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MECHANISM OF DEIODINATION OF ^{125}I-HUMAN GROWTH HORMONE *IN VIVO*

RELEVANCE TO THE STUDY OF PROTEIN DISPOSITION

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Abstract—Examination of the disposition of proteins employing ^{125}I-labeled tracers can be complicated by the *in vivo* deiodination of the tracer. The purpose of this study was to characterize the mechanism by which ^{125}I-labeled proteins are deiodinated *in vivo* using ^{125}I-human growth hormone (hGH) as a model compound. Intravenous (i.v.) administration of ^{125}I-hGH resulted in a biphasic plasma kinetic pattern, with the majority of radioactivity removed from the plasma during the first 15 min. The level of circulating radioactivity at 2 hr was similar to that 15 min after administration. Radioactivity was eliminated from the animals almost exclusively in the urine. The chemical form of radioactivity in the plasma and urine was analyzed by HPLC, and precipitation of radioactivity with silver nitrate or trichloroacetic acid. Fifteen minutes after administration of ^{125}I-hGH, 30% of the circulating radioactivity was present in the form of iodide (^{125}I$^-$). By 2 hr, the majority of radioactivity in the plasma was in the form of ^{125}I$^-$. The radioactivity in the urine was present exclusively in the form of ^{125}I$^-$. *In vivo* deiodination of ^{125}I-hGH was reflected by the accumulation of radioactivity in the thyroid glands. There was no evidence for the presence of ^{125}I-peptide intermediates in the plasma or urine of treated animals. *In vitro*, ^{125}I-hGH was degraded to ^{125}I-peptide intermediates by thyroid gland but not liver or kidney homogenates. In the absence of cofactors, ^{125}I$^-$ was not observed as an *in vitro* metabolic product. However, in the presence of dithiothreitol and NADPH as cofactors, the predominant metabolic product formed by thyroid gland homogenates was ^{125}I$^-$. The deiodination of ^{125}I-hGH by thyroid gland homogenates was inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), indicating that proteolysis of ^{125}I-hGH was required for deiodination to occur. This was supported by the observation that ^{125}I-labeled proteolytic fragments of ^{125}I-hGH, but not ^{125}I-hGH, were deiodinated by liver or kidney homogenates in the presence of these cofactors. Deiodination by thyroid gland homogenates was inhibited by the sulfhydryl-group blocking reagent, iodoacetate, in a concentration-dependent manner. The characteristics of the *in vitro* deiodination reaction suggest that a form of thyronine 5′-monodeiodinase may be involved in the *in vivo* deiodination of ^{125}I-hGH and possibly other ^{125}I-proteins. These data suggest that the disposition of proteins may be determined more accurately with ^{3}H-, ^{14}C- or ^{35}S-labeled molecules which better represent the characteristics of the native protein.

Recombinant technology has allowed the large-scale production of highly pure protein molecules which had previously been available in small quantities with questionable purity. Naturally occurring proteins such as human insulin, growth hormone and tissue plasminogen activator are now being modified in ways which can provide more desirable kinetic and pharmacologic profiles [1–3]. The development of endogenous and modified proteins as pharmaceutical agents has led to uncertainties regarding the appropriate procedures to study their pharmacokinetics and disposition.

The plasma kinetics of exogenously administered proteins have been commonly examined by radioimmunoassay, employing polyclonal or monospecific antibodies to the protein of interest [4–7]. Tissue distribution, degradation and excretion studies involving proteins have typically employed ^{125}I-labeled molecules as tracers. Previous studies have examined the disposition of an array of proteins and peptides utilizing ^{125}I-labeled material in combination with qualitative measures of protein degradation such as solubility of label in trichloroacetic acid or immunoprecipitation [8–11]. These studies have provided basic information on the disposition of proteins, but suffer from the assumption that the fate of the labeled molecule accurately reflects that of the unlabeled material. Labeling with iodine involves addition of an atom which is not part of the structure of the natural molecule and provides a potential point of metabolism not present in the unlabeled molecule. *In vivo*, ^{125}I-proteins appear to undergo deiodination which may result from destabilization of the iodine by the adjacent hydroxyl group on the phenyl ring or the ability of iodinated tyrosyl residues to act as substrates for deiodinases of thyroid hormones [11–14]. The mechanism by which deiodination occurs and how deiodination impacts upon the interpretation of studies employing iodinated tracers has not been directly addressed. The purpose of this study was to characterize the fate of ^{125}I-human growth hormone (^{125}I-hGH) in an attempt to more clearly define the mechanism by which ^{125}I-labeled proteins are deiodinated *in vivo*.

METHODS

Materials

^{125}I-Biosynthetic human growth hormone (hGH*)

was prepared by the lactoperoxidase technique [15] and purified by gel filtration chromatography. The specific activity of the ^{125}I-hGH used in these studies ranged from 40 to 55 μCi/μg and was diluted with unlabeled biosynthetic hGH (Lot 222-HG6, Eli Lilly & Company) in 0.9% saline prior to dosing. Unlabeled 3-iodo-L-tyrosine and dithiothreitol was purchased from the Sigma Chemical Co. (St. Louis, MO).

In vivo *studies*

Plasma kinetics and tissue accumulation. Male Fischer 344 rats (Harlan, Indianapolis, IN), 210–225 g, were administered ^{125}I-biosynthetic human growth hormone (166 μg/kg, 12 μCi/animal; or 20 μg/kg, 8.1 μCi/animal) intravenously by tail vein. Blood was obtained by cardiac puncture at 1, 5, 15, 30, 60 and 120 min after injection, and plasma was prepared by centrifugation at 3000 rpm for 15 min at 4°. Thyroid glands and samples of liver were obtained 2, 15, 30, 60 and 120 min after injection. Levels of ^{125}I-radioactivity were measured in a gamma counter. The data are expressed as counts per minute per gram tissue or percent of administered dose per gram tissue or milliliter plasma. The chemical nature of the labeled material in plasma was analyzed by size-exclusion HPLC (SE-HPLC) and reverse-phase HPLC (RP-HPLC).

Routes of elimination. Male Fischer 344 rats, 210–225 g, were administered ^{125}I-hGH (60 μg/kg body wt, 8–10 μCi/animal) intravenously by tail vein. The animals were placed in metabolism cages with free access to food and water. Urine and feces were collected over a 24-hr time period and levels of radioactivity were quantified in a gamma counter. Labeled excretion products in the urine were characterized by SE-HPLC and RP-HPLC.

Chromatographic analysis

SE-HPLC. Samples of plasma and urine (10–50 μL) were injected directly onto a Zorbax GF 250 column (9.2 × 250 mm). The column was eluted with 0.025 M ammonium bicarbonate buffer (pH 6.5) at 2 mL/min. Column effluent was collected and radioactivity measured in a gamma counter, or radioactivity profiles were determined with the use of an on-line Ramona 5-LS (Raytrest, U.S.A.) radiochemical detector.

RP-HPLC. Samples of plasma or urine (10–20 μL) were injected without prior preparation onto a Brownlee Aquapore RP-300 column (4.6 × 250 mm) equipped with a 30 mm guard column of the same packing material. The column was eluted with either of two gradients: (1) 15A%/85%B to 80%A/20%B in 20 min, 80%A/20%B to 95%A/5%B in 5 min; (2) 0%A/100%B to 55%A/45%B in 30 min at 1 mL/min. Solvent A = acetonitrile/0.1% trifluoroacetic acid (TFA); solvent B = water/0.1% TFA. Radioactivity profiles were determined with the use of an on-line Ramona 5-LS radiochemical detector, or column effluent was collected and radioactivity measured in a gamma counter.

Assays for degradation products of ^{125}I-*hGH*

Precipitation with trichloroacetic acid (TCA). Urine (25–100 μL) and plasma (50 μL) samples from animals treated with ^{125}I-hGH were precipitated with ice-cold 15% TCA (final concentration). The precipitated proteins were pelleted in an Eppendorf microfuge at 16,000 *g* for 10 min. The supernatant was removed and the pellet was subsequently washed two more times with 15% TCA. The radioactivity in the pellets was determined in a gamma counter, and was assumed to represent undegraded and/or large fragments of ^{125}I-hGH. Similar experiments were run with control rat urine, plasma and buffer spiked with ^{125}I-hGH or ^{125}I-NaI.

Precipitation with silver nitrate. Urine (50–100 μL) and plasma (100 μL) samples from animals treated with ^{125}I-hGH were precipitated with 0.33% silver nitrate (final concentration). The samples were allowed to sit at room temperature for 10 min and complexed material was pelleted at 16,000 *g* for 10 min. Radioactivity in the pellet was counted and considered to be inorganic ^{125}I$^-$. Similar experiments were also performed with control urine and plasma spiked with ^{125}I-NaI or ^{125}I-hGH, and buffer containing 3-monoiodo-L-tyrosine (1 mg/mL, HPLC assay as described below).

In vitro *metabolism*

Tissue preparation. Male Fischer 344 rats (210–225 g) were killed, and homogenates of liver, kidney, and thyroid glands were made to 5% (w/v) in 50 mM sodium phosphate, 1.15% KCl (pH 7.2) with a hand-held glass/glass homogenizer at 4°. Homogenates were sedimented at 2000 *g* for 2 min to pellet large debris and the supernatants were used in the assay of deiodination. Protein was determined by the method of Bradford [16] with bovine serum albumin (BSA) as standard.

Deiodination

Assay. Deiodination of ^{125}I-hGH and ^{125}I-hGH fragments was measured using a RP-HPLC method (see below). Conversion to free ^{125}I$^-$ was expressed as the increase in the percentage of total radioactivity eluting at 5 min under the conditions used. The peak at 5 min cochromatographed with ^{125}I-NaI and was precipitated completely with silver nitrate, indicating that it was inorganic iodide.

RP-HPLC analysis. After precipitation with formic acid, the reaction supernatants were applied onto an Applied Biosystems Aquapore RP-300 (7.0 × 250 mm) column equipped with a 30 mm guard column of the same packing material. The column was eluted with either of two gradients: (1) 10%A/90%B for 5 min, 10%A/90%B to 30%A/70%B in 3 min, 30%A/70%B for 5 min, 30%A/70%B to 100%A in 10 min, 100%A for 2 min; (2) 5%A/95%B for 5 min, 5%A/95%B to 45%A/55%B in 10 min, 45%A/55%B to 95%A/5%B in 10 min, 95%A for 5 min at 1.5 mL/min. Solvent A =

* Abbreviations: hGH, human growth hormone; RP-HPLC, reverse-phase HPLC; SE-HPLC, size-exclusion HPLC; 5′-MD, thyronine 5′-monodeiodinase; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; rT_3, reverse triiodothyronine; DTT, dithiothreitol; i.v., intravenous; 3-MIT, 3-monoiodo-L-tyrosine; NaI, sodium iodide; and PMSF, phenylmethylsulfonyl fluoride.

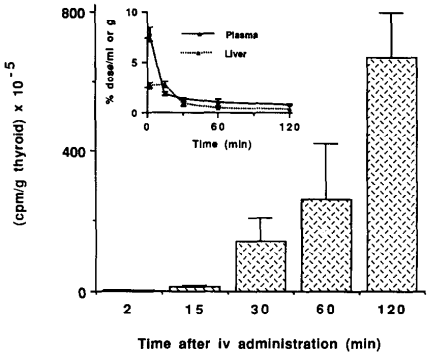

Fig. 1. Accumulation of ^{125}I-radioactivity in thyroid glands of rats after i.v. administration of ^{125}I-hGH. Rats were dosed with ^{125}I-hGH (20 μg/kg, 8.1 μCi/animal) and radioactivity was measured in thyroid glands, plasma, and liver samples. Results are expressed as cpm/g or percent dose/g tissue or mL plasma. Data are means ± SD (N = 3).

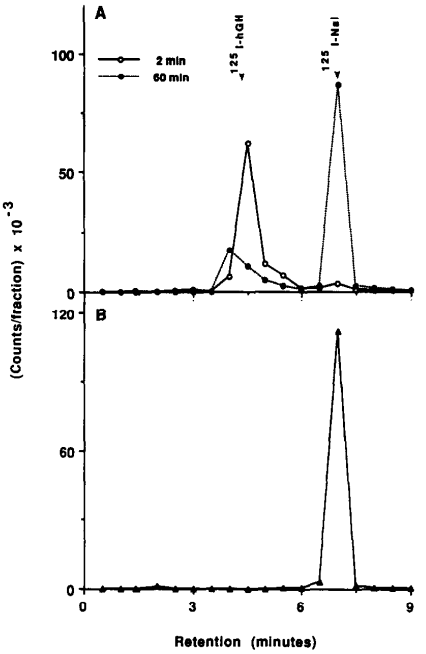

Fig. 2. SE-HPLC profiles of radioactivity in rat plasma and urine after i.v. administration of ^{125}I-hGH. (A) Plasma (2 and 60 min after i.v. dosing) and (B) urine samples were fractionated on a Zorbax GF 250 column as described in Methods. Plasma samples are from the experiment described in Fig. 1; the profile represents a typical chromatogram. The retention of standard ^{125}I-hGH and of ^{125}I-NaI are indicated.

acetonitrile/0.1% TFA; solvent B = water/0.1% TFA. Radioactivity profiles were obtained with the use of an on-line Ramona 5-LS radiochemical detector, or column effluent was collected and radioactivity measured in a gamma counter.

Studies using ^{125}I-hGH as substrate

Several experiments were performed to elucidate the mechanism of ^{125}I-hGH deiodination. All incubations were carried out in 50 mM sodium phosphate/1.15% KCl (pH 7.2), over a period of 2 hr at 37°, using a homogenate protein concentration of 3.75 mg/mL, and a ^{125}I-hGH concentration of 165 ng/mL. Dithiothreitol (DTT, 5 mM) and NADPH (1 mM) were added as cofactors as needed. When necessary, thyroid homogenates were also incubated with ^{125}I-hGH in the presence of the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), followed by an additional 60-min incubation with 5 mM dithiothreitol (DTT) and 1 mM NADPH.

Studies using ^{125}I-hGH fragments as substrate

Preparation of ^{125}I-fragments. Thyroid homogenates were incubated with ^{125}I-hGH for 2 hr as above. After addition of formic acid to 15%, precipitated protein was pelleted at 16,000 *g* for 3 min. The soluble fraction was dried in a Speed-vac (Savant), washed twice with distilled water, and redried in a Speed-vac. Radioactivity corresponding to ^{125}I-hGH fragments (assessed by RP-HPLC, Fig. 7A) was suspended in phosphate buffer and used in subsequent assays.

Incubations. Liver, kidney and thyroid homogenates (3.75 mg/mL) were incubated at 37° with ^{125}I-hGH fragments (100–150 ng equivalents/mL) in a volume of 20 μL. Reactions were carried out for 90 min in the presence of 5 mM DTT and 1 mM NADPH, and terminated by addition of formic acid to 15%. Proteins were pelleted at 16,000 *g* for 1 min, and supernatants were analyzed by RP-HPLC.

RESULTS

In vivo *studies*

Plasma kinetics and tissue accumulation. There was a biphasic decline of ^{125}I-radioactivity in plasma following i.v. administration of ^{125}I-hGH to rats (Fig. 1). The majority of radioactivity was cleared from the plasma during the first 15 min post-administration. After 15 min, an extended phase with a $T_{1/2}$ of >2 hr was observed. The levels of radioactivity in the plasma at the 15- and 120-min time points represent approximately 13 and 8% of the radioactivity present at 1 min, respectively.

Intravenous administration of ^{125}I-hGH resulted in a time-dependent increase in ^{125}I-radioactivity in the thyroid glands (Fig. 1). Two hours after administration, the level of radioactivity in the thyroid glands was 200-fold higher than the level after 2 min. In contrast to the thyroid, radioactivity in the plasma and liver decreased with time (Fig. 1,

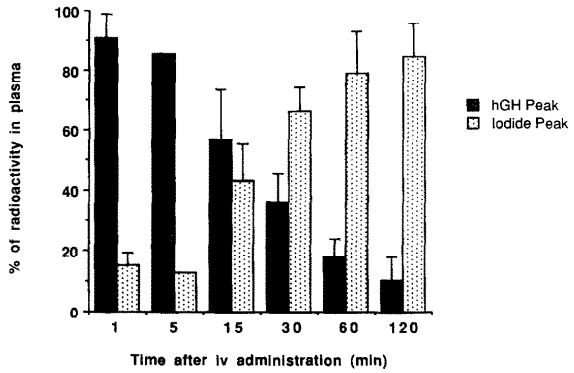

Fig. 3. SE-HPLC characterization of ^{125}I-radioactivity in rat plasma. Rats were dosed i.v. with ^{125}I-hGH and plasma samples were fractionated on a Zorbax GF 250 column. The results indicate the percent of radioactivity at each time point corresponding with the retention of standard ^{125}I-hGH or ^{125}I-NaI. Data are means ± SD (N = 4). Total radioactivity applied to the column ranged from 1000 to 7000 cpm.

inset). Only 13% of the radioactivity present in the liver at 2 min was present 2 hr after the i.v. administration. Two hours after i.v. administration, the level of ^{125}I-radioactivity/g tissue was 1150-fold greater in the thyroid gland than in liver.

The profiles of radioactivity in the plasma 2 and 60 min after i.v. administration were determined by SE-HPLC (Fig. 2A). Two minutes after dosing, the majority of the radioactivity in plasma was associated with material having a retention time corresponding to parent ^{125}I-hGH (4 min). At 60 min, however, the radioactivity was associated almost exclusively with a lower molecular weight component which had a retention time the same as ^{125}I-NaI. The radioactivity in this peak was precipitated completely by silver nitrate. The proportion of total radioactivity in plasma corresponding to ^{125}I-hGH or ^{125}I-determined by SE-HPLC indicated a time-dependent conversion of ^{125}I-radioactivity to inorganic ^{125}I$^-$ (Fig. 3). RP-HPLC analysis of radioactivity in plasma 60 min after i.v. administration also indicated that the predominant form of radioactivity at this time was ^{125}I$^-$ (Fig. 4A).

Routes of elimination. The ^{125}I-radioactivity following i.v. administration of ^{125}I-hGH was eliminated almost exclusively via the urine with >83% of the administered radioactivity recovered within 24 hr (not shown). Size-exclusion (Fig. 2B) and reverse-phase HPLC (Fig. 4B) profiles of urine from the animals demonstrated the presence of a single peak having a retention time corresponding to ^{125}I-NaI. By RP-HPLC, the radioactivity in the urine cochromatographed with ^{125}I-NaI but not with 3-monoido-L-tyrosine (not shown), providing further evidence that the radioactivity was inorganic in nature and not associated with tyrosine residues which could be liberated as a result of complete hydrolysis of ^{125}I-hGH. Radioactivity in the urine of ^{125}I-hGH-treated rats was not precipitated by 15% TCA, resembling the results with biological samples spiked with ^{125}I-NaI. Similarly, the percentage of TCA precipitable radioactivity in plasma decreased with time after i.v. administration of ^{125}I-hGH (Fig. 5). ^{125}I-Labeled material in urine was precipitated with silver nitrate, further indicating its inorganic nature (Fig. 6). Control urine spiked with ^{125}I-NaI behaved similarly. The percentage of ^{125}I-radioactivity in plasma which was precipitated by silver nitrate increased with time after i.v. administration of ^{125}I-hGH. ^{125}I-Labeled organic molecules (^{125}I-hGH and 3-monoiodo-L-tyrosine) were not precipitated by silver nitrate.

In vitro *studies*

TCA assay. Under the conditions used in the *in vitro* assay of ^{125}I-hGH degradation, the increase in TCA solubility represented proteolysis of ^{125}I-hGH (Fig. 7A) as determined by RP-HPLC.

In vitro *deiodination*. In the absence of cofactors, ^{125}I-hGH was converted by thyroid gland homogenates to ^{125}I-labeled proteolytic products having retention times of 10 to 10.5 min and 16.75 to 17.5 min (Fig. 7A). These products were not formed by liver and kidney homogenates (not shown). A representative profile of the RP-HPLC assay for the conversion of ^{125}I-labeled peptides to ^{125}I$^-$ in the presence of cofactors is shown in Fig. 7B.

After a 2-hr incubation of ^{125}I-hGH with liver, kidney or thyroid homogenates in the absence of cofactors the level of ^{125}I$^-$ increased slightly from 2% in the control to 5.4 to 6.8% of the total radioactivity (Fig. 8). Addition of the cofactors, DTT and NADPH, to the incubation stimulated the formation of ^{125}I$^-$ by thyroid homogenates to 70%, but had no effect on ^{125}I$^-$ formation by liver or kidney homogenates. The stimulatory effect of DTT and NADPH on deiodination in the thyroid homogenates was not apparent when reactions were carried out in the presence of the serine protease inhibitor, PMSF.

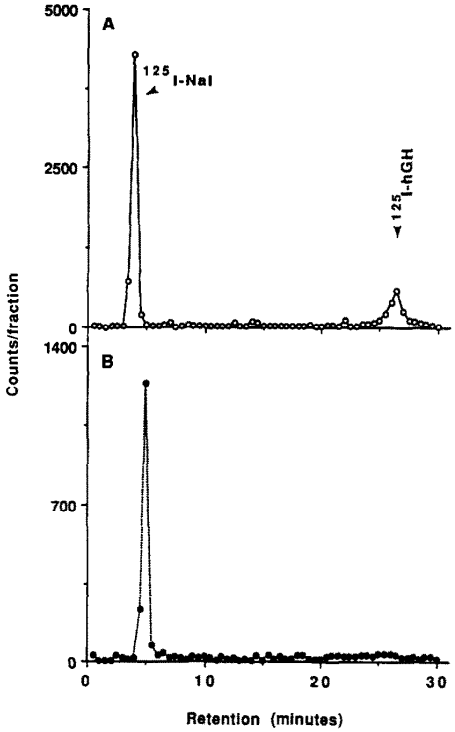

Fig. 4. RP-HPLC profile of radioactivity in plasma and urine of rats after i.v. administration of ^{125}I-hGH. (A) Plasma (60 min after i.v. dosing) and (B) urine samples were fractionated on an Aquapore RP-300 column as described in Methods, using a two-step gradient of acetonitrile/0.1% TFA. Retention of standard ^{125}I-hGH and of ^{125}I-NaI are indicated. The figure shows a representative profile. Plasma and urine were from the same animal analyzed in Fig. 2.

Although liver and kidney homogenates had poor proteolytic activity toward intact ^{125}I-hGH and low levels of deiodinase activity when ^{125}I-hGH was used as substrate, the deiodinase in these tissues was very active against ^{125}I-labeled hGH fragments in the presence of DTT and NADPH (Fig. 9).

Preincubation of thyroid homogenates with iodoacetate produced a concentration-dependent inhibition of the deiodination of ^{125}I-labeled hGH fragments (Fig. 10). The data suggest that the labeled fragments having a retention of 10 to 10.5 min were substrates for the deiodinase, while the more hydrophobic products which were present in smaller quantities appeared to be poorer substrates.

DISCUSSION

The information presented demonstrates that studies on the kinetics, metabolism and elimination of proteins or peptides which employ an ^{125}I-label should be interpreted cautiously. Whether the information obtained from the in-depth analysis of a single ^{125}I-labeled molecule (^{125}I-hGH) pertains to a majority of ^{125}I-proteins/peptides is uncertain. Differences in the molecular weight, tertiary structure, or metabolic stability of a ^{125}I-labeled protein make it difficult to generalize about the *in vivo* disposition and deiodination of these molecules. However, data from previous studies with other ^{125}I-labeled proteins [9–14], along with the results of the study described here suggest that deiodination reactions occur commonly and can obscure the interpretation of disposition studies.

In the present study, conclusions regarding the catabolism of exogenously administered hGH could not be made due to the deiodination of the tracer employed. The kinetics of ^{125}I-radioactivity after i.v. administration of ^{125}I-hGH showed a biphasic pattern which was not comparable to information obtained after i.v. administration of unlabeled hGH to rats [17]. Since iodide has been shown to have a plasma half-life approaching 30 hr in the rat [18], this difference may be related to the kinetics of ^{125}I$^-$ which was the predominant form of the radioactivity in the plasma

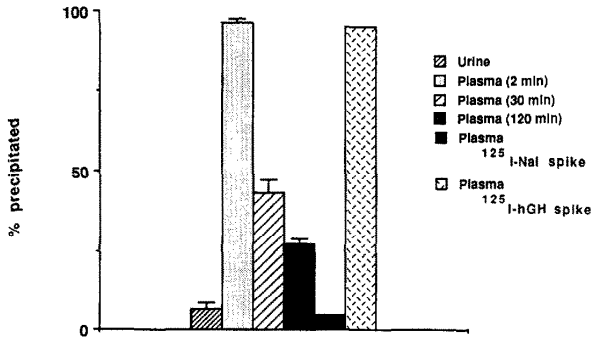

Fig. 5. Trichloroacetic acid precipitation of ^{125}I-radioactivity. Urine and plasma (2, 30 and 120 min post-dosing) from rats after i.v. administration of ^{125}I-hGH (Fig. 1), and control plasma spiked with ^{125}I-hGH or ^{125}I-NaI (5000–10,000 cpm) were precipitated with 15% TCA. The data show the percent of radioactivity in the samples that was precipitated, and represent means ± SD (N = 3) or mean values from two individual experiments.

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