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THE RELEASE OF RAT INTESTINAL CHOLECYSTOKININ AFTER ORAL TRYPSIN INHIBITOR MEASURED BY BIO-ASSAY

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SUMMARY

1. The distribution, molecular form and release of cholecystokinin (CCK)-like activity in extracts of rat small intestine was studied with an *in vitro* gall-bladder bio-assay. In contrast to the reported heterogeneity of CCK-like immunoreactivity in the intestine, only a single molecular form of CCK-like activity was detected using the bio-assay.

2. This CCK-like activity eluted from Sephadex G50 with a K_{av} of 0·69, after the triacontriapeptide of cholecystokinin (CCK_{33}) and before cholecystokinin octapeptide (CCK_8). This CCK-like activity, which corresponded to an approximate mol. wt. of 2500, may represent the 22 amino acid peptide of CCK (CCK_{22}). The bio-assay peak of CCK-like activity had pancreozymin activity and CCK/gastrin C terminal immunoreactivity. The CCK-like activity was readily extracted from the small intestine at neutral pH, but subsequent treatment with cold 0·5 M-acetic acid extracted further CCK-like activity of the same molecular form as that recovered under neutral conditions.

3. The bio-assay detected no CCK-like activity, nor was pancreozymin-like activity found in fractions corresponding to CCK_{33} or CCK_8 after Sephadex G50 chromatography of rat intestinal extracts.

4. Oral trypsin inhibitor was a potent stimulus for the release of CCK-like activity from the upper small intestine of the rat. After oral trypsin inhibitor release, CCK-like activity was rapidly resynthesized.

INTRODUCTION

Recent immunochemical studies have emphasized the molecular heterogeneity of cholecystokinin-like peptides in the proximal small intestine (Dockray, 1977; Rehfeld, 1978). It is not known, however, which of these molecular forms of cholecystokinin (CCK) is released by food in the intestine to stimulate gall-bladder contraction and pancreatic secretion. Indeed some of these immunoreactive forms may possibly be biologically inactive precursors or degradation products of the active form of the hormone. There have been very few studies which have measured the relative biological activity of the different molecular forms of CCK-like immunoreactivity identified in the intestinal extracts. A number of bio-assays exists for CCK but

probably the most specific and sensitive bio-assay suitable for such a study is the *in vitro* rabbit gall-bladder bio-assay.

Traditionally, CCK has been considered to be a gut hormone released after a meal to stimulate both gall-bladder contraction (cholecystokinin activity) and pancreatic secretion (pancreozymin activity). An essential criterion for hormonal status, however, is the demonstration of release of the hormone from an endocrine organ into the circulation to stimulate remote target tissues. For CCK, as for some other peptide hormones, it has been difficult to demonstrate either release from the intestine, or elevated plasma levels of hormone, after ingesting a meal. There are many difficulties in measuring peptide hormones in plasma, probably the most important of which are the very low circulating concentrations which are normally present and the rapid destruction of the hormones in the circulation. Radioimmunoassay is usually the method of choice for the assay of peptide hormones but the specificity and sensitivity of the techniques depend largely on the quality of the antisera used. It has proved particularly difficult to raise sensitive and specific antisera suitable for the radioimmunoassay of plasma CCK samples and as yet no widely accepted plasma CCK radioimmunoassay is available (Rehfeld, 1979). In view of these problems the release of CCK from the intestine has usually been studied indirectly, by measuring the pancreatic secretory response or gall-bladder contraction in response to luminal stimuli. In most of these studies the release of CCK has been inferred, though in a few cases a rise in pancreozymin or cholecystokinin activity in the blood has been demonstrated. Khayambashi & Lyman (1969) demonstrated elevated pancreozymin activity in plasma of trypsin inhibitor-fed rats using an isolated perfused pancreas. Trypsin inhibitor was used in this study since it is a potent stimulus of pancreatic secretion in the rat; it is thought to remove the resting inhibition of CCK release by trypsin in the intestinal lumen (Green & Lyman, 1972). Berry & Flower (1971), using an isolated superfused gall-bladder preparation, demonstrated increased circulating cholecystokinin activity after infusing digested fat and protein into the intestinal lumen of anaesthetized cats. However, these bio-assay demonstrations of CCK release have proved difficult to confirm because of the low circulating concentrations of hormones together with the possibility of non-specific interference from other kinins in plasma. Another method of demonstrating CCK release is therefore needed, and a possible technique appears to be the measurement of the reduction in intestinal CCK levels after feeding a potent stimulus such as trypsin inhibitor. Since the intestinal concentrations of CCK are much higher, the responses elicited by bio-assay would be greater and therefore more accurately quantitated. Furthermore, some of the interfering kinins might be destroyed by boiling during the extraction of intestinal CCK.

This paper reports studies on the gel filtration profile of CCK-like activity measured in intestinal extracts by an *in vitro* gall-bladder bio-assay together with the demonstration of CCK release. In contrast to the immunochemical heterogeneity of intestinal CCK, only a single molecular form of CCK-like activity was found by gel filtration of intestinal extracts, corresponding to a molecular weight intermediate between natural porcine CCK triacontriapeptide (CCK_{33}) and sulphated synthetic CCK octapeptide (CCK_8), possibly the 22 aminoacid peptide (CCK_{22}). No CCK activity was found in fractions corresponding to natural porcine CCK_{33} or sulphated synthetic

CCK_8 when extracts of rat small intestine were chromatographed on Sephadex G50. Oral trypsin inhibitor was found to be a potent stimulus of CCK release, since the CCK-like activity in intestinal extracts fell significantly after feeding trypsin inhibitor.

METHODS

Extraction of CCK

CCK was extracted from tissues by the method of Rehfeld (1978). Rats were anaesthetized with ether and killed by exsanguination. The small intestine was removed, divided into thirds and luminal contents washed out. The proximal third was then frozen on dry ice (−78 °C). The delay between death and freezing of tissues was less than 2 min. Frozen proximal small intestine was chopped into small pieces and boiled in distilled water (10 ml./g; pH 6·6) for 25 min. Tissue was then homogenized in a Sorvall Omnimixer homogenizer for 3 min, and centrifuged at 15,000 *g* for 15 min. After decanting the supernatant, the pellet was re-extracted with cold 0·5 M-acetic acid (3 % v/v; pH 2·5). Aliquots of both aqueous and acid extracts were assayed separately for CCK activity. The acid extract was lyophilized to evaporate the acetic acid and reconstituted with water before bio-assay. The efficiency of CCK extraction was assessed by the recovery of porcine cholecystokinin triacontriapeptide (CCK_{33}, G.I.H. Research Unit, Karolinska Institute, Sweden) and synthetic cholecystokinin octapeptide (CCK_8, Squibb Inc. Princeton, N.J., U.S.A.) added to tissues before boiling, and then extracted as described above.

Chromatography

Lyophilized extracts from 1–2 *g* small intestine were resuspended in 3 ml. water or acetic acid and centrifuged at 15,000 *g*. A 2 ml. aliquot was applied to a Sephadex G50 column (100 × 1·8 cm) and eluted with 0·25 M-ammonium bicarbonate (4 °C; flow 10 ml./hr). The fractions were then lyophilized to remove ammonium bicarbonate since this interfered with gall-bladder contraction. Fractions were then redissolved in 0·5 ml. Krebs–Ringer solution prepared as described by Berry & Flower (1971). The total CCK-like activity recovered after chromatography was 80–90 % of the activity applied to the column. The void volume (V_0) of the column was determined from the elution volume of blue dextran and the total volume (V_t) was measured from the conductivity peak (salt peak) after applying M-sodium chloride. In addition, coloured markers, blue dextran and vitamin B_{12} were routinely added to extracts to monitor elution volumes. The elution volumes of the known molecular forms of CCK were also determined by chromatography of purified CCK_{33} (G.I.H.) and CCK_8 and measuring CCK-like activity in the fractions by bio-assay. The elution volumes for 17 amino acid gastrin (gastrin-17) and CCK_8 were determined separately by chromatography of the ^{125}I-labelled hormones applied to the column mixed with rat intestinal extracts. The K_{av} of the labelled compound was determined from the peak of radioactivity, measured with a Packard Gamma Scintillation Spectrometer, Model 5630.

Bio-assay

New Zealand white rabbits, weighing 2–3 kg, were killed by barbiturate overdose. The gall-bladder was rapidly removed, placed in ice-cold Krebs–Ringer solution and the bile flushed out with the cold Krebs solution. Krebs–Ringer solution was prepared as described by Berry & Flower (1971). Gall-bladders were not cut into strips but were used intact, resulting in a more stable and sensitive preparation. Isolated gall-bladders were suspended in an organ bath containing Krebs–Ringer solution bubbled continuously with 95 % O_2 and 5 % CO_2. The muscle was kept under tension with a counterweight of between 0·2 and 0·7 *g*, depending on the strength of the contraction in response to CCK. Isotonic contractions were measured with a Harvard Transducer (Model 386), amplified and recorded on a rectilinear chart recorder. Standards were prepared from porcine CCK (G.I.H.) and synthetic CCK octapeptide (Squibb Inc.). G.I.H. CCK (40 Ivy dog units/ampoule), which is stated to be 25 % pure, comprises mainly CCK_{33} together with some CCK_{39} (Oliver & Harvey, 1977). Vagne & Grossman (1968) reported that 1 mg CCK_{33} was equivalent to 3000 Ivy dog units (IDU): 1 p-mole CCK_{33} would, therefore, be equivalent to 12 milli Ivy dog units (mIDU). This equivalence was assumed in this study. Since biological activity was found to vary between batches, standards were prepared throughout from the same batch (batch no. 27822).

The gall-bladder preparation was calibrated by constructing a cumulative dose–response curve

with one of the standard preparations of CCK. A dose of CCK standard just above threshold was added to the bath. This was usually about 1 p-mole CCK_{33} which would give a bath concentration of 1×10^{-10} M. When this response had plateaued, a second dose of CCK standard equal to the first was added to the bath. A third dose twice the initial dose followed, by which time the response had usually reached about 60% of the maximum. This was followed by two larger doses, sufficient to finally produce maximal contraction. For the bio-assay of unknown extracts responses were measured in the range of 20–50% of the maximal contraction, and bracketed between two cumulative standard responses to check against changes in sensitivity. To ensure reproducibility and to increase the number of samples assayed, two or three gall-bladders were set up in parallel; thus, about forty samples could be assayed in 8 hr. The volume of extract assayed was 0·1–0·2 ml. for jejunum and 0·4–1 ml. for distal jejunum and ileum, with at least two measurements of CCK-like activity being made for each extract.

Pancreozymin activity

Pancreozymin activity was determined by measuring pancreatic secretion in rats acutely anaesthetized with urethane as described by Dockray (1972). Pancreozymin activity was measured as the protein content of pancreatic juice in three 10 min collections after bolus intravenous injection of CCK standards or unknown extracts.

Preparation of radiolabelled hormones

Radiolabelled CCK was prepared by iodinating synthetic desulphated CCK_8 with ^{125}I using the chloramine T method of Hunter & Greenwood (1962). After 30 sec incubation, the reaction was stopped with sodium metabisulphite. The ^{125}I-labelled CCK was separated from the free ^{125}I by chromatography on a Biogel P2 column. A number of fractions were selected from the [^{125}I]CCK peak, and degree of suitability determined by overnight incubation with antisera to determine the non-specific activity, degree of label damage and the total percentage bound. The best fractions were then divided into aliquots and stored at −20 °C; in this way the label was stable for up to 3 months. Synthetic human gastrin I (Imperial Chemical Industries, U.K.) was also iodinated with ^{125}I by the chloramine T method. The labelled gastrin was separated from free iodide by chromatography on a Sephadex G10 column.

Radioimmunoassay

Aliquots from fractions comprising the peak of CCK-like activity identified by bio-assay were assayed blind for gastrin/CCK C-terminal immunoreactivity in another laboratory (Dr J. Hansky, Prince Henry's Hospital, Melbourne, Australia). The gastrin/CCK immunoassay used an antiserum (AB 5135) specific for the C-terminal region of CCK and gastrin. Details of this assay have been published elsewhere (Hansky & Ho, 1979). The ID_{50} (amount required to inhibit the bound to free radioactivity by 50%) is 5 f-mole for gastrin-17, 5 f-mole for CCK_8, and 25 f-mole for CCK_{33}.

Trypsin inhibitor study

Trypsin inhibitor was prepared from raw soyabean flour (Boschetti & Delay, 1978) and had an activity of 75 mg bovine trypsin inhibited per 100 mg of trypsin inhibitor preparation, as determined by the method of Kakade, Rackis, McGhee & Puski (1974). Male Wistar rats weighing 200–220 g were fasted for 18 hr. Rats were lightly anaesthetized with ether and gavaged with 100 mg of trypsin inhibitor dissolved in 2 ml. 0·15 M-saline. Groups of four rats were killed immediately, and 10, 20, 30 and 60 min after gavage with trypsin inhibitor. Control groups of rats were gavaged with 100 mg bovine serum albumin and killed at varying intervals. After killing, the small intestine was rapidly removed, and the luminal contents washed out with cold 0·1 M-imidazole buffer pH 7·4. The intestine was divided into thirds and the proximal third was frozen on dry ice. The entire pancreas was also rapidly removed and frozen on dry ice. The pancreas was then minced, homogenized in distilled water and, after dilution, sonicated in a Branson sonifier. An aliquot of the sonicate was then assayed for amylase activity by the method of Dahlqvist (1962). Intestinal washings were homogenized, centrifuged (1500 g) and aliquots of the supernatant assayed for amylase and trypsin activity. Trypsin activity of intestinal contents was measured against N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) substrate by the method of Preiser, Schmitz, Maestracci & Crane (1975), without further activation. The CCK-like activity from the mucosa of the proximal third of the small intestine was extracted and assayed as described above.

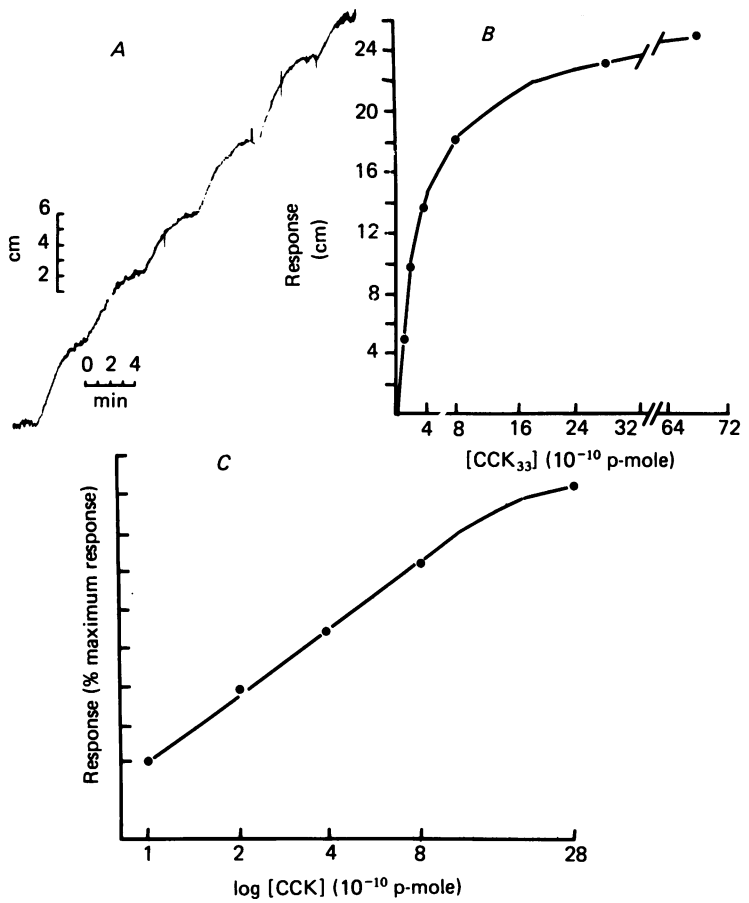

Fig. 1. The dose–response relationship of the *in vitro* gall-bladder to porcine CCK_{33} (G.I.H.). *A* shows a recording of the *in vitro* gall-bladder contractions to 1, 1, 2, 4, 20 and 40 p-mole CCK_{33}, given cumultatively as described in the Methods. *B* is the relationship of the above cumulative responses to the CCK concentration plotted on a linear scale. The response is measured as centimetres of isotonic contraction. *C* shows the relationship of the response of the log of CCK concentration, each response being expressed as a percentage of the maximal contraction. The 50 % maximal contraction was elicited by a CCK concentration of $3{\cdot}3 \times 10^{-10}$ M-CCK_{33} equivalents. The correlation coefficient of regression of the log of CCK concentration against response 20–80 percent maximum was 0·998.

Statistical methods

Data are expressed as means and standard deviations. Means were compared using Student's *t* test for unpaired data and were considered statistically significantly different if $P < 0{\cdot}05$.

RESULTS

The isolated gall-bladder was highly sensitive to CCK, measurable contractions being seen with 0·3 p-mole of porcine CCK_{33}, i.e. concentration of 3×10^{-11} M. Comparing the potency of synthetic CCK_8 with CCK (G.I.H.), 1 p-mole CCK_8 was

found to be equivalent to 17 mIDU CCK (G.I.H.). Assuming CCK (G.I.H.) to be entirely CCK_{33}, 1 p-mole CCK_8 would therefore be equivalent in potency to 1·4 p-mole porcine CCK_{33} on the *in vitro* rabbit gall-bladder. A graded increase in the strength of contraction was seen with increasing doses of CCK. Fifty percent maximal contraction was elicited by 3×10^{-10} M-CCK_{33} (Fig. 1). The strength of contraction depended on the concentration of CCK in the bath at that time, regardless of whether standard or extract was given as a single dose or a number of doses cumulatively. The dose–response curve was a rectangular hyperbola which gave a linear relationship between response and the logarithm of CCK concentration (Fig. 1).

TABLE 1. The reproducibility of CCK-like activity measured in intestinal extracts measured by *in vitro* gall-bladder bio-assay. Two intestinal extracts were assayed for CCK-like activity using three volumes of each extract on two different gall-bladders. The isotonic contraction of CCK in extracts and standards was measured in cm. The responses were measured by comparing the response elicited by extracts to those elicited by known doses of a porcine CCK_{33} standard. The concentration of CCK-like activity is expressed as p-mole CCK_{33} equivalent per ml. extract

Sample no. 1 (small intestine extract)

	Standard CCK_{33}		Extract volume (μl.)	Response (cm)	CCK-like activity (p-mole CCK_{33})	Estimated CCK-like concentration (p-mole CCK_{33}/ml.)
	Dose (p-mole)	Response (cm)				
Gall-bladder no. 1	2	8·1	200	10·1	2·2	11
	4	19·0	300	15·2	3·3	11
	6	22·8	400	17·0	4·0	10
Gall-bladder no. 2	2	8·9	200	8·9	2·1	10·5
	4	14·4	300	12·7	3·3	11
	6	17·5	400	14·2	4·2	10·5

Sample no. 2 (small intestine extract)

	Standard CCK_{33}		Extract volume (μl.)	Response (cm)	CCK-like activity (p-mole CCK_{33})	Estimated CCK-like concentration (p-mole CCK_{33}/ml.)
	Dose (p-mole)	Response (cm)				
Gall-bladder no. 1	2	8·6	200	10·1	2·5	12·5
	4	15·2	300	13·9	3·8	12·7
	6	18·5	400	16·5	5·0	12·5
Gall-bladder no. 2	2	7·6	200	7·9	2·3	11·5
	4	10·9	300	10·1	3·8	12·7

No tachyphylaxis was observed with repeated doses of up to 60 % of the maximal response. Useful responses were obtained from the gall-bladder for about 8–10 hr. Over the first few hours the gall-bladder's sensitivity to CCK gradually increased, after which it plateaued. During these changes of sensitivity, a linear log dose–response curve was maintained. Reproducible measurements of the CCK-like activity in intestinal extracts could be made (Table 1). Varying aliquots of the same extracts gave estimates of the CCK-like activity which agreed within 10 %. Extracts measured on different gall-bladders agreed within 13 %. Fiducial limits of the bio-assay

calculated for a three point bio-assay protacol were 85–118% for a 95% confidence level (Edinburgh Department of Pharmacology, 1968). The reliability of the bio-assay for measurement of CCK extracted from rat tissue was assessed by adding ^{125}I-labelled CCK to aqueous extracts of rat brain. The extract was eluted from a Sephadex G50 column and each fraction of the CCK peak was measured for radioactivity and CCK-like biological activity. The brain extract applied to the column contained approximately 200 p-mole CCK_8 and the mass of the CCK in the labelled tracer was

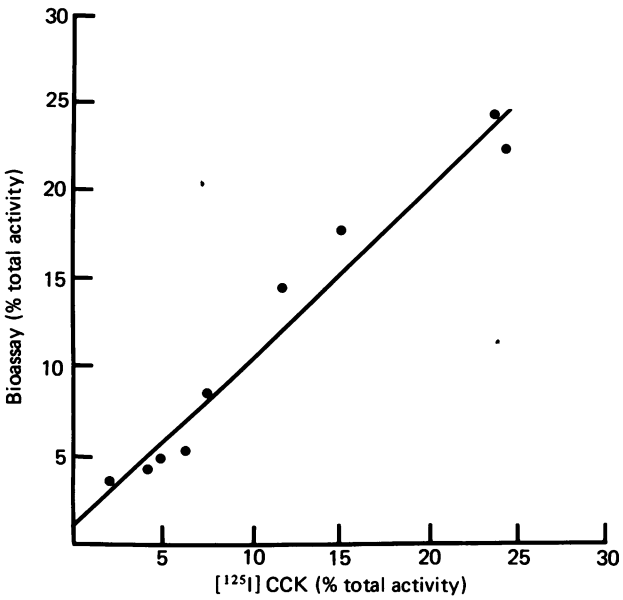

Fig. 2. Correlation between the CCK-like activity measured by bio-assay and recovery of labelled CCK_8. An extract of rat brain was dissolved in 10 ml. water and mixed with ^{125}I-labelled CCK_8. The mixture was applied to a Sephadex G50 superfine column and eluted with 0·25 M-ammonium bicarbonate buffer pH 8·4. Column dimension 70 × 1·8 cm; bed volume 110 ml., sample size 10 ml., flow 10 ml./hr, at 4 °C, fraction size 4·2 ml. The CCK-like activity in each fraction was measured by bio-assay and the radioactivity measured by liquid scintillation counting. The activities eluted from the column with the same K_{av} (1·05). The graph shows the CCK-like activity and radioactivity in each fraction, expressed as the percentage of total activity recovered. The regression line is fitted by the methods of least squares. The equation of this line is $y = 0{\cdot}98 + 0{\cdot}88$ with a correlation coefficient of 0·98.

less than 0·5% of the total CCK. The correlation between the CCK-like biological activity and the radioactivity in each fraction is shown in Fig. 2. The correlation coefficient was 0·98 and the slope of the regression line was 0·94, indicating a precise measurement of extracted CCK by the bio-assay over a tenfold range of activity.

The specificity of the response of the *in vitro* gall-bladder to CCK as determined by Berry & Flower (1971) and Johnson & McDermott (1973) was confirmed; acetylcholine stimulated contraction only above 10^{-5} M and pentagastrin (Peptavlon, I.C.I., Macclesfield, U.K.) had no effect below a concentration of 10^{-6} M. Synthetic human gastrin I (Sigma, St Louis, U.S.A.) only stimulated contraction above 10^{-8} M.

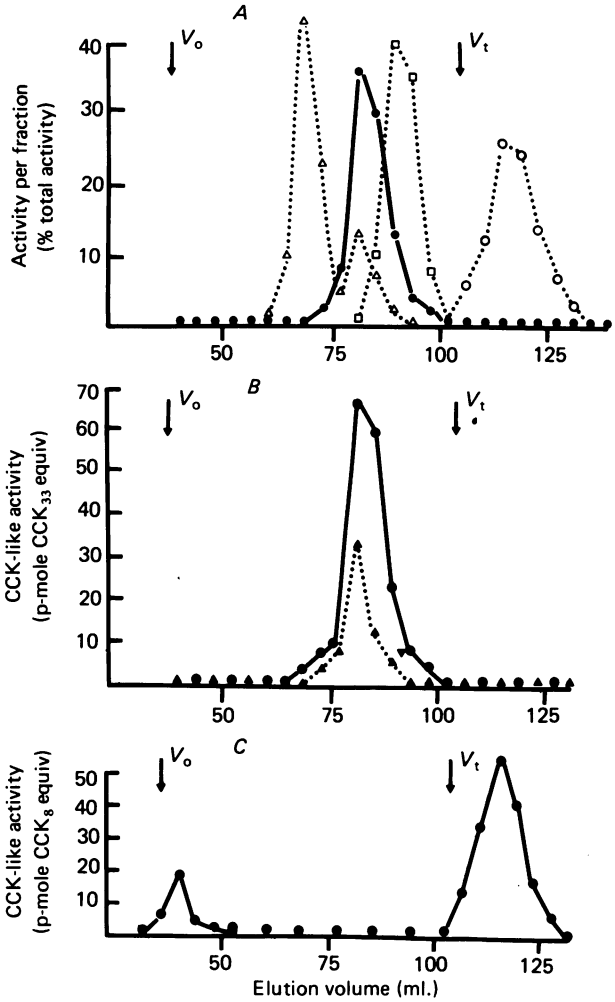

Fig. 3. Sephadex chromatography of tissue extracts. *A* shows the elution profiles for rat intestinal extracts alone (●), rat intestinal extract with added porcine CCK_{33} (△), ^{125}I-labelled human gastrin in intestinal extract (□) and ^{125}I-labelled synthetic CCK_8 in intestinal extract (○). All four samples were chromatographed separately. Fractions from chromatographs of intestinal extracts were assayed for CCK-like activity by bio-assay, and fractions from chromatography of ^{125}I-labelled hormones for radioactivity by spectroscopy. Activity in each fraction is expressed as a percentage of total activity recovered. *B* shows the elution profile of CCK-like activity measured by bio-assay of rat small intestine extracted by the initial aqueous extraction (●) and by the subsequent cold extraction with 0·5 M-acetic acid (▲). CCK-like activity in each fraction is expressed as p-mole CCK_{33} equivalent. *C* shows the elution profile of CCK-like activity measured by bio-assay in aqueous/acid extracts of rat brain. CCK-like activity is expressed as p-mole CCK_8 equivalent. In each case Sephadex G50 superfine was used. Column dimensions: 70 × 1·8 cm, bed volume 110 ml., column buffer 0·25 M-ammonium bicarbonate, sample size 2 ml., flow 10 ml./hr, fraction size 4·2 ml. at 4 °C. V_o = void volume. V_t = total volume. The profile of CCK-like activity in rat intestinal extracts after Sephadex G50 chromatography was determined ten times by bio-assay. The range of K_{av} determination for the peak of CCK-like activity was 0·67–0·71.

Synthetic substance P (Sigma, St Louis, U.S.A.) was found to stimulate the rabbit gall-bladder only above 10^{-7} M, a concentration 1000 times that required for CCK.

Only extracts of brain and small intestine showed CCK-like activity, and the time course of a gall-bladder contraction with these extracts was identical to that produced by standard CCK solutions. These tissue extracts also had marked pancreozymin activity, stimulating enzyme output when injected intravenously into anaesthetized rats, the potency being similar to the equivalent CCK standard. No CCK-like activity was found in extracts of liver, kidney, spleen or pancreas. The greater part of the CCK-like activity extracted from the rat intestine was recovered in the initial aqueous extraction. The subsequent acid extraction comprised approximately one third of the total CCK-like activity extracted. With repeated acid extraction of the pellet no further CCK-like activity was recovered. The efficiency of recovery of CCK_{33} and CCK_8 added to tissues prior to aqueous/acid extraction was 89 % for CCK_{33} and 110 % for CCK_8.

The concentration of CCK-like activity in the rat proximal small intestine was 132 ± 9 ($n = 8$) p-mole CCK_{33} equivalents per g tissue wet weight, whilst the intestinal concentrations in the rabbit and dog were 430 ± 17 ($n = 4$) p-mole CCK_{33} equiv per g and 308 ± 28 ($n = 4$) p-mole CCK_{33} equiv per g respectively. Significant concentrations of CCK-like activity were found in the antrum (40 ± 8 ($n = 3$) p-mole CCK_{33} equiv per g) of all three species examined. High concentrations of CCK-like activity were found in the dog brain, the highest concentrations being found in the cerebrum (270 ± 14 ($n = 4$) p-mole CCK_8 equiv per g) with lower concentrations in the diencephelon (100 ± 12 ($n = 4$) p-mole CCK_8 equiv per g) and medulla (19 ± 6 ($n = 4$) p-mole CCK_8 equiv per g). The cerebellum had negligible CCK-like activity. A similar distribution was found in the rabbit brain.

The molecular forms of CCK-like biological activity found in the rat were determined by Sephadex chromatography of brain and small intestinal extracts (Fig. 3). The chromatography of extracts of rat brain revealed in fractions corresponding to CCK_8 (K_{av} 1·1). Bio-assay detected only a single peak of CCK-like activity in rat intestinal extracts. This activity eluted after porcine CCK and before gastrin-17 with a K_{av} of 0·68. The peak of CCK-like activity in acid extracts of rat intestine eluted with the same K_{av} as aqueous extracts (Fig. 3). Fractions containing the peak of CCK-like activity (K_{av} 0·68) were pooled and rechromatographed on a Sephadex G50 column. The CCK-like activity eluted with the same K_{av} as in the initial fractionation (Fig. 4).

The bio-assay did not detect CCK-like activity corresponding to CCK_8 or CCK_{33} in rat intestinal extracts after chromatography on Sephadex G50. The failure to detect CCK_8 and CCK_{33} may be a consequence of CCK inhibitors co-eluting with these forms of CCK. To exclude this possibility rat intestinal extracts were eluted from Sephadex G50 and the fractions corresponding to CCK_{33} and CCK_8 were pooled. The gall-bladder response to CCK alone was compared to that elicited by CCK in the presence of these pooled fractions. The sensitivity of the gall-bladder to CCK was not reduced by fractions corresponding to CCK_{33} or CCK_8 from rat intestinal extracts. Furthermore CCK_{33} and CCK_8 were not selectively destroyed during extraction since pure preparations of these forms of CCK added prior to extraction were fully recovered after extraction. Nor did this intermediate form of CCK-like activity found in the

rat intestine result from breakdown of CCK_{33} during extraction. When porcine CCK_{33} was mixed with intestinal tissue and then extracted the added CCK was fully recovered in a peak corresponding to CCK_{33} (Fig. 4). No co-factor was found co-eluting with this intermediate form of CCK which potentiated gall-bladder contraction. Cumulative responses of the *in vitro* gall-bladder were determined to sequential doses

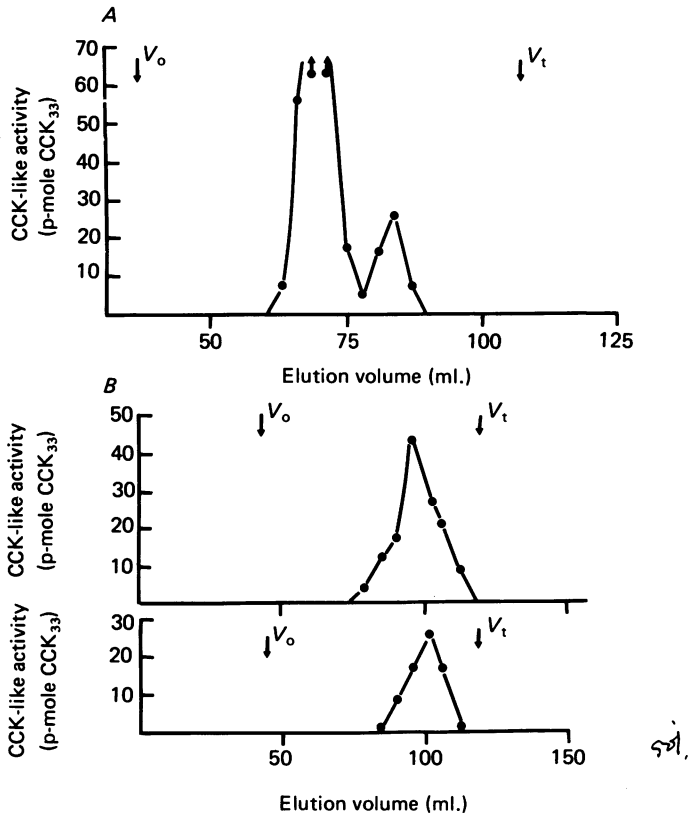

Fig. 4. *A*, chromatography of porcine CCK_{33} added to extracted rat intestine and re-extracted by boiling in water followed by cold acetic acid extraction. The CCK-like activity in each fraction was determined by bio-assay and measured in p-mole CCK_{33} equivalents. Two peaks of CCK-like activity were found: a major peak (K_{av} 0·48) and a minor peak (K_{av} 0·7). The void volume (V_o) was 33 ml. and the total volume (V_t) was 110 ml. *B*, rechromatography of rat intestinal extracts. The upper graph shows the profile of CCK-like activity (K_{av} 0·69) after chromatography on Sephadex G50 determined by bio-assay. The lower graph shows the profile of CCK-like activity (K_{av} 0·72) after the peak of activity from the upper graph was eluted from Sephadex G50 a second time. The void volume (V_o) was 45 ml. and the total volume (V_t) was 122 ml. The conditions of chromatography were the same as in Fig. 3.

of synthetic sulphated CCK_8 standard. The correlation of the log dose–response relationship over a tenfold range (1·5–15 p-mole, four points) was 0·997. After relaxation, an aliquot of a fraction from the peak of CCK-like activity found in the rat intestinal extracts was added to the organ bath and elicited a contraction equivalent to 2·8 p-mole. A dose of CCK_8 standard (3·4 p-mole) was then added and

the cumulative response elicited was equivalent to 6·4 p-mole. The estimated CCK_8 added was therefore 3·6 p-mole, which was not significantly different from the actual dose (3·2 p-mole) added, considered the limits of accuracy of the bio-assay (10–15%). This was repeated for the two other major fractions of the peak of CCK-like activity in rat intestinal extracts of Sephadex chromatography and no signficant potentiation or antagonism could be found.

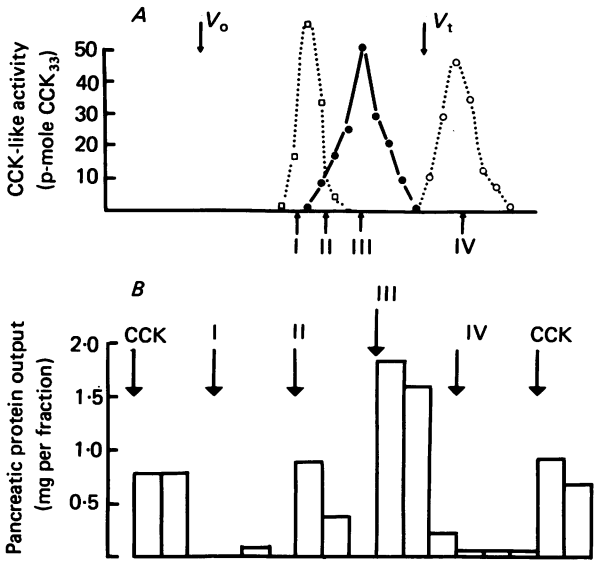

Fig. 5. The pancreozymin activity of the intermediate form of CCK-like activity in extracts of rat small intestine. *A* shows a single peak of CCK-like activity (K_{av} 0·69; ●) after chromatography of rat intestinal extracts eluted from Sephadex G50 with 0·25 M-ammonium bicarbonate. The elution profile of the pure forms of CCK are shown: porcine CCK_{33} (□), K_{av} 0·5 and synthetic CCK_8 (○), K_{av} 1·06. *B* shows the protein content in pancreatic juice from acutely anaesthetized rats collected as sequential 10 min fractions. Bolus intravenous injections of pooled fractions after chromatography of intestinal extracts were made at the points indicated. Injection I was from fractions corresponding to the elution volume of porcine CCK_{33} (K_{av} 0·5). Injection II corresponds to fractions between the CCK_{33} peak and the peak of CCK-like activity found in the rat. Injection III indicates the injection of fractions from the peak of CCK-like activity (K_{av} 0·69) found in the rat intestinal extract. Injection IV corresponds to the peak of CCK_8 (K_{av} 1·03). At the beginning and end of the collections rats received an injection of 1 IDU of CCK standard. The protein of each fraction is the mean of experiments performed on two rats.

The fractions corresponding to the peak of CCK activity as measured by bio-assay showed marked pancreozymin activity (Fig. 5). The pancreozymin activity was measured by the protein output in pancreatic juice secreted in response to injections of pooled fractions after chromatography. No activity was found in fractions corresponding to the elution volume of CCK_8 and very little in fractions corresponding to that of CCK_{33} (Fig. 5).

The peak of CCK-like activity identified by bio-assay was assayed for gastrin/CCK C-terminal immunoreactivity. There was a good correlation between the CCK-like

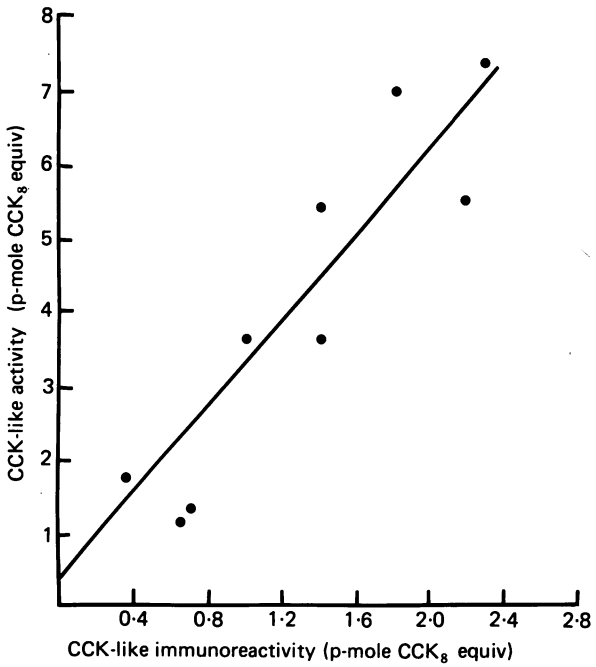

Fig. 6. The correlations between C-terminal immunoreactivity and CCK-like activity measured by the bio-assay of fractions in the peak of CCK-like activity (K_{av} 0·68) identified after chromatography of rat intestinal extracts on Sephadex G50. The ordinate shows the CCK-like biological activity measured by bio-assay in p-mole CCK_8 equivalent and the abscissa shows the corresponding C-terminal immunoreactivity of each fraction in p-mole of CCK_8 equivalent. The equation of the line of best fit is $y = 3x + 0{\cdot}3$ and the correlation coefficient was 0·908.

TABLE 2. CCK-like immunoreactivity in rat intestinal extracts corresponding to the elution volume CCK_8 (K_{av} 1·1). An aliquot (300 μl.) of rat intestinal extract was eluted from Sephadex G50 with 0·25 M-NH_4HCO_3. Fractions of 1 ml. were collected and assayed for CCK-like immunoreactivity using a CCK/gastrin C-terminal radioimmunoassay. The void volume (V_0) was 30 ml. and the total volume (V_t) was 85 ml. A larger aliquot (2 ml.) of the same extract was also eluted from Sephadex G50 under the same conditions except that 3 ml. fractions were collected. These fractions were then assayed for CCK-like biological activity using an *in vitro* rabbit gall-bladder bio-assay. n.d. = not detected

Fraction number	Immunoreactivity (p-mole CCK_8)	CCK-like biological activity
84	0·54	
85	0·95	n.d.
86	0·82	
87	1·05	
88	1·56	n.d.
89	1·32	
90	0·44	
91	—	n.d.
92	0·11	
93	0·04	

activity in each fraction of the peak measured by bio-assay and the corresponding gastrin/CCK-like immunoreactivity (Fig. 6).

Fractions from intestinal extracts corresponding to CCK_8 were also assayed for CCK/gastrin C-terminal immunoreactivity. Table 2 shows the presence of a significant peak of CCK-like immunoreactivity in rat intestinal extracts corresponding to the elution volume of labelled CCK_8. This elution volume also corresponds to the peak of both CCK-like biological activity and immunoreactivity found in brain extracts.

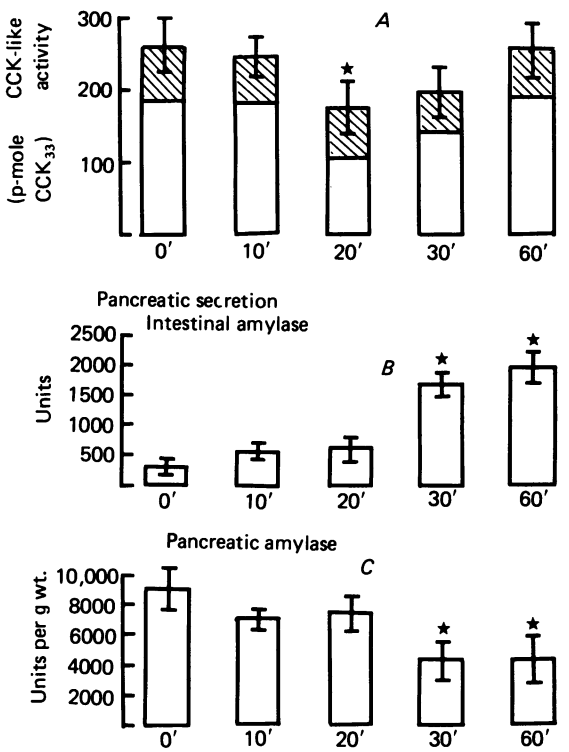

Fig. 7. Release of endogenous CCK-like activity from the intestine by oral trypsin inhibitor. *A* shows the CCK-like activity extracted from the proximal third of the intestine at various times after feeding soybean trypsin inhibitor. The CCK-like activity was measured by bio-assay and is expressed in p-mole CCK_{33} equivalent for both the aqueous extract (□) and the acid extract (▨). *B* shows the corresponding total intestinal amylase levels after feeding trypsin inhibitor, expressed as units as defined by Dahlqvist (1962). *C* shows the amylase concentration of the pancreas after feeding trypsin inhibitor expressed in units per g. Each value is the mean standard deviation of four rats.

No CCK-like biological activity was detected when fractions of this intestinal extract corresponding to the CCK_8 peak were bio-assayed although six times more extract was applied and the fractions were three times the volume. If the CCK_8 immunoreactivity had biological activity the estimated CCK in these bio-assayed fractions would be about 20 p-mole CCK_8 and readily detectable by the *in vitro* rabbit gall-bladder, which responds to doses of CCK_8 as low as 0·2 p-mole.

Oral trypsin inhibitor was a potent stimulus of the release of CCK-like activity from

the small intestine (Fig. 7). Twenty minutes after gavage intestinal CCK content was nearly halved, the reduction being confined to the initial water extract. One hour after gavage intestinal CCK content had returned to the initial basal levels, indicating rapid synthesis of endogenous CCK.

Chromatography of CCK-like activity in intestinal extracts from rats killed 20 min after feeding trypsin inhibitor, compared with that from rats killed immediately after feeding, is shown in Fig. 8. Again only a single peak of CCK-like activity (K_{av} 0·69)

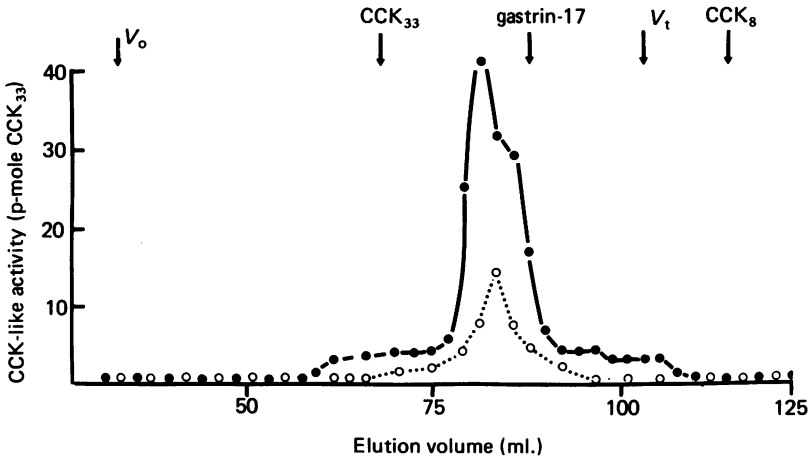

Fig. 8. The release of the intermediate form of CCK-like activity from the intestine by oral trypsin inhibitor. Aqueous/acid extracts of proximal small intestine from rats killed immediately (●) and 20 min (○) after feeding trypsin inhibitor were separately chromatographed in Sephadex G50. The conditions of chromatography were the same as in Fig. 3. The ordinate shows the CCK-like activity measured by bio-assay in p-mole CCK_{33} equivalents. The abscissa shows the elution volume, and the void volume (V_0) and the total volume (V_t) are indicated. The elution volumes of porcine CCK_{33} and synthetic CCK_8 were determined by separate calibration.

was seen, with a reduction in this peak 20 min after feeding trypsin inhibitor. Potent but brief stimulation of pancreatic secretion followed the release of CCK-like activity. Thirty minutes after gavage intestinal amylase was greatly increased, but apparently very little pancreatic secretion occurred after this since intestinal amylase levels had not changed from the 30 min values and nearly all the amylase activity was recovered in the ileum. This stimulation of secretion nearly halved the pancreatic amylase content (Fig. 8). Although oral protein did stimulate some pancreatic secretion, trypsin inhibitor was ten times more potent.

DISCUSSION

The CCK-like biological activity in extracts of rat brain was found predominantly as CCK_8, agreeing with previous immunochemical studies (Dockray, 1977; Rehfeld, 1978). The CCK-like biological activity found in extracts of rat intestine, however, differs from previous reports of CCK-like immunoreactivity in porcine and human small intestine. Only a single peak of CCK-like biological activity was found in rat

intestine regardless of whether the tissue was extracted under acid or aqueous conditions. This differs from the heterogeneity of CCK-like immunoreactivity found in extracts of porcine and human small intestine reported by Dockray (1977) and Rehfeld (1978). Furthermore this peak of biological activity found by bio-assay did not correspond to either CCK_{33} or CCK_8, the predominant forms of CCK-like immunoreactivity reported in the porcine and human small intestine. The biological activity (K_{av} 0·69) eluted after CCK_{33} and before gastrin-17, corresponds to a molecular weight of approximately 2500. It is possible that this biological activity corresponds to the 22 amino acid peptide of CCK (CCK_{22}), formed by tryptic cleavage of the lysine, 22 amino acids from the C-terminal end of CCK. Both Dockray (1977) and Rehfeld (1978) found significant amounts of this peptide in extracts of porcine small intestine, although much less than CCK_{33} or CCK_8.

It is highly unlikely that the CCK-like activity found in this study using a bio-assay is a peptide other than CCK. Studies by Onadetti, Rubin, Engel, Pluscec & Sheehan (1969), using synthetic analogues of CCK, demonstrated that gall-bladder contraction is highly specific for the sulphated heptapeptide structure which defines CCK. Consequently the specificity of the gall-bladder bio-assay is probably superior to bio-assays based on pancreatic or gastric secretion. Although intestinal extracts contain a bewildering number of peptides which stimulate smooth muscle, it is unlikely that other peptides are present in extracts at high enough concentrations to stimulate gall-bladder contraction. Substance P does not appear to be a problem. Although estimates of the gall-bladder potency of substance P have been complicated by the contamination of natural preparations of substance P with CCK (Zetler, Cannon, Power, Skrabanek & Vanderhaeghen, 1978), the present study found synthetic substance P stimulated contraction only at 10^{-7} M. Furthermore substance P is a 12 amino acid peptide and should elute after gastrin-17, rather than before it. Bombesin, which is widely distributed throughout the gut, has a similar spectrum of biological activity to CCK. Although some molecular forms of bombesin would elute from Sephadex G50 with a K_{av} similar to the CCK-like activity found in this study, it only stimulated gall-bladder contraction at 10^{-7} M (Espermer & Melchorri, 1975). Furthermore the CCK-like activity identified in the present study differs from bombesin in its distribution and extraction properties (Walsh, Wong & Dockray, 1979). Motilin is a potent stimulant of gastrointestinal smooth muscle, but it is only a weak stimulant of gall-bladder contraction, again requiring concentrations of 10^{-7} M (Strunz, Domshke, Mitznegg, Domschke, Schubert, Wunsch, Jaeger & Demling, 1975). The tissue concentrations of these three peptides and of CCK are all of the order of 100 p-mole/g (Bloom, 1978). This would give an organ bath concentration of 10^{-9} M from an aliquot of intestinal extracts. This is a hundredfold less than the concentrations needed to stimulate gall-bladder contraction for substance P, bombesin and motilin, but ten times greater than that needed for CCK, which was found to stimulate gall-bladder contractions at 10^{-10} M in the present study. The peak of CCK-like activity identified by bio-assay had gastrin/CCK C-terminal immunoreactivity, providing direct evidence that the CCK-like activity identified by bio-assay is a form of CCK. It is unlikely that gastrin contributes significantly to the CCK-like activity found in extracts of rat proximal intestine. Not only are the gastrin levels low outside of the antrum in the rat (Larsson, Hokanson, Rehfeld, Stadil & Sunder,

1974) but also the gall-bladder potency is much less than CCK on the rabbit gall-bladder (Amer, 1969).

The predominance of this intermediate form of CCK-like activity detected by bio-assay may result from a factor which potentiates the gall-bladder response and co-elutes with the intermediate form but not with the CCK_{33} or CCK_8 fractions of rat intestinal extracts. This possibility is also suggested from the findings in Fig. 6 where the CCK-like activity measured by bio-assay was greater than that measured by immunoassay. However, no potentiating factor could be found in the intermediate peak of CCK-like activity determined by bio-assay, since the gall-bladder response to pure CCK_8 was not enhanced by the presence of fractions in this peak. It is more likely that the discrepancy between bio-assay and radioimmunoassay findings results from a difference in the relative affinities of the extracted CCK for the gall-bladder receptor and the antibody. Thus the antibody used in this study has only one-fifth the affinity for CCK_{33} as for CCK_8 (ID_{50} 25 f-mole *vs.* ID_{50} 5 f-mole, respectively), whereas on gall-bladder muscle CCK_{33} has two thirds the potency of CCK_8 (1 p-mole CCK_{33} equiv, 12 mIDU and 1 p-mole CCK_8 equiv, 17 mIDU, respectively). It is probable that the intermediate form of CCK found in this study has activity relationships intermediate between CCK_{33} and CCK_8. Direct comparison of the absolute amount of CCK measured by bio-assay and radioimmunoassay is therefore complicated because of this difference in the relative affinity of the ligands for the different molecular forms of CCK. These methods of assay would be directly comparable only if this intermediate form of rat CCK were available in pure form and could be used as a standard.

The failure to find CCK-like activity corresponding to CCK_{33} and CCK_8 in rat intestinal extracts may possibly result from their activity being disguised by co-eluting physiological antagonists. Although a number of peptides inhibit CCK stimulated gall-bladder contraction, relatively high concentrations are required: glucagon 10^{-3} M (Amer, 1972), vasoactive intestinal polypeptide 10^{-6} M (Ryan & Ryave, 1978), secretin 10^{-6} M (Ryan & Cohen, 1976) and enkephalins 10^{-7} M (Zetler, 1979). These inhibitory actions are pharmacological effects and unlikely to affect CCK responses when the concentration of extracted peptide is 10^{-9} M. A cumulative effect of these peptides was excluded by the observation in this study that the gall-bladder response to CCK was unaffected by the presence of intestinal extracts. Since CCK_8 and CCK_{33} were recovered fully when added to intestinal tissue before extraction the failure to detect these forms of CCK did not result from their destruction during extraction. Furthermore extraction did not result in measurable conversion of CCK_{33} to this intermediate form of CCK-like activity. The failure to detect CCK_{33} and CCK_8 with the bio-assay may possibly result from desulphation of the C-terminal tyrosine of CCK during chromatography of Sephadex G50. This would destroy their biological activity without necessarily reducing their immunoreactivity. However, CCK_8 was readily detected after chromatography of brain extracts, and when exogenous CCK_{33} was added to rat intestinal extracts and chromatographed it was readily detected as a distinct peak of activity.

No CCK-like or pancreozymin-like biological activity was found in rat intestinal extracts corresponding to CCK_8 even though a large proportion of the immunoreactivity has been found in this form in porcine and human intestinal extracts (Dockray,

1977; Rehfeld, 1978). Dockray (1980) has also found that CCK_8 comprises a large proportion of the CCK-like immunoreactivity found in rat intestinal extracts. This study also found significant amounts of CCK_8 immunoreactivity in rat intestinal extracts; however, we could find no CCK-like or pancreozymin-like biological activity in fractions corresponding to this peak of CCK_8-like immunoreactivity. This suggests that the immunoreactive CCK_8 found in rat intestine does not possess biological activity. We also could find no evidence for a co-eluting antagonist disguising this activity. Possibly the immunoreactive CCK_8 found in the rat intestine does not have a sulphated tyrosine, and may regulate functions other than gall-bladder contraction or pancreatic secretion. However, Rehfeld (1978) found that the CCK_8 in porcine intestinal extracts bound to an antiserum specific for the sulphated form of CCK, implying that the CCK_8 in intestinal extracts in this species is sulphated.

The predominance of this intermediate form of CCK in rat small intestine may represent a species difference. The multiple molecular forms of peptide hormones like CCK probably result from the tryptic cleavage of a prohormone (Steiner, 1976). The size of these molecular forms is determined by the position of the trypsin-sensitive basic amino acids, lysine and arginine, in the peptide sequence. Since the *N*-terminus of CCK is not necessary for biological activity, it may not be conserved between species. A mutation in the CCK coding gene might therefore result in a substitution of the *N*-terminal lysine, precluding the formation of CCK_{33} from the longer precursor of that particular species. Larsson & Rehfeld (1977) noted marked differences between the immunoreactivity of rat and porcine gastrin towards antisera specific for the *N* terminus, which presumably reflect species differences in the *N*-terminal peptide sequence.

The marked fall in CCK-like activity after feeding trypsin inhibitor is one of the few demonstrations of the release of CCK from the intestine by luminal stimuli. Although a fall in CCK concentration may result from decreased synthesis relative to release, in the present study the rapid fall in CCK-like activity after feeding trypsin inhibitor is most reasonably explained by release into the circulation since this is followed by potent stimulation of pancreatic secretion. Direct demonstration of CCK release, however, awaits a reliable radioimmunoassay for CCK in plasma.

Trypsin inhibitor feeding may also provide a useful model for studying the mechanisms of CCK release from endocrine cells in the small intestine. It may also be possible to exploit the rapid resynthesis of CCK after oral trypsin inhibitor to study the biosynthesis of CCK in the intestine using the elegant methionine pulse-labelling techniques recently described for the rat cerebral cortex (Goltermann, Rehfeld & Roigaard-Petersen, 1980). However, the return of CCK-like activity to resting levels is very rapid and may possibly result from the conversion of a biologically inactive form of CCK into the active form rather than by *de novo* synthesis of the peptide. Nevertheless, labelled methionine is incorporated into CCK_8 extracted from rat cerebral cortex within 30 min of intracisternal injection, implying very rapid biosynthesis of active forms of CCK. Finally, by using the bio-assay in conjunction with these immunochemical techniques it may be possible to identify at what stage in the biosynthesis of CCK does sulphation of the C-terminal tyrosine occur, since this step is essential for significant biological activity.

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