Am J Physiol Gastrointest Liver Physiol 285: G255–G265, 2003.
First published April 9, 2003; 10.1152/ajpgi.00523.2002.

CCK-58 is the only detectable endocrine form of cholecystokinin in rat

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Submitted 11 December 2002; accepted in final form 3 April 2003

Reeve, Joseph R., Jr., Gary M. Green, Peter Chew, Viktor E. Eysselein, and David A. Keire. CCK-58 is the only detectable endocrine form of cholecystokinin in rat. *Am J Physiol Gastrointest Liver Physiol* 285: G255–G265, 2003. First published April 9, 2003; 10.1152/ajpgi.00523.2002.—CCK-58 differs from CCK-8 in patterns of expression of pancreatic secretion of fluid and amylase and gallbladder contraction. These differences have physiological relevance only if CCK-58 release is stimulated by nutrients entering the intestine and if CCK-58 circulates in sizeable amounts. In this study, we report that when radiolabeled CCK-58 is added to rat blood and plasma is formed, there is extensive loss and degradation of the radioactive peptide. Therefore, a new method was developed to minimize loss and degradation of this label. This method recovered >85% of the label with no detectable degradation. Furthermore, the optimized method recovered all unlabeled exogenous cholecystokinin molecular forms in >80% yields. Blood from fasted rats and rats in which cholecystokinin release was stimulated by the trypsin inhibitor camostat contained only CCK-58 (3.5 ± 0.5 and 17 ± 1.5 fmol/ml, respectively). Because CCK-58 predominates in the blood, this molecular form should be used in studies on the physiology and pathophysiology of cholecystokinin.

pancreas; radioimmunoassay; blood processing; plasma; molecular forms

RELEVANCE OF CCK-58 TO cholecystokinin physiology depends on two factors: *1*) CCK-58 should differ from other molecular forms of cholecystokinin in expression of its bioactivity, and *2*) CCK-58 should be present in the circulation in sizeable amounts. The observation that CCK-58 uniquely stimulates pancreatic fluid secretion in awake (26) and anesthetized (17) rat models is evidence for the first factor. In addition, CCK-58 strongly stimulates pancreatic fluid secretion (chloride rich) at doses where CCK-8 weakly stimulates or actually inhibits fluid (26). Furthermore, CCK-58 does not cause pancreatitis at levels in which CCK-8 clearly initiates this condition (27). Other actions of cholecystokinin reported to differ between CCK-8 and CCK-58 include different patterns of gallbladder contraction (22), central nerve stimulation (9), and peripheral nerve actions (12). Therefore, the remaining factor for evaluation of the physiological relevance of CCK-58 requires that the blood levels of this form be determined.

The presence and amount of CCK-58 observed has varied between laboratories. Our studies observe significant amounts of CCK-58 in the blood of dogs (5) and humans (2, 4), but the proportions in humans are disputed (4, 20). Previous reports on the endocrine forms of cholecystokinin in rats did not detect CCK-58. One study in rats in which cholecystokinin release into blood was stimulated by diversion of bile and pancreatic juice reported that CCK-22 and CCK-8 were the major forms in blood, although CCK-33/39 was the major form stored in the small intestine (8). In another study, in which cholecystokinin release was stimulated with a trypsin inhibitor, the major circulating form reported was CCK-22 (the same predominant form observed in their small intestine extracts) (11). In another work (14), the blood of fasting rats contained nearly equal amounts of CCK-8 and CCK-33, whereas rats stimulated by ad libitum feeding or suckling contained a greater proportion of CCK-8. Furthermore, in a study in which cholecystokinin release was stimulated by ethanol or soybean trypsin inhibitor, the major cholecystokinin forms detected in blood were mainly CCK-22 and CCK-8 (13).

There is evidence that in vitro enzymatic degradation of CCK-58 produces smaller forms of cholecystokinin in humans (21). CCK-58 added to human blood was degraded into smaller forms, and the degradation of CCK-58 could be inhibited by lowering the pH of blood and the plasma formed from the blood. These results led us to ask the question: is the predominance of these smaller molecular forms in rat truly a species difference in the endocrine forms of cholecystokinin

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compared with dogs (5) and humans (2, 4, 18, 20), or does the detection of these smaller forms reflect degradation of endogenous forms of cholecystokinin in vitro during collection and processing of blood?

Thus in this work, we systematically evaluated the stability and recovery of radiolabeled cholecystokinin forms at each step in the collection and processing of rat blood. The radiolabeled peptides had a radioactive iodide added to the tyrosine two amino acids from the NH_2 terminus in CCK-8 or *position 20* in CCK-58. In another set of experiments we used CCK-58 with, and iodide on, both tyrosines at *positions 20* and *52*. The results with iodinated peptides were validated with synthetic peptides that have identical structure to endogenous peptides to ensure that the iodide groups (or modifications to other amino acids occurring during the iodination process) do not alter recovery or degradation. Therefore, exogenous CCK-8, CCK-22, CCK-33, and CCK-58 were added separately to rat blood to validate methods developed with the radiolabeled analogs of cholecystokinin.

The aim of the present research was to develop a method that prevents degradation of ^{125}I-Tyr^{20}-labeled CCK-58 and ^{125}I-Tyr^{2}-labeled CCK-8 added to rat blood and yields over 80% recovery of label. The method development included evaluation of stability and total recovery of labeled CCK-8 (a short acidic peptide) and labeled CCK-58 (a long basic peptide) to all surfaces they would contact during collection and processing of blood. The resultant method yielded good recovery of exogenous molecular forms of cholecystokinin after their addition to rat blood. This new method was applied to the evaluation of the molecular forms of cholecystokinin in the blood of fasted and camostat-stimulated rats. Because the optimized method developed here yielded different results from those observed in previous studies on the endocrine form of cholecystokinin in rat, the degradation of ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 was compared for the new optimized method to a method that simulated previous studies. The new optimized method detected only CCK-58 in fasted and stimulated rat blood.

MATERIALS AND METHODS

Peptides. CCK-8 was purchased from Peninsula (Belmont, CA). Rat CCK-22, CCK-33, and CCK-58 (Fig. 1) were synthesized on an automatic peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) by using 9-fluoenylmethoxy carbonyl (FMOC) strategy. A detailed description of the synthesis of rat CCK-58 is the subject of another report (unpublished

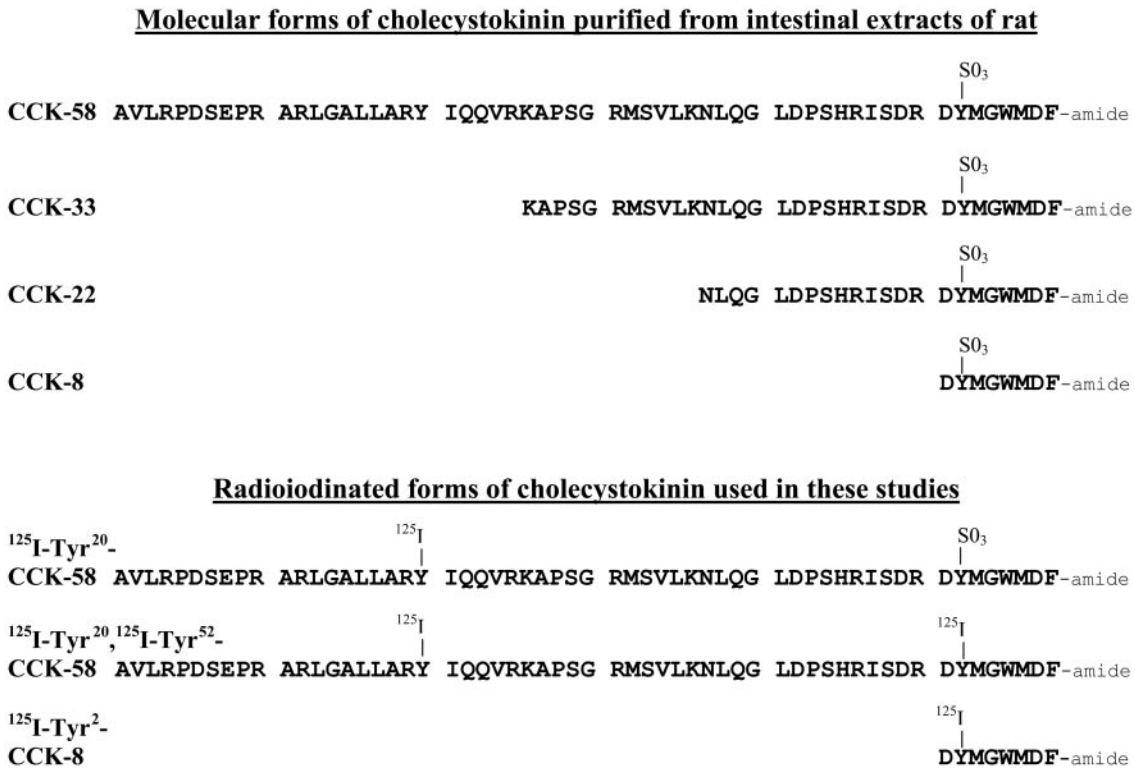

Fig. 1. Molecular forms of cholecystokinin purified from intestinal extracts of rats and radioiodinated forms of cholecystokinin used in these studies. All of these molecular forms of cholecystokinin have a sulfated tyrosine 7 amino acids from their COOH terminus. Only CCK-58 has an additional tyrosine at *position 20*. Only the nonsulfated tyrosine was iodinated by the chloramine T method, so nonsulfated CCK-8 was used to make monoiodo-CCK-8. Likewise, only the tyrosine at *position 20* was iodinated, because the tyrosine at *position 52* was sulfated. Nonsulfated CCK-58 was iodinated on Tyr^{20} and Tyr^{52}, yielding a peptide whose COOH-terminal and NH_2-terminal fragments were radiolabeled. This allows detection of formation of CCK-8 and presumably CCK-22. ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 were used in initial recovery studies, and in subsequent studies it was shown that exogenous CCK-8 and CCK-58 had similar recoveries to the iodinated peptides (Fig. 4).

observation), and similar methods were used for the synthesis, unblocking, purification, and characterization of rat CCK-22 and CCK-33.

Radiolabeling CCK-58 and nonsulfated CCK-8. The cholecystokinin analogs fall into two categories: short negatively charged forms and long positively charged forms. Recoveries can be very different for these two types of peptides. ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 (Fig. 1) were used to evaluate recovery of short acidic forms and long basic forms, respectively, of cholecystokinin from container surfaces encountered during the collection and processing of fasting and fed rat blood. These labels were also used as internal markers of recovery in all determinations of the molecular forms of cholecystokinin in fasted and stimulated rat blood and as markers to decide which fractions from SepPak should be pooled. Additionally, ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 was used to compare degradation of peptides by the method developed in this report to a method that simulates prior studies on the molecular forms of cholecystokinin in rats (8, 11, 13, 14).

Each peptide (10 μg) was dissolved in 20 μl sodium phosphate buffer (pH 7.4). Radiolabeled Na^{125}I (500 μCi) in 5 μl was then added to the peptide solution. Chloramine T (10 μg in 10 μl) was added to the mixture. After 20 s, the oxidation reaction was quenched by the addition of an equal volume of 50% acetic acid. The labeled peptide was separated from the free ^{125}I by G-10 gel-permeation chromatography. The early eluting radioactivity from gel-permeation chromatography was pooled and then purified by reverse-phase HPLC. The tubes that contained the first peak of radioactivity from the G-10 column were pooled, diluted threefold with 0.1% trifluoroacetate, and loaded onto a C-18 reverse-phase column. The ^{125}I-labeled CCK-58 was eluted with a gradient of 22.5–37.5% acetonitrile. The latest major HPLC peak eluting at ~35% acetonitrile was well separated from the unreacted peptide that eluted at ~38.5% acetonitrile. This ^{125}I-labeled CCK-58 radiolabel bound to the CCK-A receptor transfected in CHO cells (data not shown), indicating that its COOH-terminal residues had not been modified during iodination and purification of the label. This purified ^{125}I-labeled CCK-58 radiolabel was used in the recovery studies. The radiolabels made by the chloramine T method were stable for ≤2 mo for use in recovery experiments.

Loss of ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 to experimental containers. The loss of peptide to different experimental containers may account for some of the differences in the detected molecular forms of cholecystokinin. The recoveries of ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 were evaluated for all surfaces they encountered during collection and processing of blood. The containers evaluated included: syringes (20 ml; model 309661, Becton Dickinson, Franklin Lakes, NJ), centrifuge tubes (50 ml; model 14-959-22, Becton Dickinson), SepPaks (0.36 g; model WAT051910, Millipore, Medford, MA), polystyrene test tubes (culture test tube 12 × 75 mm; model 19-961-10, Fisher, Pittsburgh, PA), and culture glass test tubes (12 × 75 mm; model 14-957-12b, Fisher).

The type of test tube makes a major difference in the recovery of various molecular forms of cholecystokinin. The recovery of ^{125}I-Tyr^{2}-labeled CCK-8 from glass was 85, 94, and 98% for incubating, drying, and radioimmunoassay steps, respectively, whereas the recovery of ^{125}I-Tyr^{20}-labeled CCK-58 was considerably lower, 77, 67, and 34% for the same steps with glass tubes. However, the recovery of ^{125}I-Tyr^{2}-labeled CCK-8 from polystyrene tubes was 101, 79, and 85% for these incubating, drying, and radioimmunoassay steps, which was much closer to the recoveries of ^{125}I-Tyr^{20}-labeled CCK-58, namely 96, 78, and 70% with polystyrene tubes.

The only non-test tube surface of major difference in recovery of ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 was SepPak cartridges. In the absence of blood, iodinated CCK-8 was recovered from the SepPak cartridges (n = 10) in a much better yield than iodinated CCK-58 (96 ± 3% vs. 65 ± 3% recovery, respectively). By contrast, in the presence of blood the recovery of iodinated CCK-58 and CCK-8 was >85 ± 1 and 79 ± 1%, respectively, when it was added to blood before SepPak chromatography (Table 1).

Plasma formation method. Previous reports on the molecular forms of cholecystokinin in rat blood involved collecting blood (usually trunk blood) into heparinized or EDTA tubes and forming plasma (8, 11, 13, 14). Freely fed Sprague-Dawley rats (275–325 g) were anesthetized with pentobarbital sodium (10 mg/kg). Trunk blood (obtained by decapitation of anesthetized rats) was collected into centrifuge tubes containing EDTA and ~25,000 ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58. The samples were kept on ice for 1 h, plasma was formed by centrifugation at 3,000 *g* for 10 min at 4°C, and the plasma was frozen at −20°C for 24–72 h. The frozen plasma was thawed by placing the centrifuge tube into a beaker containing room temperature water for 1 h. The thawed sample was chromatographed by SepPak and HPLC as described below.

Development of a new method for collecting and processing blood. Most of the label (50–70%) partitioned with the red blood cells during formation of plasma with the plasma formation method. Likewise, the method used previously in our laboratory to detect CCK-58 in human (2) and dog plasma (5) did not achieve the goal of 80% recovery of labeled CCK-58 from rat blood. New methods were attempted to achieve this goal by using CCK-8 and CCK-58 labels. Whole blood (1 ml) collected by cardiac puncture was added to 9 ml of various buffers containing radiolabeled CCK-8 and CCK-58. The number of counts added to blood of iodinated ^{125}I-Tyr^{2}-labeled CCK-8 varied from 3,800 to 21,000 counts/min (cpm) and of ^{125}I-Tyr^{52}-labeled CCK-58 varied from 9,500 to 23,000 cpm. The buffers tested were 1, 2, 5, and 10% trifluoroacetate; 2, 5, and 10% acetic acid; or 0.05, 0.1, 0.3, and 0.5 M ammonium acetate (pH 3.6) containing 0.5 M NaCl. The best recovery of ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-la-

Table 1. *Cholecystokinin immunoreactivity of blood derived from fasted and camostat fed rats and recovery of ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58*

Rat	Recovery, ^{125}I-Tyr^{2}-labeled CCK-8, %	Recovery, ^{125}I-Tyr^{20}-labeled CCK 58, %	Cholecystokinin Immunoreactivity Eluting Before CCK-58 (pM), CCK-8, CCK-22, CCK-33	CCK-58 Immunoreactivity, pM
Fasted	79 ± 1	85 ± 1	0.3 ± 0.3	3.5 ± 0.5
Camostat	87 ± 3	89 ± 4	0.0 ± 0	17 ± 1.5

Values are means ± SE; n = 3 fasted rats and 6 camostat rats.

beled CCK-58 was with cold 0.1 M ammonium acetate (pH 3.6) buffer containing 0.5 M NaCl.

Acidic buffer method. Freely fed Sprague-Dawley rats (275–325 g) were anesthetized with pentobarbital sodium (10 mg/kg). Blood was collected by exposing their hearts by blunt dissection. Blood (7–12 ml) was collected by cardiac puncture and put into tubes containing EDTA and radiolabeled cholecystokinin analog(s) in cold 0.1 M ammonium acetate (pH 3.6) buffer containing 0.5 M NaCl of enzyme inhibitors from Peptide International (Louisville, KY) diprotin A, E64D, aprotinin, Ac-synthetic inhibitor metalloproteinase (SIMP)-1, and antipain (100 μg each inhibitor) and 100 μg of (1–48) CCK-58 (from the University of California Los Angeles Peptide Synthesis Facility). The number of counts added to blood of iodinated ^{125}I-Tyr^{2}-labeled CCK-8 varied from 3,800 to 21,000 cpm and of ^{125}I-Tyr^{52}-labeled CCK-58 varied from 9,500 to 23,000 cpm. Part of this acidic buffer mixture (10 ml) was in the syringe that blood was drawn into, and the remainder of the acidic buffer (80 ml) was put into a beaker on ice with magnetic stirring. As soon as the blood was taken, it was added to the beaker containing the acidic buffer. The final concentration of each inhibitor was 1 μg/ml. The mixture was centrifuged at 3,000 *g* for 10 min at 4°C. In experiments designed to compare the plasma formation and the acidic buffer methods, the ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 were mixed with blood, the mixture was allowed to sit for 1 h on ice before centrifugation, the supernatant was frozen for 24–76 h, and the frozen mixture was thawed by placing its container in room temperature water for 1 h. In all other experiments a combination of ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 labels (~20,000 cpm each) were used to evaluate stability and recovery of endogenous cholecystokinin, and centrifugation, SepPak (10 g; model WAT043345; Millipore, Medford, MA), and HPLC steps were performed as soon as possible.

Recovery of exogenous CCK-8, CCK-22, CCK-33, and CCK-58 from fasted rat blood. Fasted blood was used to evaluate the recovery of 500 fmol of CCK-8, CCK-33, and CCK-58. The blood collected by cardiac puncture (7–12 ml) was processed as quickly as possible through the HPLC step. Acidic buffer was added in two portions. The first 10 ml was in the syringe containing ~25,000 cpm of radioactivity (^{125}I-Tyr^{2}-labeled CCK-8 or ^{125}I-Tyr^{20}-labeled CCK-58) and 500 fmol of CCK-8, CCK-22, CCK-33, or CCK-58 (without the protease inhibitors described above) was added. The blood acidic buffer mixture was then added to an additional 80 ml of acidic buffer. Recovery of the radiolabels was determined by gamma counting. The counts recovered after the HPLC and drying steps were summed and divided by the total counts added initially to determine the percent recovery (Table 1). Recovery of the synthetic peptides was determined by radioimmunoassay of the fractions after they had been dried.

In a separate experiment to determine whether recovery of small amounts of CCK-8 was similar to recoveries observed for fasting levels of CCK-58, we added 25 fmol of exogenous CCK-8 to 10 ml of rat blood and processed the sample as described above. The concentration of CCK-58 in blood diluted with buffer was 0.25 pM [less than the concentration of endogenous CCK-58 in blood diluted with buffer from fasted rats (0.35 pM).

Determination of endocrine forms of cholecystokinin from fasted and trypsin inhibitor-stimulated rat blood. Radiolabeled analogs of ^{125}I-Tyr^{2}-labeled CCK-8 (3,800–21,000) and ^{125}I-Tyr^{20}-labeled CCK-58 (9,500–22,000) were used to evaluate the stability and recovery of endogenous forms of circulating cholecystokinin processed by the acidic buffer method. Blood from four rats that had been fasted for 24 h were pooled to determine the fasting forms of cholecystokinin. Cholecystokinin release was stimulated by gavage of camostat (0.1 mg/1 g weight dissolved in 2 ml water) into the stomach of rats. Blood collection was initiated 45 min after infusion of camostat.

SepPak chromatography. Plasma from the plasma formation method or the supernatant from the acidic buffer method were loaded onto a C-18 SepPak (10 g cartridges; model WAT04335, Millipore, Milford, MA), rinsed with 20 ml trifluoroacetate, and then eluted with 50 ml 50% acetonitrile containing 0.1% trifluoroacetate. Fractions (4 ml each collected during the loading, rinse, and elution steps) were evaluated for label recovery by gamma counting. The early (^{125}I-Tyr^{2}-labeled CCK-8) and late (^{125}I-Tyr^{20}-labeled CCK-58) radioactive fractions were pooled separately for HPLC chromatography. In the initial experiments with fasted rat blood we pooled all eluted fractions from the SepPak for HPLC chromatography, and we did not get distinct immunoreactive or radioactive peaks because the column was overloaded. Therefore, the early and late radioactive fractions were separated before HPLC chromatography to reduce the total amount of sample loaded on the analytical HPLC column.

HPLC chromatography. Fractions containing early (^{125}I-Tyr^{2}-labeled CCK-8) or late (^{125}I-Tyr^{20}-labeled CCK-58) radioactivity eluted from SepPak were diluted sixfold and loaded onto an analytical Vydac C-18 reverse-phase HPLC column (10 μ, 4.6 × 250 mm; model 218TP104, Western Analytical, Hesperia CA) equilibrated in 0.1% trifluoroacetate. The diluted sample was injected in 7-ml aliquots with a 10-ml injection loop. The sample was eluted with a 10-min gradient to 20% acetonitrile and then a 120-min gradient to 35% acetonitrile at a flow rate of 1 ml/min. Absorbance at 220 and 280 nm were recorded. Fractions (2 ml) were dried by vacuum centrifugation, then counted to determine recovery of radioactivity. The dried samples were dissolved in 600 μl of cholecystokinin radioimmunoassay buffer before adding antibody or tracer.

Bolton-Hunter labeling of CCK-33 for radioimmunoassay. Porcine CCK-33 was purchased from Peninsula Laboratories (Belmont, CA). The ^{125}I-labeled Bolton-Hunter reagent (~4,000 Ci/mmol) was purchased from Amersham (Piscataway, NJ). The benzene solvent with the Bolton-Hunter reagent was evaporated with a gentle stream of nitrogen gas passed through a molecular sieve filter to remove traces of water. CCK-33 (10 μg) was dissolved in 10 μl of 0.05 M acetic acid and injected into the reaction vessel. The reaction buffer (34 μl of 0.05 M borate buffer, pH 10) was added simultaneously to the reaction vessel with the peptide, and the mixture was shaken for 90 min. The unreacted Bolton-Hunter reagent was allowed to decompose by letting the mixture stand at 4°C for 1 h. The mixture was added to 100 μl of 0.1 M sodium phosphate buffer at pH 7.6 containing 0.1% BSA and 0.1% Triton X-100. This mixture was chromatographed on a G-10 Sephadex column (0.75 × 8 cm) and eluted with 0.1 M sodium phosphate buffer at pH 7.6 containing 0.1% BSA. Fractions (0.8 ml) were collected, and each fraction was counted for ^{125}I. The labeled peptide eluted in the void volume and was pooled and diluted with 3 ml 0.1% trifluoroacetate. The peptide mixture was then injected onto a reverse-phase analytical HPLC C-18 column (Vydac, 4.6 mm × 25 cm, Hesperia, CA). A gradient of 22.5–37.5% acetonitrile over 60 min was used to elute the labeled CCK-33 (which eluted at ~31% acetonitrile). The 1-ml fractions were counted by gamma counting, and the peak tubes

were diluted to give ~3,000 cpm in 200 μl in radioimmunoassay buffer.

Cholecystokinin radioimmunoassay. Standard curves were made with cholecystokinin analogs that were quantitated by amino acid analysis or absorbance at 280 nm. Antiserum RO16 (raised to sulfated CCK-10) reacts equally well with CCK-8, CCK-22, and CCK-33 (IC_{50} = 6, 7, and 5 pM), respectively, but is about fivefold less potent for CCK-58 (IC_{50} = 32 pM) The relative cross-reactivity of CCK-8 to rat CCK-58 (fivefold difference) is similar to that observed previously for CCK-8 and canine CCK-58 (fourfold difference) (24). Antiserum RO16 does not require a sulfate group on the tyrosine seven amino acids from the COOH terminus for high-affinity binding of cholecystokinin analogs (23). This antisera has negligible cross-reactivity with gastrin peptides and reacts well with oxidized cholecystokinins (23). Polystyrene tubes yielded better recovery of labeled and cold CCK-58 than glass under radioimmunoassay conditions. Antibody RO16 was used at a 1:100,000 dilution. The label used was porcine CCK-33 radiolabeled with ^{125}I-labeled Bolton- Hunter reagent. Fractions from the HPLC were dried by vacuum centrifugation to 10–50 μl. Fractions and standard curve peptides (CCK-8 and CCK-58) were diluted to 600 μl with radioimmunoassay buffer (0.1 M sodium phosphate buffer, pH 7.5, containing 0.05 M NaCl and 0.025 M disodium ethylenediaminetetracetic acid, 0.1% wt/vol RIA grade bovine serum albumin, and 0.1% Triton X-100; Sigma, St. Louis MO). The 600 μl sample was vortexed for 10 s and allowed to set at room temperature for 30 min. Then 200 μl of radioimmunoassay buffer containing the antisera and 200 μl of buffer containing ~3,000 cpm of labeled CCK-33 were added simultaneously. The final 1-ml volume was incubated with the radiolabel for 16 h at 4°C, and then the free and bound radiolabels were separated with charcoal previously equilibrated with Dextran-70 for 16 h. Standard curves were prepared by plotting the bound cpm/free cpm (B/F) percentages after nonspecific binding blank correction by using a Creative Research Immunoassay computer program. Unknown values were obtained by computer comparison of B/F percentages after nonspecific binding blank correction with standard curves to either CCK-8 (for peptides eluting in the CCK-8, CCK-22, and CCK-33 regions) or CCK-58 (for peptides eluting in the region of CCK-58).

Gastrin circulates at 10-fold higher concentrations than cholecystokinin and therefore could account for some of the observed immunoreactivity with antisera RO16. To determine whether gastrin cross-reactivity could account for any of the observed immunoreactivity, antisera 5135 (equally reactive with gastrin and CCK-8) was used to measure the HPLC fractions from stimulated rat blood. The only detected immunoreactivity with both antisera eluted in the position of synthetic CCK-58. The major form of gastrin-17 elutes in another region of the HPLC and is likely to have been lost during processing of rat blood and SepPak concentration.

RESULTS

Peptide synthesis. Rat CCK-22, CCK-33, and CCK-58 (Fig. 1) all eluted on HPLC as two major peaks (results not shown). The first peak was the nonsulfated peptide, and the second peak was the sulfated analog. Fractions of >90% purity as evaluated by high-performance capillary electrophoresis were pooled, resulting in 18–37 mg of the sulfated peptides at purities of 92–95%. Mass spectral analysis confirmed that the correct mass was obtained for each peptide. Details of the synthesis and purification of rat CCK-58 will be reported elsewhere. The percent peptide weight (72–78%) was used to calculate the dry weight of standard peptides, and stock solution concentrations were verified by their absorbance at 280 nm before diluting them twofold in 0.2% BSA in 1% acetic acid.

Collection of blood samples. Several methods listed in MATERIALS AND METHODS were evaluated by adding ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 to 1 ml blood and adding this mixture to 9 ml of buffer and determining the recovery of the label in the supernatant after centrifugation. Two buffers (trifluoroacetic and acetic acid) lysed the red blood cells and were therefore eliminated from further consideration. The best recovery of label was with cold 0.1 M ammonium acetate (pH 3.6) containing 0.5 M NaCl. This buffer had several properties designed to prevent degradation of the endogenous peptides or the exogenous labels. Importantly, this buffer had the lowest pH that did not lyse red blood cells and allowed the blood to be quickly chilled to inhibit enzymatic degradation of endogenous peptides. In addition, this method diluted the proteolytic enzymes and endogenous cholecystokinin peptides, further reducing the degradation of the peptides.

Comparison of plasma formation and acidic buffer methods: experiments with radioactive CCK-8 and CCK-58. The mean recovery was determined by adding ^{125}I-Tyr^{2}-labeled CCK-8 or ^{125}I-Tyr^{20}-labeled CCK-58 to rat blood and processing the samples by the plasma formation or the acidic buffer methods. The recovery of labeled CCK-8 and CCK-58 was 24 ± 5 and 20 ± 13%, respectively, when blood was processed by the plasma formation method. In contrast, the recovery of labeled CCK-8 and CCK-58 was 86 ± 6 and 88 ± 8%, respectively, when blood was processed by the acidic buffer method. ^{125}I-Tyr^{20}-labeled CCK-58 and unlabeled CCK-58 have different chemical and physical properties because of the iodinated tyrosine. To validate that the recovery of ^{125}I-Tyr^{20}-labeled CCK-58 is similar to the recovery of unlabeled CCK-58, we added 10 fmol of ^{125}I-Tyr^{20}-labeled CCK-58 or 500 fmol of exogenous CCK-58 to rat blood, processed them by the acidic buffer method, and measured their recovery. The recoveries (n = 3) were 102 ± 16 and 83 ± 2% for the labeled and unlabeled CCK-58 peptides; the values were similar, indicating that the radiolabeled peptide can give reasonable indications of recovery. However, the most valid recovery experiments should be done with unlabeled peptide. In these experiments, we used radiolabeled peptides for our method development because it was much faster (minutes vs. days).

Figure 2 shows the elution profile of 500 fmol of CCK-8, CCK-22, CCK-33, and CCK-58 added separately to 7–12 ml rat blood and processed by the acidic buffer method without the enzymatic inhibitors. The retention times of radiolabeled CCK-8 and CCK-58 and exogenous CCK-8, CCK-22, CCK-33, or CCK-58 were 28, 107, 55, 67, 81, and 119 min, respectively. There was only one major peak of immunoreactivity for each of the four cholecystokinin forms. This indicates that very little degradation occurs in vitro, when the pep-

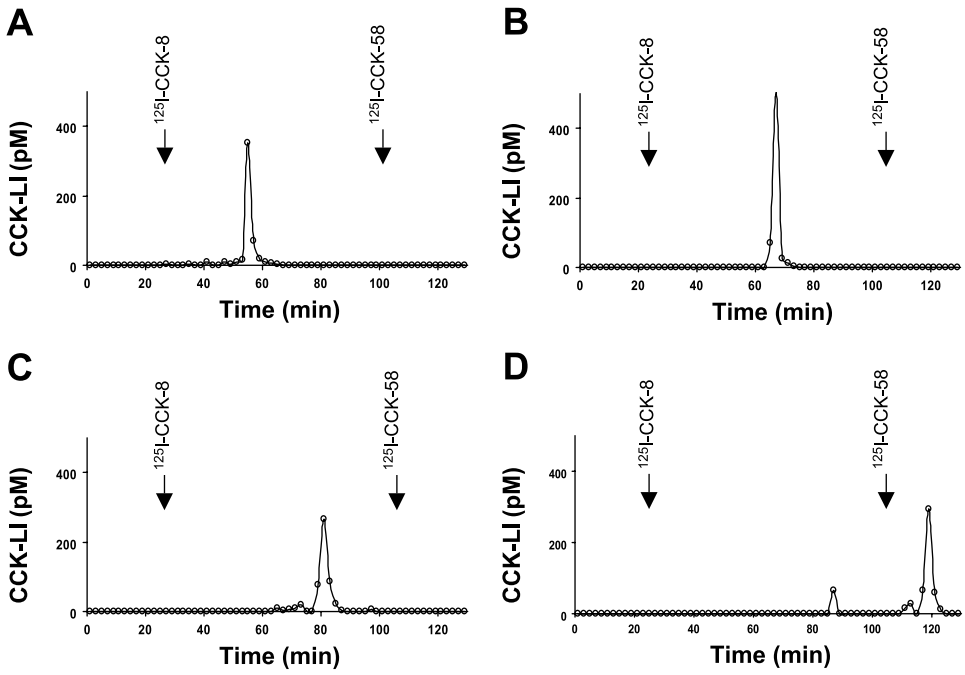

Fig. 2. Recovery of 500 fmol of exogenous cholecystokinin from rat blood by the new optimized method. Exogenous peptides were added to rat blood, and the mixture was processed as described for the acid buffer method. The amount of immunoreactive cholecystokinin for CCK-8 (*A*), CCK-22 (*B*), CCK-33 (*C*), and CCK-58 (*D*) was determined after drying the HPLC fractions and performing the radioimmunoassay in the same tubes used to collect HPLC fractions.

tides were added to blood and processed by the newly developed method. The recovery of the peptides was excellent for the four peptides. The yield was 91, 120, 90, and 86% for CCK-8, CCK-22, CCK-33, and CCK-58, respectively. The minor amount of immunoreactivity that eluted before CCK-58 did not elute in the region of any of the standards used to calibrate the column (Fig. 2*D*). Of note, the HPLC profiles of labeled and exogenous CCK-8 and CCK-58 added to a solution of 0.1% trifluoroacetic acid in water in the absence of blood show similar retention times to the exogenous and radiolabeled peptides added to blood. The same HPLC retention time of cholecystokinin analogs in the presence or absence of blood indicates that neither the endogenous nor the labeled peptides were degraded during the processing of blood.

To determine whether low concentrations of CCK-8 were lost because of nonspecific adsorption to containers or the SepPak matrix, we performed a recovery experiment with 25 fmol of exogenous CCK-8 added to 10 ml rat blood. The blood concentration of 2.5 pM CCK-8 was lower than the 3.5 pM amount of endogenous CCK-58 detected in fasted rat blood. Recovery in this experiment was 22 fmol (88% recovery). On the basis of this result, we estimate detection limits of 1.2 pM for CCK-8. We estimate that if CCK-8 is present in stimulated rat blood, its concentration is <10% of the concentration of CCK-58. Therefore, the detection of only CCK-58 was not due to the loss of CCK-8 during collection and processing of blood.

Molecular forms of cholecystokinin in fasted rat blood. Blood from three sets of four fasted rats (39–40 ml) was processed by the acidic buffer method. No radioactivity was observed in the loading and rinse fractions during SepPak concentration. The SepPak chromatography separated ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 radioactivity into two distinct peaks (Fig. 3*A*). The counts eluted from SepPak are not an accurate estimate of the total counts recovered, because the detection window of the gamma counter is optimized for 1-ml fractions. Therefore, the 4-ml fractions from SepPak allows proper pooling of fractions but gives no reliable estimate of recovery. Recoveries are on the basis of the radioactivity recovered after drying of HPLC fractions. The average recovery of the ^{125}I-Tyr^{2}-labeled CCK-8 label added to fasted blood was 79%. There was no cholecystokinin-like immunoreactivity in the SepPak fraction containing labeled CCK-8 (Fig. 3*B*). The average recovery of the ^{125}I-Tyr^{20}-labeled CCK-58 label added to blood of fasted rats was 85%. This radioactivity eluted as a single peak from the HPLC (Fig. 3*C*), indicating that there was no major breakdown of label during collection and processing of the blood. In two sets of rats, the sole cholecystokinin-like immunoreactivity eluted in the region of CCK-58. In one set of rats, there were minor peaks of cholecystokinin immunoreactivity eluting before the predominant CCK-58 peak (Fig. 3*C*). The most abundant minor components eluted in the region of CCK-8 and CCK-22 (Fig. 3*C*). The labeled rat CCK-58 (^{125}I-Tyr^{20}-labeled CCK-58) eluted before endogenous rat CCK-58. The presence of minor amounts of CCK-8 and CCK-22 could result from some degradation of endogenous CCK-58 in these samples or better recovery of these forms in this experiment. However, we have shown that recovery of low concentrations of CCK-8 is 88%. The occurrence of minor peaks in the position of CCK-8 and CCK-22 in only one of the nine samples of fasted and fed blood suggests that this minor amount of immunoreactivity resulted from incomplete inhibition of CCK-58 degradation in this one experiment. ^{125}I-Tyr^{2}-labeled CCK-8, ^{125}I-Tyr^{52}-la-

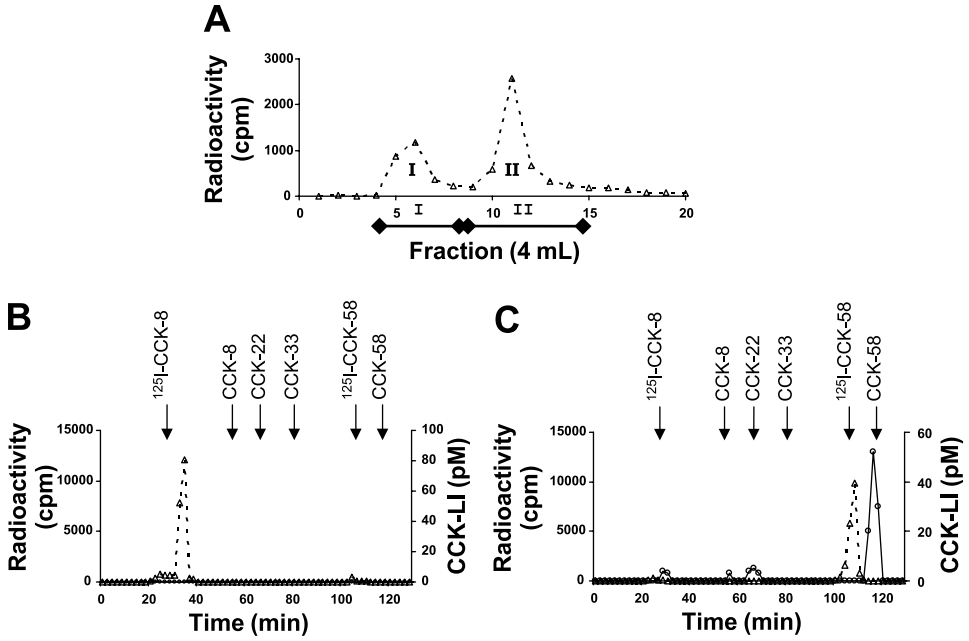

Fig. 3. Molecular forms of cholecystokinin in fasted rat blood. Blood from 4 rats were added to the ammonium acetate buffer described in *Molecular forms of cholecystokinin in fasted rat blood* containing ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58. Radioactivity (dashed lines) eluted from the SepPak in two peaks (*A*). The early radioactivity from the SepPak was diluted and chomatographed by HPLC (*B*). Radioactivity from the second SepPak peak was diluted and chromatographed by HPLC (*C*). All fractions from *B* and *C* were dried and analyzed for cholecystokinin immunoreaactivity (solid line) and radioactivity (dashed lines). Arrows show the average elution time for radiolabeled CCK-8 and CCK-58 and endogenous CCK-58. Results for two other sets of 4 fasted rats were similar to the results shown.

beled CCK-58, and CCK-58 eluted at 28 ± 5, 107 ± 4, and 119 ± 5 min (n = 8–9), respectively. All of the elution times of the radioactive and immunoreactive peaks are the same within experimental error. The variation in elution times is attributed to the amount of protein loaded on the analytical column used in these experiments. We cannot exclude that some of the differences arise from modification of the labeled and endogenous peptides (e.g., oxidation of methionine or tryptophan residues, alpha/beta aspartyl shift, or other amino acid residue alterations). However, cleavage of the endogenous or labeled peptides is unlikely, because this type of modification would cause larger shifts in elution time than those observed. CCK-58 was the predominant peak (3.5 ± 0.5 pM) of cholecystokinin immunoreactivity in fasted rat blood (Table 1).

Molecular forms of cholecystokinin in camostat-stimulated rat blood. The blood from six single rats (7–12 ml) with cholecystokinin stimulated by intragastric infusion of camostat were processed by the acidic buffer method. No radioactivity was observed in the loading and rinse fractions during the SepPak concentration step. As described in Fig. 3*A*, the percent recovery for radioactivity was determined with dried HPLC fractions. The SepPak chromatography separated ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58 into two distinct peaks (Fig. 4*A*). These peaks were chromatographed separately by HPLC. The aver-

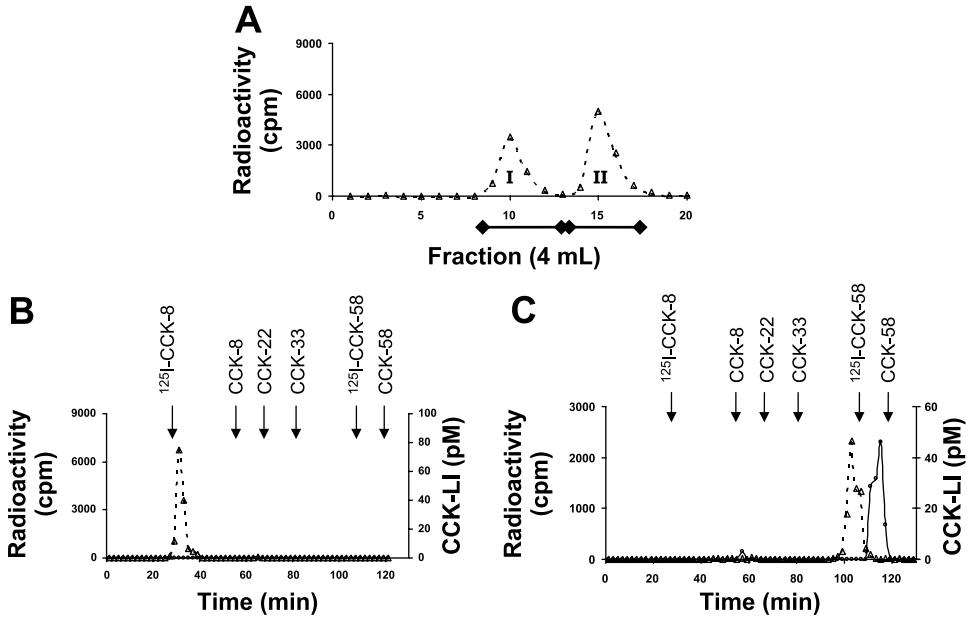

Fig. 4. Molecular forms of cholecystokinin in rat blood after stimulation of cholecystokinin release by camostat. The blood from 1 rat was added to the ammonium acetate buffer described in *Molecular forms of cholecystokinin in camostat-stimulated rat blood* containing ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20}-labeled CCK-58. Radioactivity (dashed lines) eluted from the SepPak in 2 peaks (*A*). Early radioactivity from the SepPak was diluted and chomatographed by HPLC (*B*). The second peak of radioactivity from the SepPak was diluted and chromatographed by HPLC (*C*). All fractions from *B* and *C* were dried and analyzed for cholecystokinin immunoreaactivity (solid line) and radioactivity (dashed line). Arrows show the average elution time for radiolabeled CCK-8 and CCK-58 and endogenous CCK-58. The results from 5 other rats whose cholecystokinin release was stimulated by gastric camostat are similar to the results shown in this figure.

age recovery of the ^{125}I-Tyr^{2}-labeled CCK-8 label added to blood was 87%. It eluted as a single peak during the HPLC step. There was no cholecystokinin-like immunoreactivity associated with the labeled CCK-8 (Fig. 4*B*). The average recovery of the ^{125}I-Tyr^{20}-labeled CCK-58 label was 89%. This radioactivity eluted as a single peak from the HPLC (Fig. 4*C*), indicating that there was no major breakdown during collection and processing of the blood. In all six rats, the sole cholecystokinin-like immunoreactivity eluted in the region of CCK-58. (Fig. 4*C*). The average concentration of CCK-58 in blood from fed rats was 17 ± 1.5 pM (Table 1).

Recovery of ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 after plasma formation and acidic buffer methods. We present a second method different from the acidic buffer method to suggest a possible explanation as to why other investigators see smaller molecular forms of cholecystokinin than we observed. This method formed plasma directly after collecting blood, as done in earlier studies (8, 11, 13, 14) on the molecular forms of cholecystokinin in rat blood. Our sample handling (incubation on ice for 1 h before freezing plasma and thawing plasma for 1 h) may not exactly duplicate the way samples were processed in earlier studies. Nevertheless, the plasma formation study with CCK-58 labeled on two tyrosines provides a possible reason for the differences in the amount of degradation observed between the plasma formation and acidic buffer methods.

Figure 5 compares the recovery of ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 processed by the plasma formation and acidic buffer methods. In these experiments, the blood or blood-buffer mixtures were kept on ice for 1 h before centrifugation, and the plasma and the plasma-buffer mixtures were frozen at −20°C. Samples were thawed at room temperature for 1 h before SepPak chromatography. The acidic buffer method resulted in 91% recovery of the label. The label eluted as a single peak in the same region as ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 used to calibrate the column.

In contrast, there was considerable breakdown and loss of this label during the plasma formation buffer. There was <40% recovery of radioactivity, and the label now eluted as six peaks. One of the peaks eluted in the same region as ^{125}I-Tyr^{2}-labeled CCK-8 that can be formed by digestion of the label between Arg^{50}-Asp^{51} (Fig. 1) and one in the same region as the intact label ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58. Four peaks eluted between ^{125}I-Tyr^{2}-labeled CCK-8 and ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58, and they most likely represent COOH-terminal-labeled peptides ^{125}I-Tyr^{16}-labeled CCK-22 and ^{125}I-Tyr^{27}-labeled CCK-33 or NH$_2$-terminal-labeled peptides ^{125}I-Tyr^{20}-labeled-(1–50)-CCK-58 or ^{125}I-Tyr^{20}-labeled-(1–46)-CCK-58. The radioactivity not recovered in plasma (60% of the starting radioactivity) was associated with red blood cells.

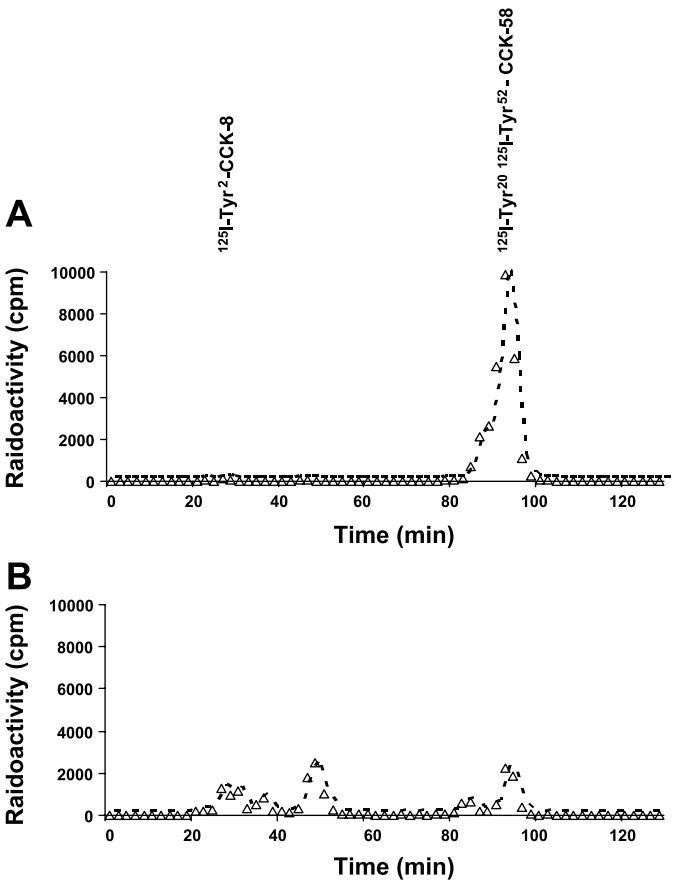

Fig. 5. Recovery of ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 after processing by the acidic buffer method and the plasma formation method. *A*: recovery with the acidic buffer method. *B*: recovery with the plasma formation method. Only CCK-58 was observed when blood was collected and processed by the optimized method reported here. These results differ from prior studies on the molecular forms of cholecystokinin in the circulation of rats in which smaller forms were detected (8, 11, 13, 14). In an attempt to reconcile these divergent results, ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 was added to rat blood, and the sample was processed in a manner that simulated prior studies (plasma formation method) and with the optimized method (acidic buffer method). The final step of the characterization, HPLC of radiolabeled fractions, showed that the label eluted as 1 peak when processed by the acidic buffer method with a recovery of 91% (*A*). In contrast, label processed by the plasma formation method had <40% recovery of label and the radioactivity eluted in 6 different peaks (*B*).

DISCUSSION

A new method of collecting and processing blood has been developed that yields similar recovery (80%) for all molecular forms of cholecystokinin that have been identified in blood (8, 11, 13, 14) or intestinal extracts (3, 25) of rat. Furthermore, unlike previous methods used to determine the endocrine form of cholecystokinin in rat (8, 11, 13, 14), this new optimized method prevented degradation of radiolabeled CCK-8 or CCK-58. The optimized method reproducibly detected only CCK-58 in blood of fasted or trypsin inhibitor-stimulated rats. When this method is used, the blood level of CCK-58 is 3.5 ± 0.5 pM in fasted rats and 17 ± 1.5 pM in stimulated rats.

A major distinction between the results reported here and previously published reports on the endocrine forms of rat cholecystokinin is that synthetic CCK-58, CCK-33, CCK-22, and CCK-8 were available for pro-

duction of label to aid in method development, evaluating recovery of exogenous peptide, and for standards in assays of chromatographed samples. Radiolabeled CCK-8 and CCK-58 allowed selection of the best surfaces for collecting and processing blood samples. The greater loss of CCK-58 compared with CCK-8 losses during the drying of HPLC fractions from glass tubes and during simulated radioimmunoassay conditions using glass tubes may partially account for previous studies (8, 11, 13, 14) in rat that did not detect CCK-58 as an endocrine form of cholecystokinin. Simulated older methods (8, 11, 13, 14) for processing blood resulted in extensive loss and degradation of ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58. At least six peaks were separated: one eluting in the position of the intact ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58, one in the position of ^{125}I-Tyr^{2}-labeled CCK-8, and four others that did not correspond to any labeled markers. These four are probably radiolabeled CCK-22 and three NH_2-terminal fragments formed during degradation of the labeled CCK-58. However, the optimized method developed in these studies produced a single peak that coeluted with the intact ^{125}I-Tyr^{20},^{125}I-Tyr^{52}-labeled CCK-58 and gave 91% total recovery. Furthermore, the stability and the good recovery of all synthetic forms of cholecystokinin added to rat blood (Fig. 2) is an important control for these types of experiments, because the iodinated forms of cholecystokinin may not duplicate recovery of endogenous cholecystokinin. The similar recovery and stability of CCK-8, CCK-22, CCK-33, and CCK-58 added to rat blood validates that the results obtained are not due to selective recovery of a single form. Because CCK-58 is not as cross-reactive with the cholecystokinin antisera RO16 compared with smaller forms, a CCK-58 standard is essential for measurement of this molecular form in blood and tissue extracts. Other antibodies (8, 11, 14) and the bioassay (13) used to detect cholecystokinin immunoreactivity in prior studies may not have detected CCK-58 as potently as shorter molecular forms of cholecystokinin. This could lead to a 5- to 10-fold underestimation of the amount of CCK-58. Loss of CCK-58 to experimental surfaces, its degradation during collection and processing of blood, and its underestimation by radioimmunoassay probably combined to give the results in earlier studies of no detectable CCK-58 in rat blood (8, 11, 13, 14).

Our laboratory previously published that CCK-58 was a predominant form in dogs (5) and humans (2, 4) comprising 63 and 36–47% of the total cholecystokinin immunoreactivity, respectively. These values were not corrected for the decreased immunoreactivity of CCK-58 with the antibody used (6), suggesting that the proportions of CCK-58 were underestimated in these species. Furthermore, the method used for determining the molecular forms of cholecystokinin in dogs (5) and humans (2) recovered only 40% of intact ^{125}I-Tyr^{20}-labeled CCK-58 added to rat blood (results not shown). The new optimized method reported here recovered >85% of labeled CCK-58 from rat blood. Reanalysis of the molecular forms of cholecystokinin by using the new method in man and dog might reveal that the only endocrine form of cholecystokinin in these species is CCK-58.

All molecular forms of cholecystokinin studied were recovered with >80% yield by using the method described in this report. The excellent recoveries were obtained although the molecular forms varied greatly in charge and size; CCK-8 and CCK-22 are short and acidic, whereas CCK-33 and CCK-58 are long and basic (Fig. 1). For example, 88% of exogenous CCK-8 was recovered from blood at a concentration 2.5 pM. This concentration was below the concentration detected for CCK-58 in fasted rats (3.5 pM), indicating that the absence of this small form in our experiments is not due to poor recovery at low concentrations.

There is an obvious discrepancy between the molecular forms of cholecystokinin detected in rat blood (only CCK-58) and the molecular forms reported in rat intestinal extracts (mostly CCK-22) (1, 11). If ^{125}I-Tyr^{20}-labeled CCK-58 is added to intestinal extracts, it is rapidly degraded into smaller forms (unpublished results). The enzymes causing this degradation could come from processing enzymes in the I cell (the cell that synthesizes intestinal cholecystokinin) or from pancreatic enzymes released into the intestinal lumen. However, we have shown a good relationship between the intestinal and blood forms of cholecystokinin in humans (2, 6) and dogs (1, 5, 16). Both tissues contain mainly CCK-58. We propose that once better methods are developed for extraction of rat intestines CCK-58 will be a major stored form. Of some interest is the fact that rat blood and intestinal extracts have proved to be the most difficult samples studied for inhibition of cholecystokinin degradation. The methods used to process blood in human and dog that detected CCK-58 did not inhibit degradation of labeled CCK-58 in rat blood. The same enzyme may cause both degradations. In brain extracts, most investigators find mainly CCK-8, but our laboratory demonstrated that 21% of the cholecystokinin immunoreactivity in brain extracts eluted in the region of CCK-58 (7).

Grossman (10) suggested that "the rigor of the scientific evidence demanded to establish a given proposition varies with the state of the art." We suggest that the method reported here sets a new rigor that should be applied when determining the forms of an endocrine peptide or for determining tissue forms of a peptide. Rehfeld (18, 19) has proposed elements of an assay needed to accurately measure plasma cholecystokinin. These elements include: *1*) sufficient peptide for immunization, *2*) an iodination reaction that does not alter binding of tracer (especially important for cholecystokinin that has two methionines and a tryptophan in its main epitope region), *3*) tracer with high specific radioactivity, *4*) antibodies of high affinity that can measure a low pico/femtomolar concentration, and *5*) antibodies should be specific for cholecystokinin with little cross-reactivity with gastrin. To this list of requirements we would add: *6*) synthetic standards for every form of cholecystokinin to be measured in the assay, *7*) demonstration that peptides are not lost in containers used

for collection and processing of blood, *8*) determination that all molecular forms of cholecystokinin are not degraded in vitro during collection and processing of blood, and *9*) similar high recovery for all forms of cholecystokinin likely to be present in blood. The fulfillment of all of these criteria supports the result that CCK-58 is the major endocrine form in rat. These criteria were only partially fulfilled when the endocrine forms of cholecystokinin were determined in humans and dogs. Thus CCK-58 may be the only endocrine form in humans and dogs.

These criteria should be applied to other endocrine peptides that have a net positive charge (e.g., peptide YY and secretin). Studies with radiolabeled peptides may show that other peptides partition with red blood cells (as with CCK-58) if forming plasma is the first step in processing blood. The key to this acidic buffer method are acidification and dilution of blood that leaves 85–95% of labeled CCK-58 in the supernatant above the red blood cells. Furthermore, working with cold buffer (4°C) may help to prevent degradation of large endocrine peptides during the collection and processing of blood as demonstrated for CCK-58. Using the method reported here may lead to new results on the concentrations and molecular forms of other endocrine peptides.

Grossman (10) also suggested that evidence needed to establish a hormonal mechanism for a gastrointestinal physiological event included that "[t]he effect is produced by infusing exogenous hormone in amounts and molecular forms that copy the increase in blood concentration seen after the stimulus for endogenous release." Therefore, it is important to study the endocrine actions of cholecystokinin with CCK-58.

Presently, most investigators have been taught that all molecular forms of cholecystokinin have the same bioactivity and that CCK-8 is the major endocrine form of cholecystokinin (15). On the basis of results presented here and preliminary studies in conscious (26) rats, neither of these conclusions are correct. CCK-58 is a major endocrine form of cholecystokinin in rat and dog (5). CCK-58 has different physiological properties from other forms of cholecystokinin (17). The presence of synthetic CCK-58 will allow new studies into the physiological and pathophysiological actions of cholecystokinin.

We thank J. Rehfeld (Copenhagen, Denmark) for helpful discussion.

DISCLOSURES

The study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-33850 (to J. R. Reeve), DK-37482 (G. M. Green), and DK-41301 and by the Veterans Affairs Research Service. Support from the Peptidomics and Antibody Cores is gratefully acknowledged.

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