on Con A, 8.7 $\pm$ 1.5 % of radioactivity from kidney and 25.4 $\pm$ 5.3 % from liver homogenate (n = 4 each) bound to the column. A control experiment, performed by adding the original complex to a freshly prepared control liver or kidney homogenate, revealed that tissue protein itself did not interfere with binding of the complex to Con A. Thus 84.8 % and 83.5 % respectively of radioactivity added to liver and kidney homogenates bound to Con A, compared with 84.9 % of radioactivity from the original complex in buffer. Furthermore, thrombin–hirudin complex was found by e.l.i.s.a. only in fractions eluted from Con A by methyl α -D-mannopyranoside. No immunologically detectable complex was found at 48 h in liver or kidney although approx. 50 % of recovered radioactivity remained in the tissues (results not shown).

By 1 h after injection of ¹²⁵I-DLT-hirudin, 6.8 $\pm$ 2.2 % (n = 4) of the injected dose was recovered in urine, compared with about 1 % for complexes labelled in either the thrombin or hirudin component (n = 3). Urinary radioactivity from ¹²⁵I-DLT-hirudin-injected animals was 96.2 $\pm$ 2.9 % acetone-precipitable, similar to radioactivity derived from thrombin–¹²⁵I-DLT-hirudin (84.6 $\pm$ 11.9 %); however, only 11.2 $\pm$ 2.3 % of urinary radio-

activity from ¹³¹I-DLT-thrombin–hirudin was precipitable with trichloroacetic acid (n = 2 each).

DISCUSSION

The residualizing label ¹²⁵I-DLT is useful for identifying the cells active in catabolism of a protein, because after uptake of the protein from the circulation, the label accumulates at the cellular site of degradation [13]. Conjugation of ¹²⁵I-DLT to hirudin did not influence its pharmacokinetic behaviour, on the basis of the comparable kinetics of plasma clearance of ¹²⁵I-DLT- and ¹³¹I-hirudin and hirudin thrombin-inhibitory activity. As expected, because hirudin is distributed essentially instantaneously in the whole extracellular compartment [28], only 30 % of the administered dose was recovered in the 100 s blood sample. By 1 h after injection of ¹²⁵I-DLT-hirudin, the majority of radioactivity recovered in tissues was in kidney, and only 40 % was in an acetone-precipitable form, indicating that the majority of hirudin was degraded in kidney. In agreement with previous studies, there was excretion of ¹²⁵I-DLT-hirudin via the kidney, although to a lesser extent in rats than in other experimental animals [9,10] or humans [5–8]. In the present experiments, the comparatively small fraction (< 10 %) of hirudin excreted in urine up to 1 h after the injection was almost completely acetone-precipitable, indicating passage through the kidney in a minimally degraded form.

The hirudin variant used in this study has four amino groups available for attachment of ¹²⁵I-DLT, one from the amino group of the N-terminal valine residue and three from intrachain lysine residues [29]. However, among the many naturally occurring hirudin isoforms sequenced so far, no single lysine residue is conserved [4], suggesting that lysine residues are not critical for thrombin–hirudin complex-formation. In contrast, kinetic [30] and X-ray [31] studies have shown that the N-terminal α -amino group of hirudin and the hydrophobic nature of the N-terminal two amino acid residues are crucial for its interaction with thrombin. α -Amino groups on peptides have a lower pK_a and are more nucleophilic than ϵ -amino groups of lysine residues, and therefore may be more readily modified by DLT. Thus there was concern that derivatization of hirudin with the very hydrophilic ¹²⁵I-DLT might interfere with its ability to bind to thrombin.

On the basis of the binding of only 25 % of the ¹²⁵I-DLT-hirudin–thrombin mixture to the Con A affinity column, labelling with ¹²⁵I-DLT did, in fact, affect the efficiency of the hirudin–thrombin interaction. We do not know which or how many amino groups of hirudin were modified by ¹²⁵I-DLT, but on the basis of the results cited above, it is likely that those molecules conjugated with ¹²⁵I-DLT at the N-terminus would not bind to thrombin. Importantly, however, the radiolabelled thrombin–¹²⁵I-DLT-hirudin complexes isolated from Con A, and used for *in vivo* studies, were largely immunoprecipitable by anti-thrombin antibody. Labelling thrombin with ¹²⁵I-DLT did not significantly interfere with its ability to form a complex with hirudin, as demonstrated by immunoprecipitation with anti-hirudin IgG, and the similarity of plasma clearance of ¹²⁵I-DLT-thrombin–hirudin and thrombin–¹²⁵I-DLT-hirudin complexes.

Radioactivity from ¹³¹I-DLT-thrombin–hirudin and thrombin–¹²⁵I-DLT-hirudin was unequally distributed between liver and kidney, with a higher portion of thrombin radioactivity in liver and of hirudin radioactivity in kidney. These results suggest possible dissociation of the complex *in vivo* with release of unbound ¹²⁵I-DLT-hirudin into the circulation and uptake in kidney. Consistent with this finding is the observation that the Con A-bound plasma radioactivity from ¹²⁵I-DLT-hirudin–thrombin decreased slightly with time. Indeed, after injection of

thrombin-hirudin complex in monkeys, a transient prolongation of activated partial thromboplastin time was observed [26], indicating increased anticoagulant activity in plasma possibly coming from released hirudin. The Con A-bound fraction of thrombin-¹²⁵I-DLT-hirudin was significantly less in kidney than in liver homogenates, consistent with increased degradation of free ¹²⁵I-DLT-hirudin. Interestingly, about 15% of radioactivity from *I-DLT-thrombin-hirudin (and from *I-DLT-thrombin-AT) but not from thrombin-*I-DLT-hirudin was recovered in lung. Thus thrombin released from the complex might be cleared by this organ and/or bind to rat AT in the circulation. In earlier work by Lollar and Owen [32], radioactivity from active-site-inactivated *I-thrombin injected into rabbits was recovered largely (73%) in the lung.

Preliminary studies revealed that as much as 30% of radioactivity from thrombin-*I-DLT-hirudin was recovered in hepatic non-parenchymal cells, whereas thrombin-AT was found primarily in parenchymal cells [33]. The hepatic clearance of thrombin-AT is thought to be mediated via a receptor which recognizes a C-terminal sequence of the inhibitor molecule, exposed on complex-formation [34]. The hirudin molecule displays no structural identity with this sequence, and our preliminary data indicate that a different mechanism of uptake may be involved in thrombin-hirudin clearance.

In summary, our results show that *I-DLT is a useful residualizing label for studying the plasma clearance of even small proteins such as hirudin and enzyme-inhibitor complexes. We have shown that injected hirudin which is not excreted in urine is largely catabolized in the kidney, whereas hirudin administered as a complex with thrombin is largely degraded in liver and kidney. Thus the data show that this protein pharmaceutical should not accumulate, but will be rapidly degraded, in tissues.

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