Metabolism of ^{125}I -labelled trypsin in man: evidence for recirculation

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SUMMARY ^{125}I -labelled human trypsin metabolism has been investigated in man. Three subjects received ^{125}I -trypsin and ^{131}I -albumin intravenously. Against a background secretin infusion (1U/kg/h), trypsin decayed biexponentially from the serum with half-lives of 17.5, 21, and 24 minutes for the rapid disappearance phase and 520, 540, and 560 minutes for the slow phase. Between 13% and 38% of the ^{125}I injected was recovered from duodenal juice aspirated continuously over 300 minutes. In contrast, less than 1% of the ^{131}I -albumin injected was recovered. When bile and pure pancreatic juice were collected at endoscopic retrograde cholangiopancreatography after intravenous ^{125}I -trypsin in a fourth subject ^{125}I radioactivity was found to be secreted *via* both these routes. After ^{125}I -trypsin infusion into the duodenum 11% of the total dose was found to be present in the circulation after 75 minutes. These results support the concept that recirculation of trypsin exists in man.

There is evidence for a recirculation of pancreatic digestive enzymes in rabbits. The magnitude of this recirculation suggests a conservation mechanism for intact proteins.^{1,2} Approximately 4% of daily energy expenditure is directed towards protein manufacture.³ Pancreatic enzyme recirculation may therefore represent a method of conserving body energy.

We have previously shown that circulating serum trypsin, an enzyme derived solely from the pancreas, enters the blood stream *via* the superior mesenteric vein, implying that it originates from intestinal reabsorption.⁴ In this study evidence is presented that human ^{125}I -trypsin can be absorbed from the gut and, injected intravenously, human trypsin can be recovered in duodenal juice in man. This demonstration affords additional support for the existence of a recirculation of trypsin.

Methods

PATIENTS

Five patients, four males and one female, aged between 21 and 63 years, were studied. All had normal pancreatic function as assessed by trypsin output in duodenal aspirate after stimulation by

cholecystokinin-pancreozymin (CCK-PZ) and secretin.⁵

Patient 1 had haemochromatosis but a glucose tolerance test was normal. Patient 2 had a hiatus hernia and patient 3, Gilbert's disease. Patients 4 and 5 had obscure abdominal pain.

The studies were approved by the Royal Free Hospital Ethical Practices Committee and informed consent obtained from each patient.

TECHNIQUES

^{125}I -labelled human trypsin was a gift from Hoechst Pharmaceutical, UK. It had been isolated from human pancreas according to the method of Temler and Kägi⁶ and was iodinated using sodium (^{125}I) iodine as described by McConahey and Dixon.⁷ The specific enzymatic activity of the purified trypsin, 119 Katal/kg, was not altered by radioiodination. Free iodine was removed by gel filtration on Sephadex G100 (Pharmacia, Uppsala, Sweden). The column was equilibrated and eluted with 0.5M phosphate buffer, pH 7.4, and trypsin stabilised by addition of 0.2% human plasma protein fraction. The specific activity of the radioactive human trypsin was 40 $\mu\text{Ci}/\mu\text{g}$. The labelled protein was immunoprecipitable with specific rabbit anti-trypsin. Similarly, protein precipitation with equal volumes of trichloroacetic acid (TCA) (20% w/v) yielded 98% of the radioactivity in the precipitate. The

purity of the protein preparation was additionally confirmed by polyacrylamide gel electrophoresis.⁸ The radioactive iodine peak was shown to be confined to trypsin (Fig. 1). ^{131}I -albumin was obtained from the Radiochemical Centre, Amersham, Bucks. The addition of either ^{125}I -trypsin or ^{131}I -albumin to whole blood *in vitro* did not cause free iodine to be liberated, as up to 98% of the radioactivity was still recoverable in the precipitate formed with TCA.

Fig. 1 ^{125}I -Trypsin electrophoresis. After purification by gel filtration through Sephadex G100, ^{125}I radioactivity is confined to a single peak.

CHROMATOGRAPHIC TECHNIQUES

Gel filtration was carried out on a Sephadex G25 column (4×30 cm) (Pharmacia, Uppsala), equilibrated and eluted with 0.2M acetic acid at 4°C.⁹ Duodenal aspirate samples were lyophilised and adjusted to pH2 with 6M acetic acid before application to the column. Fractions of 3 ml were collected. Chromatography of duodenal juice mixed with sodium (^{125}I) iodine established that free iodine did not bind non-specifically with the protein. The electrophoretic mobility of the duodenal juice radioactivity was compared with that of authentic radiolabelled human trypsin on polyacrylamide gel prepared by the method of Reisfeld *et al.*⁸

ANALYTICAL METHODS

Radioactivity was measured on an LKB Wallac Ultragamma 1280-1 Spectrometer. The acid precipitable radioactivity in 1 ml serum was estimated by counting the radioactivity in the precipitate after the addition of an equal volume of TCA. Urine (1 ml) was treated similarly, except that 500 μl albumin solution (4% w/v) was added as a carrier.

Acid-precipitable radioactivity in duodenal juice was estimated after the addition of phosphotungstic acid (PTA) (5% w/v in 2M HCl) with 0.1 M sodium iodide as a carrier.¹⁰

Because of the large amounts of trypsin present in duodenal juice, complete precipitation with anti serum was not possible. The radioactivity associated with immunoreactive trypsin in duodenal juice was therefore estimated as follows. Firstly, 500 μl of the sample was added to 1 ml of a rabbit anti-human trypsin antibody followed by 500 μl of anti-rabbit gamma globulin under the incubation conditions described for the trypsin assay.¹¹ Radioactivity in the precipitate was counted. Secondly, to assess the fraction of total radiolabelled trypsin thus precipitated, a measured dose of authentic trypsin (10 000 counts per minute) (cpm) was added to parallel samples of duodenal juice which were then submitted to the same procedure.

DESIGN OF STUDY

Iodine uptake by the thyroid was blocked throughout the study with Lugol's iodine. After a normal breakfast a duodenal tube was positioned in the third part of the duodenum under fluoroscopic control. A continuous intravenous infusion of secretin (Boots Co. Ltd, Nottingham) (1 Crick-Harper-Raper (CHR) unit/kg/h) was administered throughout the study. Thirty minutes before the start of the study a single injection of CCK-PZ (Boots), 1 CHR unit/kg, was given intravenously to empty the gallbladder and thus minimise contamination of duodenal juice with bile. Trypsin output in the duodenal juice aspirated over this period was used to assess pancreatic function.⁵ ^{125}I -trypsin (0.5–3.1 μCi) and ^{131}I albumin (0.1–0.3 μCi) were injected intravenously at 0 minute. Albumin was given as a control macromolecule to assess non-specific protein leakage into the gut. Blood samples were taken at one minute intervals after the injection of the labelled proteins for five minutes, then at five minute intervals for the next 25 minutes, at half hourly intervals up to 300 minutes, and then daily for the next four days. Duodenal juice was aspirated continuously and pooled into 15 minute aliquots for up to 300 minutes. Additional injections of CCK-PZ, 1 unit/kg, were administered at 120 and 240 minutes. Urine and stools were collected over five days.

In patient 4, ^{125}I -trypsin was injected intravenously and both pancreatic juice and bile collected separately at endoscopic retrograde cholangiopancreatography.

^{125}I -trypsin (4.9 μCi) was infused into the duodenum in patient 5 and serial blood samples collected from a peripheral vein. ^{131}I albumin (0.12 μCi) was injected intravenously to determine plasma volume. After 120 minutes the duodenal contents were washed out with six exchanges (30 ml each) of normal saline. The radioactivity removed with each

wash was counted (Fig. 8). A bolus intravenous injection of CCK-PZ (100 units) and secretin (100 units) was then given and duodenal juice aspirated and pooled into 10 minute aliquots for 30 minutes.

The effect of duodenal juice on ^{125}I -trypsin was assessed in two separate experiments. ^{125}I -trypsin ($0.002\ \mu\text{Ci}$) was incubated with 500 ml of fresh duodenal juice at 18°C for 30 minutes. Free ^{125}I -iodine released from trypsin was estimated by counting the radioactivity appearing in the supernatant after precipitation with TCA.

Fig. 2 ^{125}I -trypsin, measured as protein bound ^{125}I activity, plotted semi-logarithmically against time (minutes) in the first subject. A best-fit curve has been constructed. Graphical analysis yields two exponentials with half-lives of 18 and 540 minutes.

Results

TRYPSIN DISAPPEARANCE FROM SERUM (Fig. 2)
The disappearance of ^{125}I -trypsin, measured as TCA precipitable ^{125}I activity, from the serum in patient 3 is shown in Fig. 2. The data were analysed graphically using a curve stripping technique based on the multiexponential method of analysis of Matthews.¹² Two components were readily stripped and no improvement in resolution was seen by attempting to strip a third exponential. The half-lives of the two exponentials were 18 and 540 minutes for the fast and slow components respectively. Similar results were obtained in the other two studies, the half-lives of the initial rapid disappearance phase being 17.5 and 21 minutes and 560 and 520 for the slow phase in patients 1 and 2 respectively.

APPEARANCE OF ^{125}I IN DUODENAL JUICE

The cumulative appearance of ^{125}I radioactivity in the duodenal juice of patient 3 is shown in Fig. 3.

Fig. 3 Appearance of ^{125}I activity in duodenal aspirate after intravenous ^{125}I -trypsin in subject 3. The 15 minute sample contained 0.7% of the total dose infused; this increased exponentially to 38% after 240 minutes. After a bolus injection of CCK-PZ, ^{125}I activity in the next 15 minutes collection was increased. In contrast, less than 1% of ^{131}I activity was recovered in 240 minutes.

Within 15 minutes of the radiolabelled trypsin injection, 0.7% of the total dose infused had appeared in the duodenal aspirate. This increased to 38% by 240 minutes. Similar results were obtained in patients 1 and 2 where 27% and 13% of the total infused dose was recovered respectively. In contrast, less than 1% of the ^{131}I -albumin infused was recovered in the duodenal juice in any of the three patients. Injections of CCK-PZ, which increase protein secretion from the pancreas,¹³ also increased the total ^{125}I activity in the duodenal juice in all three patients. This was due partly to an increase in the volume of juice recovered and also to a higher concentration of radioactivity.

NATURE OF RADIOACTIVITY IN DUODENAL ASPIRATE

The protein-bound ^{125}I activity in duodenal juice was estimated by precipitation with PTA and by chromatography on Sephadex G25. In subjects 1, 2, and 3, a cumulative total of 1.9%, 3.4%, and 9.3% of the ^{125}I infused was recovered in the PTA precipitable fraction of duodenal juice. This represented 12.5%, 14.5%, and 24.4% of the total ^{125}I

Fig. 4 Chromatographic characterisation of radioactivity in duodenal juice after IV ^{125}I -trypsin (Sephadex G25). In this sample taken from subject 3, 12% of the radioactivity elutes with the protein fraction. The majority of the radioactivity elutes with free iodine or iodotyrosine.

activity recovered in the duodenal aspirate in these subjects.

Sephadex G25 chromatography of the sample of duodenal juice from patient 3 collected within the first 15 minutes is shown in Fig. 4. In this sample 12% of the radioactivity eluted with protein and 63% with either free iodine or iodotyrosine; a small amount of radioactivity eluted with oligopeptides. The protein fractions eluted from the column were lyophilised and subjected to polyacrylamide electrophoresis. The mobility of the ^{125}I -labelled protein in the duodenal aspirate was similar to that of the authentic ^{125}I human trypsin stabilised in a solution of bovine serum albumin (10 mg/ml) (Fig. 5).

Fig. 5 Polyacrylamide gel electrophoresis of ^{125}I -labelled protein in duodenal juice after intravenous injection of ^{125}I -trypsin. The mobility of ^{125}I -trypsin in albumin (10 mg/ml) is shown for comparison. The gels were cut into 0.5 cm sections and the radioactive (hatched area) counted. The ^{125}I radioactivity in duodenal juice is not equally distributed among the protein bands. The mobility of ^{125}I -trypsin in albumin is similar to that of the ^{125}I -protein in duodenal juice. The observed heterogeneity may be related to trypsin autodegradation.

Fig. 6 Appearance of ^{125}I activity in pure pancreatic juice and bile collected at ERCP after IV ^{125}I -trypsin. Total amounts of radioactivity recovered in pancreatic juice or bile per minute are shown. Significant amounts of activity—that is, at least three times background—was recovered within two minutes. Large quantities of radioactivity were also recovered in bile.

APPEARANCE OF ^{125}I IN PURE PANCREATIC JUICE AND BILE

In patient 4, after endoscopic retrograde cannulation of the pancreatic duct, pancreatic juice was collected into 1 or 2 min aliquots. Radioactivity (85 cpm) appeared in pancreatic juice within five minutes of the intravenous injection of the ^{125}I -labelled trypsin (Fig. 6). During the study the cannula was passed into the bile duct. The ^{125}I activity was greater in the samples of bile than in the pancreatic juice, although the secretion rate of the pancreatic juice (0.5–4.0 ml/min) was similar to that of bile (1.2 ml/min). However, only 0.1% of the total dose infused was recovered in the pancreatic juice samples during the 30 minutes of collection. This may be related to the administration of hyoscine methyl-

bromide, given in this single experiment to inhibit duodenal motility, which also inhibits pancreatic exocrine secretion.¹⁴ In view of the low total counts recovered, the radioactivity in the PTA precipitates were not sufficiently above background to be meaningful.

Fig. 7 Appearance of ^{125}I activity in serum after the introduction of ^{125}I -trypsin into the duodenum. CCK-PZ injected intravenously at 120 minutes is associated with a rise in serum ^{125}I radioactivity.

^{125}I -TRYPSIN ABSORPTION FROM THE GUT

After the introduction of ^{125}I -trypsin ($4.9 \mu\text{Ci}$) into the duodenum, significant amounts of radioactivity appeared in the circulation within four minutes and increased to a maximum by 75 minutes (Fig. 7). Based on a plasma volume of 3.2 l (calculated from the dilution of the ^{131}I -albumin injection) the total ^{125}I radioactivity circulating within the plasma at 75 minutes was $0.53 \mu\text{Ci}$ (10.8% of the infused dose). Protein-bound ^{125}I activity, which was measured as TCA precipitable ^{125}I activity, ranged from 24% – 47% of total activity in the serum

samples (mean 33.4%). Chromatographic separation of the pooled 75 minute and 105 minute samples on G25 Sephadex yielded 68% of the total radioactivity in the protein fraction, 24% with small peptides and only 8% with free iodine. The lower recoveries of radioactive protein by TCA precipitation suggests that this material might be associated with the glycoprotein inhibitors in serum. Glycoproteins are incompletely precipitated by TCA.

Figure 8 summarises the second part of the ^{125}I -trypsin absorption experiment. Duodenal washouts were started 120 minutes after intraluminal administration of ^{125}I -trypsin. The ^{125}I radioactivity recovered with each of the six saline exchanges (approximately 30 ml each) are shown. A large amount of radioactivity ($18 \times 10^4\text{ cpm}$) was recovered in the second wash, but this decreased to only $0.9 \times 10^4\text{ cpm}$ in the final wash. CCK-PZ (1 CHR unit/kg) was injected intravenously and duodenal juice aspirated continuously for 30 minutes and 10 minutes aliquots pooled. Significantly more ^{125}I radioactivity was recovered in the duodenal juice samples, (5.3×10^4 , $3.4 \times 10^4\text{ cpm}$, and $4.4 \times 10^4\text{ cpm}$ respectively) than in any of the final four saline washes. The duodenal juice aliquots measured 112, 24, and 24 ml respectively.

Of the total ^{125}I radioactivity in the pooled duodenal juice samples, 66% eluted protein on G25 chromatography and 58% could be precipitated with specific antiserum.

EFFECT OF DUODENAL JUICE ON ^{125}I -TRYPSIN

After the incubation of ^{125}I -trypsin with duodenal juice for up to 30 minutes more than 95% of the label was still precipitable by TCA. The deiodination of labelled trypsin therefore is not due to duodenal juice *per se*.

Fig. 8 ^{125}I -trypsin was introduced into the duodenum at the start of the study. The duodenum was then washed out after 120 minutes with six saline exchanges (30 ml) and the radioactivity removed with each, counted. CCK-PZ ($1\mu\text{g/kg}$) was then injected intravenously and three 10 minute collections of duodenal juice pooled. The ^{125}I radioactivity in the duodenal juice samples is shown. 58.4% of the radioactivity in the pooled samples was immunoprecipitable with specific antiserum.

^{125}I IN URINE AND FAECES

Most of the ^{125}I activity not recovered in duodenal aspirate was excreted in urine, the majority within the first 24 hours. Only minimal amounts of this radioactivity was precipitable with acid. Less than 0.5% of the total ^{125}I injected intravenously in any of the four patients appeared in the faeces over five days. However, in patient 5, 5% of the total ^{125}I infused into the duodenum was recovered in faeces within the next 24 hours.

Discussion

These results show that the radioactivity from ^{125}I -labelled pure human trypsin introduced into the circulation in man rapidly disappears from the serum and reappears in duodenal juice. Similarly, after the introduction of ^{125}I -labelled human trypsin in the duodenum, radioactive material is absorbed into serum and is further recirculated into duodenal juice. The trypsin-derived radioactivity can be shown to be associated with polypeptides by both gel chromatography and acid precipitation.

The ^{125}I -labelled polypeptide in duodenal juice can be precipitated by specific human trypsin antiserum and has similar mobility on polyacrylamide gel electrophoresis to authentic trypsin. The evidence therefore, suggests that this material might indeed be trypsin.

The mechanism whereby radioactivity derived from ^{125}I -trypsin enters the duodenum from the serum is not clear. When both pancreatic juice and bile were collected separately at endoscopic retrograde cholangiopancreatography, circulating trypsin was found to be cleared *via* both these routes. Non-specific leakage through the intestinal wall is improbable, as less than 1% of the intravenously injected control macromolecule, albumin (molecular weight 60 000 daltons), was recovered in the duodenum. Trypsin bound to the glycoprotein inhibitors in serum has a molecular weight in excess of 76 000 daltons.

The presence of trypsin-derived radioactivity in bile was unexpected. Donaldson *et al.*¹⁵ recently demonstrated the presence of serum-derived amylase in bile. It is possible therefore that bile may be an important route of excretion of circulatory pancreatic enzymes. Significant amounts of immunoreactive trypsin have been shown to be excreted daily in bile.¹⁶

The possibility that the ^{125}I -labelled protein recovered in duodenal juice after intravenous ^{125}I -trypsin injection had been synthesised from amino acid products of trypsin catabolism is unlikely for two reasons. Firstly, incorporation of ^{75}Se -selenomethionine from serum into stimulated

pancreatic juice proteins takes at least 30 minutes.¹⁷ In the present studies ^{125}I radioactivity appeared in pancreatic juice within five minutes and in duodenal juice within 15 minutes. Secondly, radioactivity bound to protein in duodenal juice appears to be confined mainly to trypsin as judged by the mobility on polyacrylamide gel electrophoresis. If the ^{125}I radioactivity had been derived from labelled amino acids, it would be distributed throughout all the newly synthesised pancreatic enzyme proteins (Fig. 5).

Free iodine in serum can be secreted into the alimentary tract *via* the salivary gland and stomach.¹⁸ Caution is therefore required in the interpretation of the results using ^{125}I tracer material. In the present studies, over 98% of the ^{125}I -trypsin injected was acid precipitable and was not deiodinated in the presence of whole blood. Free iodine is therefore unlikely to have been introduced as a contaminant. The large amounts of non-protein bound iodine appearing in duodenal juice after ^{125}I -trypsin injection must therefore be related to the metabolism of trypsin.

After the instillation of ^{125}I -trypsin into the duodenum, 11% of the total dose was present in the circulation at 75 minutes. Over two-thirds of this was associated with protein. Intestinal absorption of pancreatic enzymes has been previously demonstrated in adult mammals, including man, for chymotrypsin,^{19 20} elastase,^{21 22} and trypsin.^{23–25} Endogenous trypsin also appears to be reabsorbed in man.⁴ Pancreatic enzyme reabsorption may therefore be a normal physiological process. In the intra-duodenal ^{125}I -trypsin experiment, ^{125}I radioactivity associated with immunoreactive trypsin reappeared in the duodenum after CCK-PZ stimulation. This suggests that the ^{125}I radioactivity absorbed previously from the duodenum had been recirculated. An alternative explanation is that ^{125}I -trypsin absorbed to intestinal epithelium^{26 27} may have been displaced by endogenous trypsin, thus raising the intraluminal ^{125}I -trypsin concentration. The concomitant increase in serum ^{125}I radioactivity at 120 minutes (Fig. 7) makes this unlikely. Displacement with unlabelled enzyme should result in decreased ^{125}I radioactivity appearing in serum. The rise in serum ^{125}I radioactivity is best explained by absorption of recirculated labelled protein. Absorption is probably the initial event in macromolecular protein absorption.

Most of the radioactivity in the circulation was finally recovered in urine. This is the usual excretory route for iodine. Only minimal amounts of this radioactivity were associated with protein, which is consistent with the observation that trypsin is not normally excreted in urine.^{29 30}

In summary, ^{125}I -labelled protein appears in serum after the administration of ^{125}I -trypsin into the duodenum. Similarly, ^{125}I -labelled protein appears in duodenal juice *via* both biliary and pancreatic secretions after the introduction of ^{125}I -trypsin into the systemic circulation. The evidence suggests that this ^{125}I -labelled protein might indeed be trypsin and is consistent with the hypothesis that a recirculation of trypsin exists in man.

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