

# Cholecystokinin Bioactivity in Human Plasma

## Molecular Forms, Responses to Feeding, and Relationship to Gallbladder Contraction

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### Abstract

A sensitive and specific bioassay for the measurement of cholecystokinin (CCK) in human plasma was developed to determine the molecular forms of CCK in circulation, CCK responses to feeding, and the physiologic role of CCK in gallbladder contraction. First, plasma was quantitatively extracted and concentrated with octadecylsilylsilica, and the extracts were then assayed for their ability to stimulate amylase release from isolated rat pancreatic acini. Acini were highly sensitive to CCK whereas gastrin reacted only weakly in this system. With the assay, plasma levels of cholecystokinin octapeptide (CCK-8) bioactivity as low as 0.2 pM were detectable. CCK bioactivity in plasma was inhibited by the CCK antagonist, dibutylryl cyclic guanosine monophosphate, and was eliminated by immunoadsorption with an antibody directed against the carboxyl terminus of CCK. Detection of fasting levels of CCK was possible in all individuals tested and averaged  $1.0 \pm 0.2$  pM (mean  $\pm$  SE,  $n = 22$ ) CCK-8 equivalents. Plasma CCK biological activity was normal in patients with gastrin-secreting tumors. After being fed a mixed liquid meal, CCK levels rose within 15 min to  $6.0 \pm 1.6$  pM. The individual food components fat, protein, and amino acids were all potent stimulants of CCK secretion; in contrast, glucose caused a significant but smaller elevation in plasma CCK levels. Gel filtration studies identified three major forms of CCK bioactivity in human plasma: an abundant form that eluted with CCK-33, a smaller form that eluted with CCK-8, and an intermediate form that eluted between CCK-33 and CCK-8. Ultrasonic measurements of gallbladder volume indicated that this organ decreased 51% in size 30 min after feeding a mixed liquid meal. This contraction occurred coincidentally with the increase in plasma CCK levels. Next CCK-8 was infused to obtain CCK levels similar to postprandial levels. This infusion caused a decrease in gallbladder volume, similar to that seen with a meal. The present studies indicate, therefore, that CCK can be bioassayed in fasting and postprandial human plasma. These studies also suggest that CCK may be an important regulator of gallbladder contraction.

A preliminary report of this study was presented at the Annual Meeting of the American Gastroenterological Association in New Orleans, LA, 1984, and has appeared in abstract form (1984. *Gastroenterology*. 86: 1163).

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### Introduction

Since its discovery by Ivy and Oldberg (1) in 1928, cholecystokinin (CCK)<sup>1</sup> has generally been accepted to be a major hormonal regulator of gallbladder contraction. Harper and Raper (2) in 1943 extracted from the small intestine a peptide that stimulated pancreatic enzyme secretion and named it pancreozymin. It is now known that CCK and pancreozymin are the same molecule that has both gallbladder contractile and pancreatic stimulatory properties (3). Cholecystokinin-pancreozymin, now termed cholecystokinin, was originally identified in porcine intestine as a 33-amino acid peptide (CCK-33) that shares an identical carboxyl terminal pentapeptide sequence with gastrin. Other molecular forms of CCK have been identified in intestinal extracts, brain, and plasma of various species (4-11). CCK-39 was characterized from hog intestine as a hexapeptide extension on the amino terminus of CCK-33 (4, 5). A larger molecular form, CCK-58, has been extracted from dog intestine and partially characterized (6). Forms of CCK similar in size to CCK-12, CCK-8, and CCK-4 have been characterized immunochemically in intestinal extracts (10, 11).

The ability to study the circulating forms of CCK and the regulation of CCK secretion has been hampered by the lack of a rapid, sensitive, and specific assay for the hormone. In general, prior bioassays for CCK have not been sensitive enough to measure circulating levels of the hormone (12, 13), and radioimmunoassays have been hampered by crossreactivity with gastrinlike substances. Estimations of circulating CCK levels by radioimmunoassay have been useful, but to distinguish CCK from gastrin, either two antibodies that differ in their ability to recognize CCK and gastrin must be used, or the plasma must be chromatographed to separate CCK from gastrin (14-16). This need for processing may account for the wide variation in CCK levels that have been reported (14-24). In addition, CCK exists in multiple molecular forms, and antibodies directed against one portion of one molecular form may not recognize another molecular form. This immunovariability may also account for some of the variability of molecular forms of CCK that has been reported.

Recently, it has even been questioned whether CCK is a primary physiologic regulator of gallbladder contraction (25, 26). Similarly, it has been suggested that physiologic postprandial CCK levels alone cannot account for postprandial pancreatic enzyme secretion (15). Reliable measurements of CCK in plasma are necessary, therefore, to determine the hormonal role of CCK in normal and pathologic states.

1. Abbreviations used in this paper: CBZ-L-tryptophan, *N*-carbobenzoxy-L-tryptophan; cGMP, guanosine 3'-5'-cyclic monophosphate; KHB, Krebs-Henseleit bicarbonate (buffer); TR, Tris-Ringer (buffer); VIP, vasoactive intestinal polypeptide.

We have now developed a method for measuring human plasma CCK based on the ability of CCK in plasma extracts to stimulate amylase release from isolated rat pancreatic acini. These acini respond to CCK concentrations as low as 1 pM, and with the ability to concentrate plasma circulating CCK, levels as low as 0.2 pM can be measured. This assay has allowed us to measure both fasting and postprandial CCK levels, the relative contribution of various food components to CCK release, and the molecular forms of CCK in plasma.

## Methods

The following substances were purchased: soybean trypsin inhibitor (types I-S and II-S), atropine sulfate,  $N^2O^2$ -dibutyl guanosine 3':5'-cyclic monophosphate (dibutyl cGMP); *N*-carbobenzoxy-L-tryptophan (*N*-CBZ-L-tryptophan), carbamylcholine (carbachol), and Sephadex G-50 superfine from Sigma Chemical Co., St. Louis, MO; chromatographically purified collagenase from Worthington Biochemical Corp., Freehold, NJ; minimal Eagle's medium amino acid supplement from Gibco Laboratories, Grand Island, NY; bovine serum albumin, fraction V, from Miles Laboratories, Inc., Elkhart, IN; procion yellow dye from Polysciences, Inc., Warrington, PA; *Staphylococcus aureus* 10% cell suspension from New England Enzyme Center, Boston, MA; octadecylsilylsilica (SEP-PAK C-18) cartridges from Waters Associates, Milpore Corp., Milford, MA; instant breakfast supplement from Carnation Co., Los Angeles, CA; vasoactive intestinal polypeptide (VIP) and synthetic human gastrin-17 I from Bachem, Inc., Torrance, CA and Sincalide (CCK-8) from E. R. Squibb & Sons, Inc., Princeton, NJ.

The following substances were gifts: CCK-8 from Dr. Miguel Ondetti of the Squibb Institute for Medical Research, Princeton, NJ; purified porcine CCK-33 from the Gastrointestinal Hormone Laboratory, Karolinska Institute, Stockholm, Sweden; gastrinoma-derived gastrin-17 II and gastrin antibody 1611 from Dr. John Walsh of University of California, Los Angeles and Center For Ulcer Research and Education, Los Angeles, CA; CCK/gastrin antibody RSB70 directed at the carboxyl terminus of CCK and gastrin from Dr. Margery Beinfeld, St. Louis University, St. Louis, MO;  $^{125}$ I-gastrin-17 from Dr. Steven Vigna, University of Oregon, Eugene, OR; Lipomul emulsion consisting of 71% corn oil (88% oleic and linoleic acids) from the Upjohn Co., Kalamazoo, MI; Casec, casein powder from Mead Johnson and Co., Evansville, IN; and Nutrisource mixed L-amino acids consisting of 7% isoleucine, 14% leucine, 7% valine, 1.5% tryptophan, 8% phenylalanine, 3% methionine, 7% lysine, 5% threonine, 5.5% arginine, 0.7% tyrosine, 1% cysteine, 7% alanine, 11.8% glutamic acid, 5.5% aspartic acid, 3% histidine, 2% serine, 6% glycine, and 5% proline from Sandoz, Minneapolis, MN.

## Bioassay of CCK

*Preparation of isolated pancreatic acini and measurement of amylase release.* The buffer used to prepare isolated acini was modified Krebs-Henseleit bicarbonate buffer (KHB), enriched with minimal Eagle's medium amino acid supplement and 0.1 mg/ml purified soybean trypsin inhibitor. KHB buffer was equilibrated to pH 7.4 with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (27).

The incubation buffer was Tris-Ringer (TR) that contained 40 mM Tris (hydroxymethyl)aminomethane, 103 mM NaCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 4.7 mM KCl, 1.28 mM CaCl<sub>2</sub>, 0.56 mM MgCl<sub>2</sub>, 11.1 mM glucose, 0.1 mg/ml soybean trypsin inhibitor, minimal Eagle's medium amino acid supplement, and 5 mg/ml bovine serum albumin (BSA). TR buffer was equilibrated with 100% O<sub>2</sub> and adjusted to pH 7.4 at 37°C.

Isolated pancreatic acini were prepared from 180–200-g female Sprague-Dawley rats by collagenase digestion of pancreas in KHB as previously described (27, 28). Acini were then incubated with plasma extracts or standard CCK-8 concentrations for 30 min at 37°C (28). Amylase released into the medium was assayed using procion yellow coupled starch as substrate (29). Amylase release expressed as percent of total amylase content, was compared with a dose-response curve

for CCK-8 in order to calculate the CCK content of plasma expressed as CCK-8 equivalents.

In this preparation of isolated pancreatic acini, CCK-8 is the most potent stimulus for amylase release (30). CCK-8 is threefold more potent than CCK-33. In contrast, gastrins are much less potent than either CCK-8 or CCK-33. Compared with CCK-8, the relative potencies of gastrins I and II are 0.00046 and 0.0025 to 1, respectively. In addition, the C-terminal pentapeptide of CCK, CCK-5, is 5,000 times less active than CCK-8 on a molar basis (30).

*Feeding and collection of plasma.* Human subjects for all studies were healthy volunteers between 21 and 43 yr of age. Subjects underwent an overnight 12–15-h fast prior to each study performed. Blood samples were drawn from an indwelling 19-gauge butterfly catheter in the antecubital fossa during the 2–3-h course of the study. Blood was collected into iced heparinized tubes and immediately centrifuged at 1,000 g to obtain plasma for CCK determinations. Blood for gastrin determination was collected into nonheparinized tubes, at room temperature, for recovery of serum.

Subjects were fed orally liquid diets of either a mixed meal or various food components (fat, protein, amino acids, or glucose). The mixed liquid meal was made of Carnation instant breakfast supplement, one egg, and "half and half" milk and cream, totaling 1.5 cal/ml and consisted of 40% fat, 20% protein, and 40% carbohydrate. This meal was given as 5.6 ml/kg body wt and was consumed over a 1–2-min period. Blood was drawn for CCK level determinations at various times up to 2 h after feeding.

Other food components included 25% solutions (100 g/400 ml of water) of either glucose, amino acids, fat (in the form of corn oil, Lipomul), or protein (given as casein). Separate control diets tested included: 400 ml of either water, 0.9% sodium chloride, or 2% sodium chloride. The osmolality of these solutions ranged from 0 to 600 mosmol.

## Extraction of CCK from plasma

CCK was extracted from plasma by adsorption onto SEP-PAK cartridges previously washed with 5 ml of methanol and 20 ml of water. The cartridges were then washed again with 20 ml of water and the CCK was eluted with 1 ml of 80% ethanol and 0.2% trifluoroacetic acid. The eluants were collected in 30-ml flat-bottomed incubation vials and dried under a nitrogen stream at 45°C. These vials were subsequently used for incubation with 1 ml of acini suspended in TR. CCK was concentrated up to sixfold by adsorbing up to 6 ml of plasma through a single cartridge and eluting the CCK into a single vial.

Recoveries of CCK standards were measured by adding known amounts of CCK-8 or CCK-33 (dissolved in either saline or 50 mM acetic acid containing 5 mg/ml BSA) to plasma from fasting subjects or charcoal-stripped plasma. These plasma samples were then processed through SEP-PAK cartridges as described above and assayed for CCK-like activity by comparing the bioactivity of plasma samples with those of standard curves of CCK-8 and CCK-33. Concentrations of CCK-8 and CCK-33 ranging from 10 to 100 fmol, were added to plasma and yielded recoveries of 92±8% (mean±SD,  $n = 14$ ) for CCK-8 and 85±10% ( $n = 10$ ) for CCK-33. Samples were usually assayed on the day of collection, however, it was found that recovery of plasma CCK bioactivity was unchanged if plasma was stored at –20°C in SEP-PAK cartridges for up to 10 d.

Gastrin radioimmunoassays were kindly performed by Dr. Clifford Deveney by the method of Stadil and Rehfeld (31). Antibody 1611, previously characterized (15), was used in a concentration of 1:500,000. Gastrin 17 was used as a standard, and the assay had a sensitivity of 0.1 fmol/ml. The normal range of basal serum gastrin values with this assay is 0–50 pM.

## Immunoprecipitation of CCK

*Staphylococcus aureus* (0.5 ml of a 10% suspension) was washed and resuspended in 1 ml of incubation buffer. 10 µl of either saline, normal rabbit serum, or anti-CCK antibody RSB70 were added and incubated 2 h at 4°C. The mixture was then washed twice, resuspended in TR

buffer, incubated 2 h at 4°C with either CCK-8 or plasma extract and then centrifuged (32). The supernates were bioassayed for CCK activity.

In addition, to determine if an inhibitor of CCK was present in plasma, these supernates were added to various concentrations of CCK-8 or carbamylcholine and incubated with 1 ml of pancreatic acini and assayed as described above. Final concentrations of these plasma extracts in the incubation were 1–2 ml of plasma/ml of acini.

### Column chromatography

Plasma samples were collected and extracted. The extracted material was resuspended in a buffer of 0.25 M ammonium carbonate, pH 8.2, with 0.2% BSA at 4°C and chromatographed on a Sephadex G-50 superfine column, 0.9 × 58 cm. 1-ml fractions from the column were then passed through separate SEP-PAK cartridges. These samples were eluted and bioassayed for CCK activity. Recovery of biological activity from plasma extracts subjected to column chromatography averaged 65% ( $n = 5$ ). To determine if CCK-8 aggregated in plasma, 200 fmol CCK-8 was added to 15 ml of plasma from a fasted subject. This enriched plasma was incubated for 10 min at 37°C, extracted through SEP-PAK cartridges, chromatographed, and bioassayed.

### CCK infusion and gallbladder ultrasonography

After an overnight (12–15 h) fast five male subjects underwent intravenous infusion of CCK-8.<sup>2</sup> CCK-8 (Sincalide) was diluted to appropriate concentrations in a total volume of 20 cm<sup>3</sup> normal saline. By use of a Harvard pump (Harvard Apparatus Co., Inc., S. Natick, MA), CCK was infused through an indwelling butterfly catheter in the antecubital vein at a rate of 14 pmol/kg per h. The actual infusion rate was determined by measuring the CCK concentration of the infusate taken from the delivery system. This measurement corrects for losses of CCK by adsorption to syringes and intravenous tubing. Blood was collected before and during the infusion through another indwelling intravenous catheter in the opposite antecubital vein.

Determinations of gallbladder volumes were made by abdominal ultrasonography as described by Everson et al. (38). Longitudinal sonograms of the gallbladder were recorded on film with a commercially available Advanced Technological Laboratories (Bellvue, WA) real-time scanner utilizing a 3.5-MH or 5-MH transducer. Scans were obtained with subjects in the upright position. Long axis views were obtained by manipulating the transducer so that it followed the appropriate long axis of the gallbladder and the largest gallbladder dimensions at each time were recorded. All of the subjects' gallbladders were found in the usual subhepatic position and orientation. No gallstones, wall thickening, or other pathology was identified. After base-line blood samples were collected and base-line gallbladder sonograms obtained, subjects either drank a mixed liquid meal (as described above) or were infused with CCK-8. Blood samples for CCK level determinations and simultaneous gallbladder sonograms were obtained at various times over a 2-h period. Gallbladder volumes were calculated by the sum of cylinders method (38).

This study was approved by the Committee on the Protection of Human Subjects of Mt. Zion Hospital and the Committee on Human Research of the University of California, San Francisco. Informed consent was obtained from each subject.

### Statistical analysis

All values from a single experiment are expressed as the mean ± 1 SD and values from pooled experiments as the mean ± 1 SEM. Comparison

2. For several reasons CCK-8 was chosen for the infusion studies rather than CCK-33. First, CCK-8 is present in human plasma. Second, CCK-8 has all of the biological activities of CCK-33 in gallbladder and other tissues; moreover, in the gallbladder CCK-8 has been reported to be either equipotent (33, 34) or more potent (35, 36) than CCK-33. Third, human CCK-8 and porcine CCK-8 are identical; in contrast, human CCK-33 and porcine CCK-33 are different by two amino acids (37). Fourth, CCK-8 is available for human use whereas human CCK-33 is not available.

of responses were made by analysis of variance with repeated measures (39). Post-hoc analysis of the difference between points was carried out by means of the Newman-Keuls test. Differences with a  $P$  value of <0.05 were considered significant.

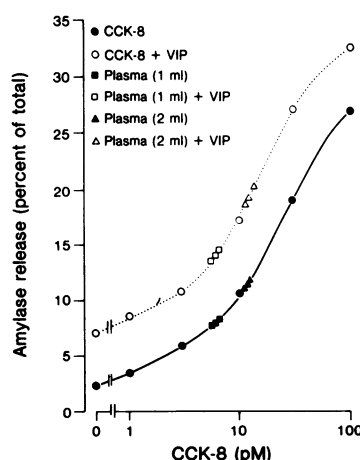
## Results

*Specificity, sensitivity, and validation of the pancreatic acini bioassay.* In the bioassay system employed, a detectable effect of CCK-8 was seen at 1 pM and maximal effects were seen at 100 pM (Fig. 1). Assay sensitivity was defined as the amount of CCK-8 that produced a statistically different response in amylase release (a response that differed by 2 SD from that observed with no hormone). In all assays performed, incubation of acini with 1 pM CCK-8 resulted in higher amylase release values than acini incubated in the absence of hormone, and in 26 of 30 consecutive experiments this difference was statistically significant. In no individual experiment was a given plasma CCK concentration calculated unless the value was statistically different from basal.

In this system plasma extracts stimulated amylase release from pancreatic acini. The dose-response curve of stimulated amylase release with postprandial plasma from subjects fed a mixed liquid meal paralleled that of the CCK-8 standards (Fig. 1). Similar parallelism was seen for standards of CCK-33.

Plasma extracts were then incubated with dibutyl cGMP, a known antagonist of CCK action (40). Dibutyl cGMP suppressed plasma-stimulated amylase release in a dose-dependent manner similar to that which was shown in rat plasma (30). The inhibition curves of amylase release by dibutyl cGMP for both the hormones and plasma were parallel. The concentrations of dibutyl cGMP that completely suppressed stimulated amylase release were 0.3–1 mM. In addition, a different antagonist of CCK, CBZ-tryptophan (41), was incubated with plasma extracts. Similarly to dibutyl cGMP, CBZ-tryptophan also inhibited plasma activity and CCK-stimulated amylase release in a parallel dose-dependent manner.

Trifluoroacetic acid used in eluting CCK from SEP-PAK cartridges did not interfere with the bioassay. There was no difference in either basal or CCK-stimulated amylase release



**Figure 1.** CCK bioactivity of plasma extracts and the effect of exogenous VIP. Postprandial plasma extracts equivalent to 1 ml (□) or 2 ml (Δ) of plasma were incubated with isolated pancreatic acini and the resultant amylase release was compared with a standard curve of CCK-8 (lower curve). Similar amounts of plasma extracts or CCK-8 standards were incubated with acini to which a maximally-stimulating dose of VIP (1 nM) had been added (upper curve). This resulted in a shift upward of the standard curve and plasma samples but did not change the calculated amount of CCK in plasma.

between control vials and vials to which 1 ml of ethanol: trifluoroacetic acid had been added and dried under nitrogen.

To test the immunoreactivity of the plasma CCK bioactivity, both plasma extracts and a CCK-8 standard were incubated with either a suspension of *S. aureus* to which normal rabbit serum was added, or a suspension of *S. aureus* to which antibody RSB70 (directed against the carboxyl terminus of CCK) had been added. After centrifugation the supernates were assayed for CCK biological activity. Incubation of the plasma extracts with *S. aureus* plus anti-CCK antibody (but not normal rabbit serum) completely removed CCK bioactivity. This result is similar to that which has been previously demonstrated for bioassayable CCK in rat plasma (30).

To investigate the possibility that an inhibitor of CCK-stimulated amylase release might be present in plasma extracts and thereby interfere in the bioassay, plasma extracts were treated with anti-CCK antibody RSB70 bound to *S. aureus* as described above. This process removed all CCK bioactivity from plasma extracts. These "CCK-free" plasma extracts were then incubated with acini to which either CCK or carbamylcholine standards were added (Fig. 2). Plasma extracts stripped of CCK had no inhibitory effect on either CCK- or carbamylcholine-stimulated amylase release.

Like CCK, muscarinic cholinergic analogues stimulate amylase release via the mobilization of intracellular calcium (42). To exclude the possibility that acetylcholine or a similar agent in plasma was interfering in this assay, acini were incubated with plasma extracts in the presence and absence of 5  $\mu$ M atropine. No change in the CCK biological activity of the plasma was seen with atropine (Table I).

In addition, VIP and secretin are two secretagogues that either stimulate or potentiate amylase release from the pancreas via the generation of cAMP (43). To test for the possibility that these hormones may have been present in significant concentrations to influence the bioassay of CCK, 1 nM VIP (a maximally stimulating concentration) was added to both the plasma extracts and the CCK standards. VIP increased amylase release to an equal degree in both plasma extracts and CCK-8 standards (Fig. 1). The net effect was a shift in the standard curve upward. The slope of the curve was unchanged.

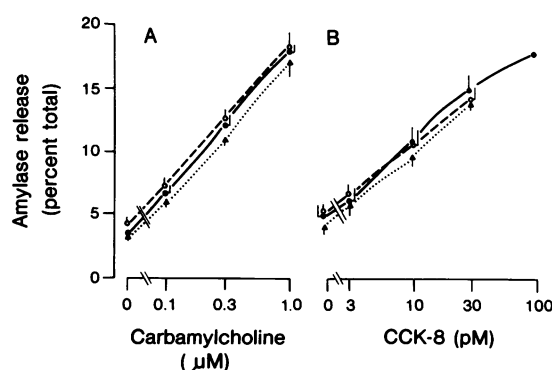


Figure 2. Influence of CCK-free plasma on amylase release from pancreatic acini. Plasma extracts, collected 15 min (○) and 60 min (▲) after feeding, were incubated with 10  $\mu$ l of antibody RSB70 for 2 h. *S. aureus* was then added to precipitate the antibody-antigen complex, and supernates were incubated with pancreatic acini containing various concentrations of (A) carbamylcholine (B) CCK-8 or standards (●). Values are the means  $\pm$  SD of triplicate determinations. A representative of two experiments is shown.

Table I. Lack of Effects of Atropine and VIP on the CCK-like Bioactivity of Plasma

	Plasma	Plasma plus 5 $\mu$ M atropine	Plasma plus 1 nM VIP
CCK-8 equivalents (pM)	12.0 $\pm$ 0.8	10.8 $\pm$ 1.3	12.6 $\pm$ 1.7

Pancreatic acini were incubated with 2 ml of plasma extracts in the presence or absence of either 5  $\mu$ M atropine or 1 nM VIP. In the case of VIP, 1 nM VIP was added to each of the CCK-8 standards as well as the plasma extracts. The CCK concentration (mean  $\pm$  SD,  $n = 6$ ) in plasma was then calculated from this standard curve. Data are compiled from two separate experiments, with triplicate determinations.

Therefore, VIP did not change the calculated amounts of CCK present in plasma samples (Table I).

Because gastrin, in very high concentrations, can stimulate amylase release from pancreatic acini, it was important to ascertain the possible interference of gastrin in this assay system. Fasting blood samples were collected from four patients with documented gastrinomas from which serum gastrin and plasma CCK levels were determined (Table II). Fasting gastrin levels were consistent with the diagnosis of Zollinger–Ellison syndrome and ranged from 205 to 724 pM gastrin 17 equivalents.<sup>3</sup> In contrast, fasting CCK levels were normal ranging from 0.7 to 1.5 pM CCK-8 equivalents.

**Plasma CCK response to feeding.** The plasma CCK responses to feeding a mixed liquid meal were then studied (Fig. 3). 12 men and 10 women were fed a liquid meal of 5.6 ml/kg and plasma CCK levels measured for up to 2 h. Fasting levels of CCK averaged 0.9  $\pm$  0.2 pM for the men and were slightly greater for the women, averaging 1.2  $\pm$  0.2 pM, but this difference was not statistically significant. There was a prompt rise in plasma CCK levels to 7.3  $\pm$  1.9 pM in male subjects and 6.2  $\pm$  0.8 in female subjects within 7.5–15 min after feeding. CCK levels fell within 60 min but remained significantly elevated for up to 2 h after feeding ( $P < 0.05$ ). To determine the effect of both volume and osmolality on CCK release, control male subjects were fed either water, normal saline, or 2% NaCl (600 mosmol, the same osmolality as the mixed meal). There was a slight but not statistically significant elevation in plasma CCK levels 7.5 min after saline ingestion (Fig. 3). Peak postprandial CCK levels after water and 2% NaCl were 1.6  $\pm$  0.6 pM ( $n = 3$ ) and 1.5  $\pm$  0.4 pM ( $n = 3$ ) CCK-8 equivalents, respectively. These levels were not statistically different from basal ( $P > 0.2$ ).

To determine the relative contributions of protein, fat, carbohydrate, and amino acids to CCK secretion, plasma levels of CCK were measured after the ingestion of 100 g of either casein, corn oil, glucose, or mixed amino acids (Fig. 4). Plasma CCK levels were measured in response to feeding each of the separate food components in the same five male subjects on different days. Each of the food components stimulated CCK release ( $P < 0.05$ ). Fat, protein, and amino acids were the

3. Gastrin concentrations are often expressed as picograms per milliliter, because gastrin-17 has a molecular weight of  $\sim$ 2,100, 100 pg/ml of gastrin is 48 pM.

Table II. Plasma CCK Bioactivity in Patients with Gastrinomas

Patient	Serum gastrin-17 equivalents	Plasma CCK-8 equivalents
	pM	pM
A	724	0.8
B	252	0.7
C	714	1.0
D	205	1.5

Immunoreactive serum gastrin and bioactive plasma CCK concentrations were determined in fasting blood samples from four subjects (A-D) with documented gastrinomas. Serum gastrin was measured with antibody 1611 directed against the midportion of gastrin as described in Methods.

most potent stimulants of CCK secretion causing a four- to sevenfold increase above fasting CCK concentrations. The integrated response for 2 h after feeding each food is shown (Table III). Fat, protein, and amino acids caused a greater increase in plasma CCK than did glucose, which resulted in a small and transient elevation of CCK levels.

**Molecular forms of plasma CCK.** To determine the molecular forms of CCK in plasma, postprandial samples were collected from four male and four female subjects 15 min after ingesting a mixed liquid meal. 30 ml of plasma were concentrated onto SEP-PAK cartridges, chromatographed over a Sephadex G-50 superfine column, and compared with CCK-33 and CCK-8 standards (Fig. 5). Three peaks of CCK bioactivity were detected in all subjects. The first and most prominent peak of biologically active material (peak *a*) had a molecular size similar to that of CCK-33. The smallest and least prominent peak (peak *c*) eluted in a position similar to that of CCK-8, while a peak intermediate in size between CCK-33 and CCK-8 (peak *b*) was also identified. The percentage of total CCK bioactivity present in each peak is shown (Table IV). In all subjects, peak *a* contained the greatest amount of CCK bioactivity. A similar profile but with smaller peaks was also observed when 100 ml of plasma from two fasting subjects was chromatographed.

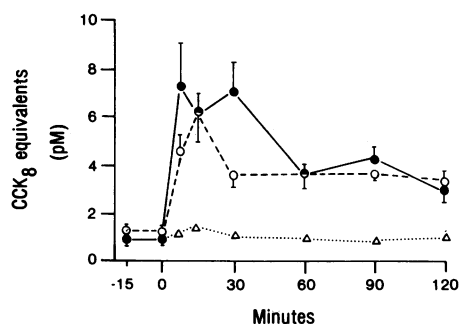


Figure 3. Plasma CCK response to feeding. After an overnight fast 12 male (●) and 10 female (○) subjects were fed 5.6 ml/kg of a mixed liquid meal or normal saline (Δ). At the times indicated, plasma was collected and extracted. These extracts were then assayed for CCK-8 bioactivity, expressed as CCK-8 equivalents. Each value is the mean±SE (*n* = 12 men, mixed meal; *n* = 10 women, mixed meal; and *n* = 4 mean; saline). Postprandial values for the mixed meal in both males and females were statistically different from basal (*P* < 0.05). There was no significant elevation in CCK after the ingestion of saline.

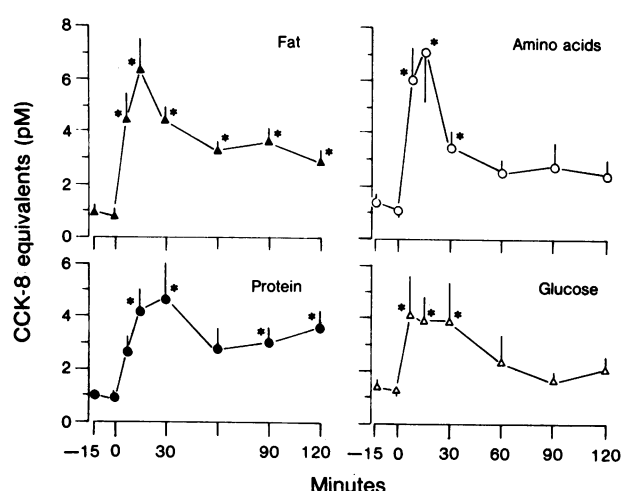


Figure 4. CCK responses to feeding fat, protein, amino acids, or glucose. After an overnight fast, five male subjects were fed 100 g of protein (●, as casein), fat (▲, as Lipomul), mixed amino acids (○), or glucose (Δ). Plasma was collected at the times indicated and extracts were assayed for CCK-8 bioactivity. On separate days the same five subjects drank each of the four food components studied. Each food was given as a 25% solution (5–5.7 ml/kg) in a volume of 400 ml. Values are the CCK levels of the five subjects (mean±SE).

To determine if the large molecular forms of CCK resulted from aggregation of smaller forms, CCK-8 was incubated with fasting plasma and chromatographed. A single peak of CCK bioactivity, eluting in the position of CCK-8 was recovered, indicating that the larger molecular forms are not aggregated CCK-8 fragments.

**Relation of plasma CCK to gallbladder contraction.** To establish whether CCK has a physiologic role in regulating gallbladder contraction, two sets of experiments were performed. First, the relationship between plasma CCK levels and gallbladder volume, measured ultrasonographically was examined (Fig. 6). Simultaneous measurements of plasma CCK and gallbladder volumes were obtained in five male subjects before and after feeding a mixed liquid meal. Plasma CCK levels rose to  $5.0 \pm 1.2$  pM within 15 min, declined by 60 min but remained elevated for up to 2 h. Coincidental with this rise in CCK, gallbladder volumes decreased to 51.4% of fasting volumes within 30 min of feeding and to 29.7% after 2 h.

To determine whether CCK could account for this degree

Table III. Integrated Plasma CCK Responses to Feeding Fat, Protein, Amino Acids, and Glucose

Food	Integrated CCK response over 2 h
	pM min
Fat	358.8±33.8
Protein	326.9±88.1
Amino acids	255.9±54.9
Glucose	173.8±54.5

The area under the curve for the CCK responses to feeding fat, protein, amino acids, and glucose shown in Fig. 5 are expressed as picomolar minutes. The integrated response is calculated from the area under the curve of CCK responses after feeding minus base line.

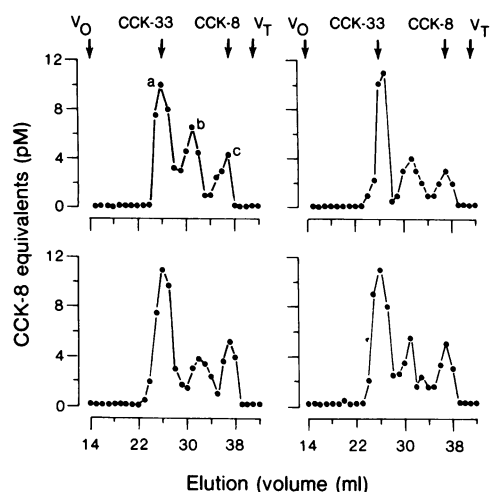


Figure 5. Molecular forms of CCK in plasma. 30 ml of plasma were collected from each subject at 15 min after feeding a mixed liquid meal. Plasma extracts were prepared as described in Methods, and reconstituted in ammonium carbonate buffer. This material was then chromatographed over Sephadex G-50 and 1-ml fractions were collected. The elution profile is shown in relation to the void ( $V_0$ ) and total volumes ( $V_T$ ) and the elution position of CCK-33 and CCK-8 standards. Profiles of two female subjects are shown on the left hand panels and profiles of two male subjects are shown on the right. *a*, *b*, and *c* denote the three peaks of CCK activity identified.

of gallbladder contraction, the same five subjects underwent similar ultrasonographic examinations of gallbladder volumes and plasma CCK measurements before and during the intravenous administration of synthetic CCK-8 (Fig. 7). Plasma CCK levels increased promptly within 10 min of beginning the infusion and remained constant at  $5.0 \pm 0.8$  pM for the 2-h period. Similar to the decrease in volume after feeding, gallbladder volumes after 30 min of steady state CCK infusion diminished to 50.7% of fasting volumes. After 2 h, gallbladder volumes decreased to 13% of fasting baseline volumes in response to CCK infusion.

To estimate the threshold for the effect of CCK on gallbladder contraction a lower dose of CCK-8 was infused into

Table IV. Molecular Forms of CCK in Plasma

	Percent of total CCK bioactivity		
	Peak <i>a</i>	Peak <i>b</i>	Peak <i>c</i>
	%	%	%
Males			
( <i>n</i> = 4)	64.5 $\pm$ 9.3	26.3 $\pm$ 2.6	9.3 $\pm$ 2.4
Females			
( <i>n</i> = 4)	57.6 $\pm$ 6.6	26.5 $\pm$ 7.6	15.9 $\pm$ 1.6

CCK activity was measured in plasma collected 15 min after feeding a mixed liquid meal. 30 ml of plasma were extracted and chromatographed on Sephadex G-50 and 1-ml fractions were collected. Each fraction was measured for CCK activity. Four male and four female subjects were studied and in each case three peaks (*a*, *b* and *c*) of CCK activity were identified as shown in Fig. 5. The percent of total CCK bioactivity present in each peak is shown above. Values are means $\pm$ SE, *n* = 4 males and 4 females.

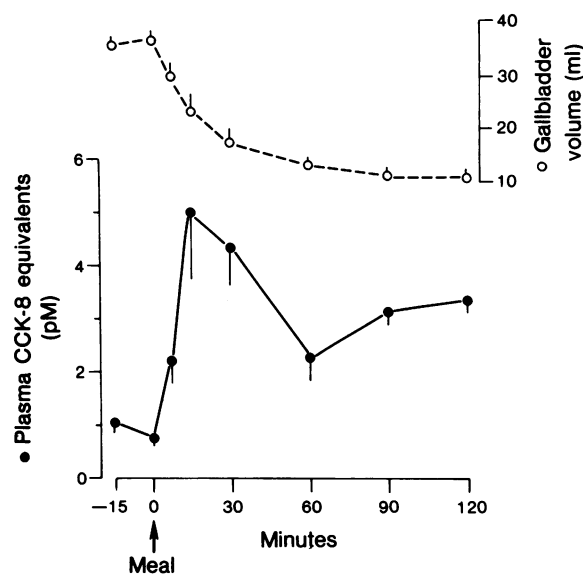


Figure 6. Relationship between plasma CCK and gallbladder contraction after feeding. Plasma CCK levels were measured in five male subjects, at the times shown, before and after drinking a mixed liquid meal. Simultaneous measurements of gallbladder volumes were determined by ultrasonography. Values are means $\pm$ SE of five subjects studied.

three subjects to achieve plasma levels of  $2.3 \pm 0.3$  (*n* = 3) pM CCK-8 equivalents. These levels decreased gallbladder volumes by 9% (from  $40 \pm 3.5$  to  $36.4 \pm 3.9$  ml) at 30 min and 28% (to  $28.8 \pm 4.2$  ml) at 2 h.

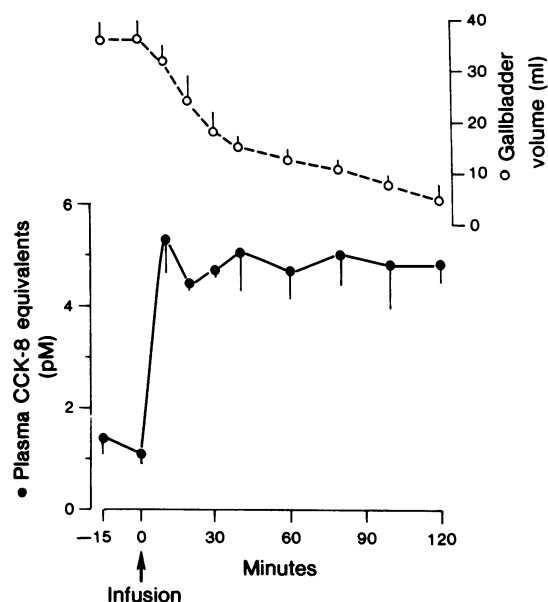


Figure 7. Relationship between plasma CCK and gallbladder contraction during CCK infusions. Simultaneous plasma CCK concentrations and gallbladder volumes were determined in five male subjects during the infusion of CCK-8. Synthetic CCK-8 was infused intravenously over a 2-h period while blood for CCK determinations was collected from the opposite arm. Gallbladder volumes were measured ultrasonographically at the times indicated. The same five subjects were used for studies of gallbladder volume after feeding (Fig. 6) and CCK infusion. Values are the means $\pm$ SE of the five subjects studied.

## Discussion

In the present study we have measured CCK activity in plasma by a sensitive and specific bioassay. We find that fasting plasma CCK levels in humans are low, on the order of 1 pM, and rise promptly after feeding. Previous estimates of plasma CCK levels have been made by gallbladder contraction bioassays and radioimmunoassays (12–24). Marshall et al. (13), using a less sensitive rabbit gallbladder contraction bioassay, reported that fasting levels of CCK averaged 2.4 nM; this value is over 2,000-fold higher than that in the present bioassay. More recently, radioimmunoassay estimates of CCK values in humans have been reported (14–24). Fasting CCK values measured by various radioimmunoassays range from undetectable to 55 pM. Although these radioimmunoassay values are lower than those of the gallbladder bioassay, a wide variation for reported CCK values still exists. One reason for this variation may be that different antisera vary in their ability to recognize heterogeneous molecular forms of CCK. Based on this study and previous work with isolated acini (44), the present bioassay has the advantage of detecting the biologically active forms of CCK, i.e., those containing the carboxyl-terminal heptapeptide sequence of CCK.

Three separate types of evidence indicate that CCK biological activity was being measured in the present assay. First, serial dilutions of plasma produced dose-response curves for amylase release that paralleled those of CCK-8. Second, the bioactivity of plasma was completely inhibited by the CCK antagonists, dibutyl cGMP and CBZ-tryptophan. Third, an antibody directed against the carboxyl terminus of CCK completely removed the CCK-like bioactivity from plasma extracts.

Other hormones and neurotransmitters, known to stimulate amylase release from pancreatic acini, also did not interfere in this assay system. Secretin and VIP stimulate amylase release by increasing intracellular cyclic adenosine monophosphate (43); these secretagogues also augment the secretory response to CCK (45). However, the addition of a maximally stimulating concentration of VIP did not influence the calculated concentration of CCK present in plasma, indicating that neither VIP nor secretin contributed to measured amylase release. Muscarinic cholinergic agents stimulate amylase release by increasing intracellular calcium (similar to the action of CCK) (42). When atropine was added to plasma extracts, there was no inhibition of biological activity, indicating that muscarinic agents were not contributing to the bioactivity of plasma extracts. Other potential secretagogues which act via the mobilization of intracellular calcium, such as bombesin and substance P (43), are not present in plasma in sufficient concentrations to elicit amylase release, their effects are not blocked by dibutyl cGMP (40, 46) and would not be expected to be inhibited by anti-CCK antibody.

The isolated pancreatic acini employed in this assay react only weakly with gastrin. Fasting gastrin levels in human plasma measured by radioimmunoassay range between 5 and 25 pM and seldom exceed 50 pM (47). Postprandial levels rarely exceed 100 pM. It is, therefore, possible to estimate the potential interference of gastrin in this CCK assay. If it is assumed that all of the gastrinlike material behaves like gastrin-17 II (the most biologically active form of gastrin in our assay system), and because in our assay gastrin II is 400 times weaker than CCK-8, fasting levels of 50 pM gastrin could be expected to account for 0.13 pM of the 1-pM fasting levels of

CCK, and postprandial levels of 100 pM gastrin II could be expected to account for 0.25 pM of the 6-pM postprandial levels of CCK. Because only one-half of the circulating gastrin is the sulfated form of gastrin-17, then the actual contributions of gastrin interference are probably less than these estimates. In patients with gastrinomas and high circulating gastrin levels as measured by radioimmunoassay, fasting levels of CCK were normal by bioassay. These data provide further evidence that gastrin does not cause significant interference in the present CCK bioassay.<sup>4</sup> Because of the selectivity of this assay system for CCK over gastrin, plasma levels of CCK are measurable with little interference from gastrin even though circulating gastrin levels may be much higher than those of CCK.

The relative potencies of various food components in stimulating CCK secretion has not previously been directly evaluated. Indirect evidence has been accumulated by measuring the ability of various foods to stimulate pancreatic secretion (48). In the present study we find that fat, protein, and amino acids are potent stimulants of CCK release. The CCK stimulatory effect appears to be an intrinsic property of the specific food and is not a function of either osmolality or volume as equal volumes of water, saline, and hypertonic sodium chloride had no effect on CCK levels. After instilling amino acids, fat, and glucose in the duodenum of humans, Go et al. (48) reported that amino acids and fat were the primary stimulants of pancreatic enzyme secretion and concluded therefore, in agreement with the present data, that these agents were the primary determinants of CCK secretion.

Interestingly, in the present study, oral glucose feeding stimulated CCK secretion, an effect that has not been appreciated previously. Oral glucose caused a prompt increase in plasma CCK levels but the effect was more transient than that seen with fat, amino acids, or protein. Our finding agrees with the observations of Go et al. (48) who reported that intraduodenal glucose caused a transient increase in pancreatic trypsin secretion.

Three forms of circulating CCK were detected by gel filtration analysis. Forms similar in size to porcine CCK-33 and CCK-8 were identified as well as an intermediate form. Earlier reports suggested that CCK-8 was the primary form of CCK in human plasma (14, 15, 20). More recently, however, others have reported immunoreactive forms larger than CCK-8 in postprandial plasma (16, 22) but the bioactivities of these forms have not been established. It has been suggested that there is a gradient of CCK forms from the proximal to distal small intestine (49). By extracting CCK from the intestine and measuring CCK concentrations by radioimmunoassay, the ratio of CCK-8/CCK-33 appeared to be greatest in the proximal intestine and decreased by mid-intestine. It may be that different forms of CCK are released at different times after feeding reflecting the CCK stimulation by food entering first the proximal and then the distal intestine. At 15 min after feeding, however, quantitatively most of the CCK bioactivity in human plasma appears to be the larger molecular form, similar in size to CCK-33. A similar profile was seen in fasting plasma. It is possible, therefore, that the intermediate or

4. The reason for the lack of elevated CCK bioactivity in these sera containing very high levels of immunoreactive gastrin is unknown. One possibility is that most of the gastrin is unsulfated. Another possibility is that a portion of this gastrin immunoreactivity is biologically inactive.



smaller forms of CCK are either directly secreted from the intestine or are derived from cleavage of the larger form.

Recently, we have reported a bioassay for plasma CCK in the rat (30). In this animal, basal levels of CCK ( $0.3 \pm 0.1$  pM) are slightly lower than in humans. In the rat there are two forms of CCK in the circulation. One form is similar in size to CCK-8 and the other is intermediate in size between CCK-33 and CCK-8. No form corresponding in size to CCK-33 was detected. Thus, there appears to be species differences in the forms of circulating CCK.

Although it is well established that exogenous administration of CCK stimulates gallbladder contraction, it has recently come into question whether CCK has a hormonal role in gallbladder contraction and whether other neural or hormonal factors account for gallbladder contraction after feeding (25). Others employing radioimmunoassays have reported an inverse correlation between gallbladder volume and plasma CCK levels estimated after a fatty meal (50, 51). However, it had not been established whether CCK alone infused under steady-state conditions and approximating physiologic concentrations could account for gallbladder contraction. In the present study, ingestion of a mixed meal caused a prompt fivefold increase in plasma CCK levels to  $5.0 \pm 1.2$  pM and levels remained elevated for 30 min before declining. Gallbladder volume in turn decreased by 51.4% within 30 min of feeding. Accordingly, CCK-8 was infused to reproduce plasma levels of  $5.0 \pm 0.8$  pM. This infusion resulted in a decrease in gallbladder volume of 50.7% within 30 min. Because the decrease in gallbladder volume after infusion of CCK (to levels that occurred endogenously) was almost identical to the decrease in gallbladder volume that occurred after feeding, a hormonal role for CCK is strongly suggested. Although gallbladder contraction was greater after the complete 2-h period of CCK infusion than after feeding, it is likely that this difference was due to higher CCK levels maintained after infusion of CCK than those which occurred after eating. Moreover, infusion of CCK-8 to achieve only a 2.5-fold increase in hormone levels also induced gallbladder contraction but at a more gradual rate.

The exact hormonal and neural regulation of gallbladder contraction after a mixed meal is unknown. The present studies, however, suggest that in normal individuals CCK has a major role in gallbladder regulation. This conclusion does not imply that other neural or hormonal factors may not participate in maintaining gallbladder tone or even stimulate gallbladder contraction under certain circumstances. However, in the population studied, CCK in physiologic concentrations appears to account for the postprandial gallbladder contraction observed.

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