DISTRIBUTION AND METABOLISM OF SECRETIN AND APROTININ IN ISOLATED PERFUSED RAT PANCREAS

RYOHEI HORI,* SEIGO IWAKAWA** AND KATSUHIKO OKUMURA*

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University,* Sakyō-ku, Kyoto 606, Japan and Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,** Kasumi-1-2-3, Minami-ku, Hiroshima 734, Japan

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The distribution and metabolism of secretin and aprotinin were studied in isolated perfused rat pancreas, which were kept under physiological conditions. At $^{125}$I-[Tyr$^1$]secretin perfusion study the radioactivity appeared in the effluent, rapidly attaining an equilibrium with the perfusate. While the intact secretin concentration decreased to 55% of the perfusate, the distribution space of secretin was estimated to be about 0.11 ml/g pancreas. The ratio of intact secretin to degraded products in the pancreas increased concomitantly with the perfusion of 82 nM secretin. When unlabeled secretin was perfused, immunoreactive secretin concentration in the portal effluent reached also a rapid equilibrium to about 60% of the perfusate. These results suggested that pancreas might clear about 40% of the entering secretin. In the case of $^{125}$I-aperotinin perfusion, intact aprotinin fraction was predominant both in the effluent and in the pancreas. The apparent distribution space was about 40% of the volume of pancreas. Meanwhile, when $^{125}$I-reduced aprotinin or $^{125}$I-[S-carboxamidomethyl]aprotinin was perfused, the decomposed products were significantly increased. These findings indicate that the disulfide bonds in aprotinin molecule play an important role for its stability as well as its antiproteolytic activity.

**Keywords**— Secretin; aprotinin; drug metabolism; isolated perfused rat pancreas; gel filtration; radioimmunoassay

Secretin, a basic heptacosapeptide without disulfide bond and one of the major stimulants of pancreatic secretion, has been used for the diagnosis of exocrine function of the pancreas and gastrinoma as well as for the therapy of duodenal ulcers.$^{1}$ Aprotinin, a basic single chain polypeptide containing 58 amino acid residues crosslinked by three disulfide bridges, is a potent protease inhibitor and it has been used in the treatment of acute pancreatitis and shock.$^{2}$

Nevertheless, these drugs as secretin and aprotinin decompose with a different way in the blood and tissues.$^{3}$ therefore, it would be more than important for clinical trial to know about the disposition of this kind of drugs in target organs or tissues. Little has been studied about the fate of peptide drugs in the target tissue, except that for insulin.$^{4}$ For these studies, in vivo or in vitro perfusion system are necessary because in vitro cell-free preparations do not accurately reflect the intact organs within living organism.

In the present paper, the distribution and metabolism of secretin and aprotinin in the pancreas, the target tissue, were studied, using organ perfusion technique, a suitable approach for studying drug disposition within the organ as previously reported.$^{5,6}$

**MATERIALS AND METHODS**

**Materials**—Highly purified secretin (16000 CHR*** U/mg), synthetic [Tyr$^1$]secretin and purified secretin (3110 CHR U/mg) free from

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*** Crick, Haper and Raper unit.
other gastrointestinal hormones were supplied by Eisai Co., Tokyo. Aprotinin was obtained from Boehringer, Mannheim; carrier-free Na$^{125}$I from The Radiochemical Centre, Amersham, bovine albumin fraction V from Sigma Chemical Co., St. Louis, Sephadex G-25,* Dextran Blue 2000 and Dextran T-70 from Pharmacia Fine Chemicals, Uppsala, Toyopearl HW-55** from Toyo Soda Mfg. Co., Tokyo. Radioimmunoassay kit for secretin was donated by Daiichi Radioisotope Laboratory, Tokyo. Reduced aprotinin and S-carboxamidomethyl]aprotinin were prepared according to the method of Liu and Meienhofer.7)

$^{125}$I-Labeled peptide was prepared by the method of Hunter and Greenwood9) and purified by gel filtration on Sephadex G-25 in 0.1 M acetic acid. It is recognized that secretin is one of unstable peptides even in a lyophilized powder.9) Small degraded products of $^{125}$I-[Tyr]$^1$secretin was observed in the sample, just after the purification by gel filtration. So, $^{125}$I-[Tyr]$^1$secretin used in this study contained about 10% of small peptide fragments, mainly $^{125}$I-tyrosine. The labeled peptide fraction was lyophilized and stored at $-35^\circ$C. Its specific activity was about 100 $\mu$Ci/µg.

Perfusion of Pancreas — Wistar male rats, weighing 250—300 g, were anesthetized with pentobarbital and the pancreas was isolated and perfused by the modified method of Penhos et al.10) as described previously.9) All blood vessels between the pancreas and duodenum were ligated to prevent the endogenous gastrointestinal peptides from affecting the disposition of exogenous peptide. The inlets of the perfusate were from the superior mesenteric and celiac arteries, and the effluent was collected from the portal vein. The pancreas-duodenum was isolated and perfused at a constant temperature (37°C). The perfusion medium was Krebs-Ringer bicarbonate buffer solution supplemented with glucose (5.8 mM), bovine serum albumin (0.5 %) and Dextran T-70 (4.6 %). The perfusate was oxygenated by carbogen gas (O$_2$ 95 %, CO$_2$ 5 %) through a fiber type oxygenator (Microporous Hollow Fiber, Mitsubishi Rayon, Tokyo) and maintained at pH 7.4. The perfusion was performed at the rate of 2.0 ml/min in a nonrecirculating system. For conditioning the pancreas was initially perfused with Krebs-Ringer buffer for 15 min. The pancreatic juice was collected from the duodenal end of the common bile duct.

Pancreatic juice flow was monitored by the method of Tachibana11) and the amylase of the pancreatic juice was determined by the method of Caraway.12)

Metabolism of Secretin and Aprotinin in the Perfused Pancreas — Degradation of peptide in the pancreas perfused as $^{125}$I-labeled peptide (about 0.1 nM) was analyzed by gel filtration of tissue sample extract. After the termination of perfusion, the pancreas was quickly dissected from the duodenum and adipose tissue, then, it was immersed in an extraction solution containing 5.4 M guanidine-HCl and 2.4 M formic acid (5 ml), weighed, minced and homogenized. The homogenate was centrifuged at 20000 × g for 10 min. All these procedures were carried out at 0—4°C. The recovery of tissue-associated radioactivity in the supernatant was 80—90 %. Samples of tissue extract and portal effluents were applied on 1.0 × 70 cm Sephadex G-25 column in the case of secretin perfusion and on 1.5 × 90 cm Toyopearl HW-55 column in the case of aprotinin perfusion, and eluted with 3 M guanidine-HCl and 2.4 M formic acid at 4°C. The collected volume was 3 ml and the flow rate was 20 ml/h. More than 90 % of the radioactivity applied was recovered in the fractions. The column was calibrated with Dextran Blue 2000 as a void volume marker ($V_0$), and with standard $^{125}$I-labeled peptide and $^{125}$I-tyrosine as an internal volume marker ($V_i$).

When unlabeled secretin was perfused, portal immunoreactive secretin concentration was determined by radioimmunoassay kit. To examine the extracellular volume of the perfused
pancreas, 3 mg/ml inulin was perfused for 5 min. The content of inulin in effluents and pancreas extract was determined by the method of Dishe and Borenfreund.\textsuperscript{130}

\textit{Interaction of} \textsuperscript{125}I-Labeled Modified Aprotinin with Trypsin —\textsuperscript{125}I-Modified aprotinin (about 1 nM) was incubated with 14 \(\mu\)M trypsin (Sigma, Type IV from hog pancreas) at 25°C for 5 min. The mixture was polymerized and subjected to disc electrophoresis using 15 \% polyacrylamide, pH 4.3, according to the method of Reisfeld et al.\textsuperscript{140} At the end, the gel was sliced by gel slicer (Miles Lab. Inc., Elkhart) and the radioactivity in the sliced gel was counted.

RESULTS

\textit{Effect of Secretin and Pancreozymin on Pancreatic Exocrine Secretion}

The changes of pancreatic juice flow induced by secretin were monitored, in order to assure the physiological function of the perfused pancreas. Pancreatic juice flow was stimulated by 5 min perfusion of secretin (0.06—16 nM) as shown in Fig. 1. The flow rate reached a peak within 15 min and gradually diminished (Fig. 1A), similar to the response observed as secretin was intravenously injected.\textsuperscript{111} The dose-response relationship of secretin concentration and total juice volume for 30 min is shown in Fig. 1B. There was a successive increase in juice volume upon the increment of secretin concentration.

Figure 2 shows pancreozymin-induced amylase output in the perfused pancreas. The isolated pancreas was previously perfused for 10 min with the perfusion medium after the conditioning period, and then, perfusate was supplemented with 50 CHR mU/ml pancreozymin for the following 40 min. Amylase output and juice volume significantly increased by the effect of pancreozymin, and maintained almost constant during its perfusion.

These results indicated that the perfusion system used was able to keep the physiological condition at least 50 min, so, it would be adequate for the study of drug disposition in the pancreas. \textit{Metabolism of} \textsuperscript{125}I-[Tyr\textsuperscript{1}]secretin and \textsuperscript{125}I-Aprotinin in the Perfused Pancreas

It has been reported that [Tyr\textsuperscript{1}]secretin shows

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Effect of Secretin on Pancreatic Juice Flow in Isolated Perfused Rat Pancreas}
\textit{Pancreatic juice flow was stimulated by the perfusion of secretin (0.06 — 16 nM) for 5 min. A: Changes of flow rate induced by the perfusion of secretin. \textbullet: 0.06 nM, \textcircled{O}: 0.24 nM, \textblack{△}: 1.0 nM, \textblack{△}: 4.0 nM, ◼: 16 nM. B: Relationship of secretin concentration and total juice volume for 30 min. Each point represents the mean value of 3 — 4 experiments. The vertical lines give the SEM.}
\end{figure}
a complete cross-reactivity to antisecretin antiserum and has a potency on exocrine pancreatic secretion.\textsuperscript{16} \textsuperscript{125}I-[Tyr\textsuperscript{1}]secretin was used as the tracer of secretin. Fig. 3 shows representative gel filtration profiles of radioactivity in portal effluent and pancreas extract of samples taken 10 min after the perfusion of radiiodinated secretin or aprotinin (about 0.1 nM). In a preliminary study using Sephadex G-50, it was observed that the elution position of immunoreactive secretin in effluent and tissue extract was almost equal to the position of \textsuperscript{125}I-[Tyr\textsuperscript{1}]secretin. The intact peptide content was calculated by the summation of the radioactivity eluted in the standard peptide fraction. The intact secretin fraction in the effluent decreased to 57.6±6.6 % (mean±S.E.M.) of the perfusate, and only 9.8±1.8 % of radioactivity in the pancreas extract was eluted at the position of the native secretin. When unlabeled secretin (16 or 82 nM) was concomitantly perfused with \textsuperscript{125}I-[Tyr\textsuperscript{1}]secretin, the intact fraction showed little changes in the effluent, but that of the pancreas extract increased to 20.2 % upon the addition of 82 nM secretin (Table I). In the case of \textsuperscript{125}I-aprotinin perfusion, more than 90 % of the radioactivity in the effluent as well as in the pancreas extract was detected at the position of the intact aprotinin. This value was little affected by the addition of unlabeled aprotinin, 0.23 or 2.3 \(\mu\)M (Table II).

In order to elucidate the role of disulfide bonds in the aprotinin molecule on its fate in the target

![Graph](https://via.placeholder.com/150)

**FIG. 2. Effect of Pancreozymin on Pancreatic Juice Flow and Amylase Secretion in Isolated Perfused Rat Pancreas**

Pancreatic juice flow and amylase secretion were stimulated by the perfusion of pancreozymin (Boots; 50 CHR mU/ml) during 40 min. Each point represents the mean of 3 experiments with SEM indicated.

![Graph](https://via.placeholder.com/150)

**FIG. 3. Gel Filtration Profiles of Effluents and Tissue Extracts form \textsuperscript{125}I-[Tyr\textsuperscript{1}]Secretin (A,B) and \textsuperscript{125}I-Aprotinin (C,D) Perfusion**

Samples taken 10 min after the beginning of the perfusion of \textsuperscript{125}I-[Tyr\textsuperscript{1}]secretin or \textsuperscript{125}I-aprotinin were subjected to gel filtration on 1.0 × 70 cm Sephadex G-25 column or 1.5 × 90 cm Toyopearl HW-55 column, respectively. The broken line represents the gel filtration profile of the standard tracer of secretin or aprotinin. A,C: effluent, B,D: tissue extract.
tissue, reduced aprotinin and [S-carboxamidomethyl]aprotinin were prepared and iodinated. Prior to the perfusion study with these derivatives, their binding activity to trypsin was studied by disc electrophoresis. More than 92% of radioactivity migrated to the position of trypsin as 125I-unmodified aprotinin was used. While, the radioactivity of 125I-reduced aprotinin or 125I-[S-carboxamidomethyl]aprotinin bound to trypsin was only 30 or 62% of the total radioactivity, respectively. It is known that the inhibitory effect of aprotinin on trypsin activity is the result from the formation of trypsin-aprotinin complex. These results indicated that the modification of disulfide bonds significantly deteriorate the inhibitory potency to proteases. Figure 4 shows the gel filtration profiles of radioactivity in portal effluent and pancreas extract of samples taken 10 min after the perfusion of 125I-modified aprotinin. In the case of 125I-reduced aprotinin, there was a significant increase of radioactivity eluted at the high molecular weight position between the void volume and the aprotinin fraction, along with an increase of small molecular fragments both in effluent and in pancreas extract. When 125I-[S-carboxamidomethyl]aprotinin was perfused, the ratio of small molecular fragments was significantly augmented compared to that of native aprotinin. In both cases of modified aprotinin the ratio of parent peptide in effluent and in pancreas extract significantly decreased as compared with that of 125I-aprotinin (Table III).

### Table I. Effect of Unlabeled Secretin on the Metabolism of 125I-[Tyr1]secretin in the Perfused Pancreas

<table>
<thead>
<tr>
<th>Unlabeled secretin (nM)</th>
<th>Intact secretin fraction (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Portal effluent</th>
<th>Pancreas extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.6±6.6</td>
<td>9.8±1.8</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>67.6±3.9</td>
<td>15.8±4.4</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>60.1±0.3</td>
<td>20.2±3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SEM of 3–5 experiments.
<sup>b</sup> Significantly different from control (p < 0.05).
Perfusion time: 10 min.

### Table II. Effect of Unlabeled Aprotinin on the Metabolism of 125I-aprotinin in the Perfused Pancreas

<table>
<thead>
<tr>
<th>Unlabeled aprotinin (μM)</th>
<th>Intact aprotinin fraction (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Portal effluent</th>
<th>Pancreas extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91.4±2.4</td>
<td>94.9±3.8</td>
<td></td>
</tr>
<tr>
<td>0.23</td>
<td>96.2±1.0</td>
<td>94.3±2.3</td>
<td></td>
</tr>
<tr>
<td>2.30</td>
<td>92.0±3.1</td>
<td>94.4±3.0</td>
<td></td>
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</table>

<sup>a</sup> Mean ± SEM of 3 experiments.
Perfusion time: 10 min.

![Gel Filtration Profiles](image)

**FIG. 4. Gel Filtration Profiles of Effluents and Tissue Extracts from 125I-Reduced Aprotinin (A,B) and 125I-[S-carboxamidomethyl]Aprotinin (C,D) Perfusion**

Samples taken 10 min after the beginning of the perfusion of 125I-modified aprotinin were subjected to gel filtration on 1.5 × 90 cm Toyopearl HW-55 column. The broken line represents the gel filtration profile of the tracer of modified aprotinin. A,C: effluent, B,D: tissue extract.
Distribution of Secretin and Aprotinin in the Perfused Pancreas

The time course of the distribution of $^{125}$I-[Tyr$^1$]secretin and $^{125}$I-aprotinin is presented in Fig. 5. The radioactivity in the effluent rapidly attained an equilibrium, within a few minutes. From the study of gel filtration, the concentration ratio of effluent to perfusate was $0.55 \pm 0.07$ as $^{125}$I-[Tyr$^1$]secretin was perfused for 10 min, that of $^{125}$I-aprotinin was $0.87 \pm 0.02$. The intact concentration in the pancreas was $10.8 \pm 2.1\%$ of the perfusate for secretin at 10 min, and $38.6 \pm 2.1\%$ for aprotinin. Although radioactivity of small molecular fragments increased in the pancreas with time perfused, little effect on the measurement during the experiment was observed for intact secretin. Radioactivity in the pancreatic juice during the perfusion for 10 min was low.

As for the perfusion of unlabeled secretin, 70 pM or 350 pM, detection of its concentration in the effluent was performed by immunoassay. A fast concentration increase was followed by a rapid equilibrium (Fig. 6). The concentration ratio of effluent to perfusate was $0.59 \pm 0.07$ at 70 pM or $0.67 \pm 0.02$ at 350 pM after the steady state was established ($1.5 - 5.0$ min).

These results indicated that approximately 40\% of the secretin entering the pancreas might be removed and metabolized, while only about 10\% of the aprotinin might be cleared. The extracellular space in the perfused pancreas was tested by infusing 3 mg/ml of inulin, its concentration in pancreas was $18.5 \pm 1.2\%$ ($n = 3$) of the perfusate after the equilibrium. Thus, the distribution space of these peptides was thought to have a limited extracellular space, and some part of them would bind to cell surface.

**DISCUSSION**

the disposition of secretin and aprotinin in the pancreas, the target tissue, was demonstrated with accuracy, by the use of organ perfusion technique. The biological half-life of secretin has been estimated to be about 2 min in man, and Thompson et al$^{18}$ have reported that about 40\% of the secretin entering the kidney, hind leg, or head has been removed in a nonspecific process during the infusion of secretin at 0.4 clinical U/kg/h in dogs. In the present study using tracer technique and radioimmunoassay, secretin distributed rapidly in the pancreas and catabolized at a constant rate, approximately 40\% of the perfused secretin, which was a compatible rate to that of Thompson et al$^{18}$ However, the blood supply to pancreas is relatively small, so that the inactivation of secretin in the pancreas might not be a major determinant in the rapid body clearance of secretin as compared with that in the kidney. In our recent gel filtration study (unpublished data), it appears that $^{125}$I-[Tyr$^1$]secretin specifically binds to pancreatic plasma membranes in an intact form and that the displaced radioactivity, by the unlabeled secretin present in the perfused pancreas, elutes at the position of intact secretin. Thus, for secretin, the metabolic process and the receptor binding process in the

<table>
<thead>
<tr>
<th>Parent peptide fraction (%)$^{a)}$</th>
<th>Portal effluent</th>
<th>Pancreas extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91.1$\pm$ 2.4$^{b)}$</td>
<td>949$\pm$ 3.8</td>
</tr>
<tr>
<td>Reduced aprotinin</td>
<td>28.3$\pm$ 3.4$^{b)}$</td>
<td>12.7$\pm$ 4.9$^{b)}$</td>
</tr>
<tr>
<td>[S-carboxamidomethyl]aprotinin</td>
<td>57.5$\pm$ 5.3$^{b)}$</td>
<td>25.4$\pm$ 3.2$^{b)}$</td>
</tr>
</tbody>
</table>

$^{a)}$ Mean$\pm$ SEM of 3 experiments.

$^{b)}$ Significantly different from control ($p < 0.05$).

Perfusion time: 10 min.
pancreas might be independent.

It has been demonstrated that aprotinin concentrates in kidney of rats and mice and that the decrease of the serum concentration can be fitted by two exponential terms, exhibiting half-life of 0.7 and 7 hours in man.\textsuperscript{20} The present study also showed a slow inactivation of aprotinin in the pancreas, while its modified derivatives containing few disulfide bonds were readily degraded. This low clearance rate might be ascribed as due to the compact structure resistant to most enzymes. These results suggest that the intrachain disulfide bridges of aprotinin play an important role in its stability and that an enzyme such as glutathione-insulin transhydrogenase (disulfide interchange enzyme, EC 1.8.4.2.)\textsuperscript{21} might participate in the initial cleavage of aprotinin molecule. The high molecular products detected in the perfusion of \textsuperscript{125}I-reduced aprotinin might be the result of intermolecular disulfide bridges with endogenous thiol-containing substances; thus, in the case of \textsuperscript{125}I-\textsuperscript{[S-carboxamido-methyl]}aprotinin the ratio of high molecular products was reduced compared to that of \textsuperscript{125}I-reduced aprotinin. Aprotinin is known to form complexes with serine proteases\textsuperscript{22} and polysaccharides.\textsuperscript{23} Its distribution volume was larger than that of secretin in the perfused pancreas, so, this might be explained by a possible binding of aprotinin to serine proteases and/or mucosubstances in the extracellular space of pancreas.

This study documents the importance of the molecular structure of peptides with regard to their metabolic fate as well as their activity and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Distribution of \textsuperscript{125}I-[Tyr\textsuperscript{1}]Secretin (A) and \textsuperscript{125}I-Aprotinin (B) in Isolated Perfused Rat Pancreas}
\textbf{Solid circle represents the total radioactivity of effluent, total height of open column represents the total radioactivity in the pancreas, and the hatched column is the intact secretin concentration, determined by gel filtration study, in the pancreas. Each point is the mean value of 3 experiments. The vertical lines give the SEM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Appearance of Immunoreactive Secretin in the Effluent from the Perfused Pancreas during the Infusion of Secretin (\textbullet{}: 70 pM, \textcircled{O}: 350 pM as Perfusate Concentration)}
\textbf{Each point represents the mean ± SEM of 3 experiments.}
\end{figure}
that the distribution and inactivation of peptide drug at the target tissue should be better understood in order to provide a more rational therapeutic use of peptidic medicals.

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REFERENCES