

A gastrin releasing peptide from the porcine non-antral gastric tissue

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SUMMARY This paper presents evidence for the existence in extracts from porcine non-antral gastric tissue of a peptide capable of causing substantial rises of plasma immunoreactive gastrin levels in a dose dependent manner and of stimulation of gastric acid and pepsin secretion. Obtained data show that the peptide is basic and that its gastrin releasing properties are at least partially resistant to atropinisation and β -receptor blockade. Antrectomy almost eliminates the rise in plasma IRGa when the peptide is administered. The possible relationship of this peptide to amphibian bombesin is discussed.

The extraction of peptides from the gastric antral or intestinal tissues (Edkins, 1905; Gregory and Tracy, 1964; Mutt 1976) has yielded the recognised or putative gastrointestinal hormones (Grossman *et al.*, 1974), but little investigation, with rare exceptions (Edkins, 1906; Sutherland and de Duve, 1948; Emås and Fyrö, 1968), has been directed towards the extraction of peptides from the non-antral gastric tissue. However, the presence of cells with endocrine characteristics (Pearse *et al.*, 1970; Vassallo *et al.*, 1971), and previously characterised peptides (Adrian *et al.*, 1976; Polak *et al.*, 1975; Carraway and Leeman, 1976; Kühl *et al.*, 1976; Larsson *et al.*, 1976; Morita *et al.*, 1976; Walsh and Holmquist, 1976; Nilsson and Brodin, 1977) has been reported in non-antral gastric tissue using histological and radioimmunological techniques. In no case, however, has any such peptide been isolated in an essentially pure form from such tissue. We have begun a systematic search for previously unrecognised peptides with endocrine or paracrine function in porcine non-antral gastric tissue. During the course of this study, a fraction was obtained which on administration to test animals causes substantial rises in plasma immunoreactive gastrin (IRGa) and gastric secretion of acid and pepsin (Mutt, 1978). This report gives a preliminary description of the active peptide component of the fraction and discusses its possible relationship to

bombesin, a tetradecapeptide isolated from frog skin (Erspamer and Melchiorri, 1975; Polak *et al.*, 1976; Walsh and Holmquist, 1976)

Methods

MATERIALS

All reagents used were of analytical grade. Alginic acid was from Ed. Mendell Co. Sephadex G-25 (fine) was from Pharmacia, and carboxymethylcellulose (CM-22) from Whatman. TLCK treated α -chymotrypsin was from Merck. Synthetic human gastrin-I (SHG-1) and Inderal (propranolol) were from Imperial Chemical Industries. Atropine sulphate was from ACO. Synthetic bombesin was from Farmitalia.

PURIFICATION AND EXTRACTION TECHNIQUES

The purification methods used in this study were adapted from those previously developed for the extraction of intestinal peptides, and which have been described in detail (Mutt, 1959; 1976). A brief description of the methods is given below.

After a wide excision of the antrum and lesser curvature from the gastric tissues of freshly slaughtered pigs, the remaining tissue (comprising the entire gastric wall) was rinsed in ice-cold water, immersed in boiling water for 10 minutes, and then frozen until extraction. At extraction the tissue was minced, extracted with 0.5 N acetic acid for 16-18

hours under constant stirring at 4°C, filtered, and the peptides in the filtrate adsorbed to alginic acid. The peptides eluted from the alginic acid with 0.2 M HCl were precipitated by NaCl at saturation, collected by filtration, and dissolved in an aqueous-ethanol (1:2) solution. On adjusting the pH to 7.2 a resulting precipitate was removed and the clear filtrate was diluted with water and adsorbed with alginic acid. The peptides eluted from the alginic acid were again precipitated by NaCl at saturation and the gastric peptide concentrate obtained was stored at -80°C. From 365 kg of boiled porcine non-antral gastric tissue, approximately 28 g of this gastric peptide concentrate is obtained.

The gastric peptide concentrate (5.6 g dissolved in 0.2 M acetic acid) was chromatographed on a Sephadex G-25 (fine) column (6.5 × 185 cm) equilibrated and eluted with 0.2 M acetic acid, and the protein elution pattern (Fig. 1) was followed by measuring the light absorbance at 280 nm. Pooled fractions were lyophilised.

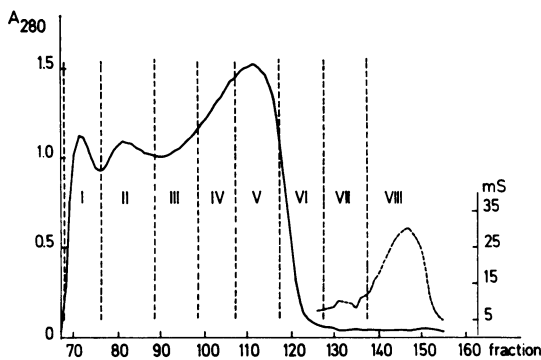


Fig. 1 Gel filtration chromatography of 5.0 g gastric peptide concentrate on a Sephadex G-25 (fine) column (6.5 × 185 cm) equilibrated and eluted with 0.2 N acetic acid. The protein elution pattern was followed by measurement of the light absorbance at 280 nm (—). The salt peak was detected by measurement of the conductivity in milli Siemens (---). Fraction volume was 35 ml. Fractions were pooled as shown and lyophilised.

The yield of fraction V from 25.2 g of gastric peptide concentrate was 2.9 g. Fraction V, containing the activity being investigated, was subjected to a methanol extraction essentially as previously described (Mutt, 1959). The gastrin releasing activity was found in the acid and neutral methanol soluble portion of the extraction mixture and 0.16 g of this active portion was obtained from 2.2 g of fraction V. The acid and neutral methanol soluble sub-fraction was applied to a carboxymethylcellulose (CMC) column (0.85 × 22 cm) equilibrated with 0.02 M

NH₄HCO₃ pH 8.0, and eluted by stepwise changes of NH₄HCO₃ solutions of increasing strength as shown (Fig. 2). The protein elution pattern was followed by measuring the light absorbance at 230 nm and pooled fractions were lyophilised and stored at -20°C until assay. From 125 mg of the acid and neutral methanol soluble fraction applied to the CMC column, 4.2 mg of fraction G and 3.3 mg of fraction H were obtained.

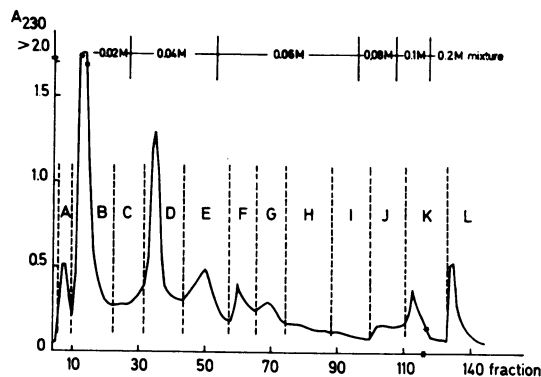


Fig. 2 Chromatography of 20 mg of the acid and neutral methanol soluble sub-fraction of fraction V (Fig. 1) on a carboxymethylcellulose column (0.85 × 22 cm) equilibrated with 0.02 M NH₄HCO₃, pH 8.0. Elution was performed with stepwise increases of NH₄HCO₃ concentrations as shown with the final eluting solution being a mixture of 0.2 M NH₃ and 0.2 M NH₄HCO₃ (1:1). The protein elution pattern was followed by measurement of the light absorbance at 230 nm (—). Fraction volume was 1.9 ml. Fractions were pooled as shown and lyophilised.

ASSAY AND CHEMICAL TECHNIQUES

The procedures used for the radioimmunological determination of plasma gastrin have been described (Nilsson, 1975). SHG-1 was used as standard and for the preparation of gastrin-1²⁵ (Stadil and Rehfeld, 1972). The antiserum used was number 4562 (a generous gift of Dr. Jens Rehfeld) which reacts with equimolar potency with gastrin-17 and gastrin-34, recognises component 1, and has minimal $\left(\frac{[\text{ID}_{50}\text{-Gastrin-17}]}{[\text{ID}_{50}\text{-CCK-33}]} < 0.005 \right)$ cross-reactivity with porcine PZ-CCK (Rehfeld, 1977). The assay is sensitive to 1 pg SHG-1 per assay tube. Blood for IRGa determinations was collected from indwelling venous catheters into lightly heparinised tubes (10 U/ml) and kept on ice until separation of plasma from the red blood cells. Plasma was stored at -20°C or assayed immediately for IRGa content. Tissue extracts were assayed for parathyroid

hormone-like immunoreactivity (IRPTH) by a commercial laboratory (Stockholms Immun-Laboratorium AB).

Tissue extracts were assayed for bombesin-like immunoreactivity (IRB) (by M. G. and S. R. B.) using an anti-serum raised in rabbits to synthetic amphibian tetradecapeptide bombesin (coupled to bovine serum albumin by carbodiimide). N-tyrosyl bombesin after iodination by the chloramine-T method was used as label. The assay was sensitive to 5 pg bombesin per assay tube. There is no cross-reaction with the known gastrointestinal hormones.

Determination of acid and pepsin content of the gastric secretions in cats provided with chronic gastric fistulae was as previously described (Vagne *et al.*, 1974; Vagne and Perret, 1976).

The effect of chymotrypsin treatment on the gastrin releasing properties was determined by incubating the active fraction for one hour at room temperature in 1% NH_4HCO_3 with α -chymotrypsin at a concentration of 25 μg chymotrypsin per mg protein of the test fraction. As a control, the same test fraction was incubated with the chymotrypsin vehicle under the same conditions. Control and chymotrypsin treated tubes were lyophilised, re-dissolved in water, heated in a boiling water bath for six minutes, acidified with 0.2 N acetic acid, and re-lyophilised. The lyophilised material was reconstituted with sterile saline just before testing.

EXPERIMENTAL DESIGNS

Gastrin releasing activity was determined by following the plasma IRGa levels before and after the infusion of test fractions in healthy mongrel dogs weighing 18-30 kg. After an overnight fast, two indwelling venous catheters were placed in separate limbs for test fraction infusion and blood sample collection. The stimulation of gastric acid and pepsin secretion was determined by intravenous infusion of test fractions into chronic gastric fistula cats as previously described (Vagne *et al.*, 1974; Vagne and Perret, 1976). All test fractions were dissolved in sterile saline just before infusion.

Series A

The dose response relationship between administration of the active extract and rise in plasma IRGa was investigated by sequential 30 minute intravenous infusions of 50, 150, and 450 $\mu\text{g}/\text{kg}$ body weight (b.w.) of fraction V from the Sephadex G-25 column (Fig. 1). Preliminary experiments had shown that a recovery period of 90 minutes between infusions of different doses ensured re-establishment of basal or near basal plasma IRGa levels. In some experiments, the reverse sequence of 450, 150, and

50 μg fraction V/kg b.w. was used. In each experiment three basal blood samples were drawn at 10 minute intervals. The 30 minute infusion was commenced at time 0, and further blood samples were drawn at 5, 10, 15, 20, 30, 45, and 70 minutes. A similar protocol was followed in infusing 450 μg fraction V/kg b.w. in dogs four weeks post-antrectomy and in infusing 4-8 $\mu\text{g}/\text{kg}$ b.w. of more highly purified extracts into normal dogs.

Series B

In overnight fasted chronic gastric fistula cats one hour infusions of 400 μg fraction V/kg b.w. or 20 $\mu\text{g}/\text{kg}$ b.w. of the more purified active extracts (fraction H from CMC chromatography) were performed. Fifteen minute gastric secretion samples were taken one half hour before and for two hours after the start of the infusion. Gastric juice volume was recorded and the acid and pepsin contents assayed.

Series C

These experiments were designed to investigate the effect of prior treatment with atropine sulphate on the plasma IRGa response to infusion of fraction V. After collection of basal samples as described in series A, a 30 minute control infusion of 200 μg fraction V/kg b.w. was commenced at time 0 and blood samples collected at 5, 10, 15, 20, 25, 30, 31, 32, 33, 35, and 45 minutes. An intravenous dose of 0.1 mg/kg b.w. of atropine was given after the 45 minute blood sample and after a further 45 minute period an identical infusion of fraction V was commenced, blood samples being drawn as in the control infusion. Preliminary experiments on two dogs were carried out according to the same protocol but 200 $\mu\text{g}/\text{kg}$ b.w. of Inderal was administered instead of atropine.

Series D

This experiment was undertaken to investigate the effect of enzymatic treatment on the gastrin releasing properties of the active extract. After two basal blood samples, taken at 10 minute intervals, an intravenous bolus injection of either the control or chymotrypsin treated test fraction was given and blood samples drawn at 2, 3, 5, 10, 15, and 20 minutes after the injection.

Results

PREPARATIVE RESULTS

The protein elution pattern of the gastric peptide concentrate on Sephadex G-25 (Fig. 1) was found to be similar to that of the corresponding intestinal material produced in this laboratory (Mutt, 1976).

Eight pooled fractions were collected with fraction VIII containing the salt peak. Fractions I-VII were assayed for IRGa. Detectable IRGa was found only in fraction VI and VII (240 and 90 pg IRGa/mg protein respectively). No detectable IRPTH was found in fraction V. Initial experiments revealed that fractions III-VI contained the active peptide which caused rises in plasma IRGa and gastric acid secretion, with fraction V being the most potent. This fraction was chosen for the initial animal experiments and for further purification.

Substantial further purification of the active peptide in fraction V was accomplished by extraction into methanol followed by chromatography of the acid and neutral methanol soluble fraction on CMC. The protein elution pattern and elution conditions of this fractionation are shown in Fig. 2.

ACTIVITY RESULTS

Series A

Figure 3 illustrates the obvious dose response relationship of the plasma IRGa to the amount of fraction V infused. This relationship was found to be independent of the order of dose infusion. Each infusate was checked for IRGa content, which was either not detectable or present in negligible amounts. A rise in plasma IRGa was seen five minutes after initiation of the infusion and maximal levels were generally reached at 10 minutes. A tendency for the IRGa level to fall from maximal levels before the infusion was finished was evident in most experiments. Plasma IRGa levels fell to basal or near basal levels between 15-40 minutes after cessation of the infusion.

A high dose of fraction V (450 $\mu\text{g}/\text{kg}$ b.w.) infused into four antrectomised dogs caused little change in plasma IRGa. All four dogs had responded well to fraction V infusion preoperatively.

All fractions from the CMC chromatography were checked for activity which was consistently found in fraction H and to a lesser extent in fraction G, both fractions being eluted with 0.06 M NH_4HCO_3 . Figure 4 shows the substantial rises in plasma IRGa resulting from 30 minute infusions, at doses of 4-8 $\mu\text{g}/\text{kg}$ b.w., of fractions G and H but with little change on infusion of fraction I. The results from fractions obtained in three different preparations are shown. Detectable but low amounts of IRB were found in the CMC fractions, being maximal in fraction G and H. IRB levels, given as pg IRB/ μg protein, in the CMC fractions from preparations 1 and 3 (Fig. 4) respectively were: E-45, 200; F-265, 600; G-815, 1010; H-740, 1500; I-75, 235 and J-45, 65. It seems from these results that fraction H from preparations 1 and 3 contained

only 0.074 and 0.15% IRB respectively, yet gave substantial rises in plasma IRGa on 30 minute infusions at doses of 4 and 8 $\mu\text{g}/\text{kg}$ b.w. respectively.

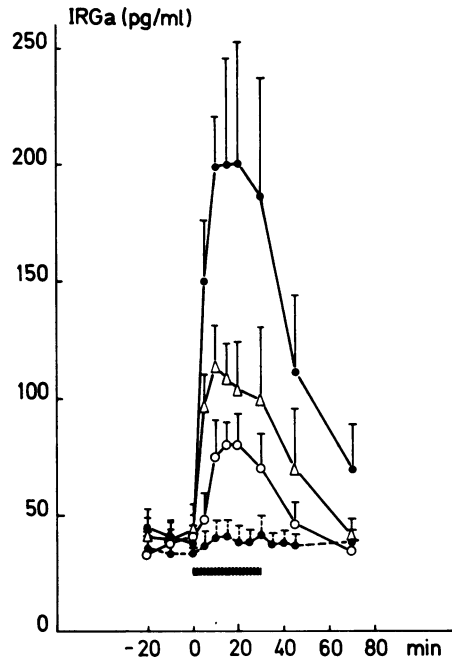


Fig. 3 Plasma IRGa levels (mean \pm SEM) in pg/ml before and after continuous 30 minute intravenous infusions (bar) of fraction V at doses of 50 $\mu\text{g}/\text{kg}$ b.w. (\circ — \circ), 150 $\mu\text{g}/\text{kg}$ b.w. (Δ — Δ) in five normal dogs and at 450 $\mu\text{g}/\text{kg}$ b.w., in five normal dogs (\bullet — \bullet) and in four antrectomised dogs (\bullet — $---$ \bullet).

Series B

In the gastric fistula cat, infusion of 400 μg fraction V/kg b.w. for one hour caused substantial increases in gastric secretory volume and acid and pepsin output (Fig. 5). The increase in all three parameters was seen in the second 15 minute period during the infusion and remained at high levels throughout infusion. On cessation of the infusion, levels of all parameters fell and had reattained basal or near basal values in the second post-infusion period. Similar but smaller (approximately 50% of the increments shown in Fig. 5) increases in the three parameters were seen during an hour long infusion of the CMC fraction H preparation at a dose of 20 $\mu\text{g}/\text{kg}$ b.w.

Series C

The effect of atropinisation on the plasma IRGa response to fraction V was investigated in eight unoperated dogs (Fig. 6). Atropine did not signifi-

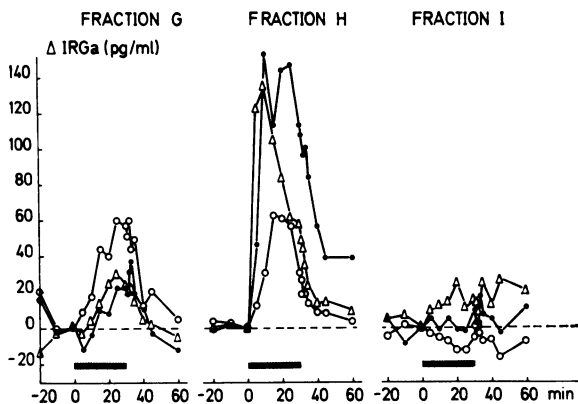


Fig. 4 Plasma IRGa level changes in pg/ml before and after 30 minutes' continuous intravenous infusion (bars) into normal dogs of the carboxymethylcellulose chromatography fractions G, H, and I. Three different chromatographic preparations are shown: preparation 1 (Δ --- Δ) 4 μ g/kg b.w., preparation 2 (\circ — \circ) 8 μ g/kg b.w., and preparation 3 (\bullet — \bullet) 8 μ g/kg b.w. Plasma IRGa at time 0 was chosen as reference level and preceding and succeeding values are shown as changes from this value.

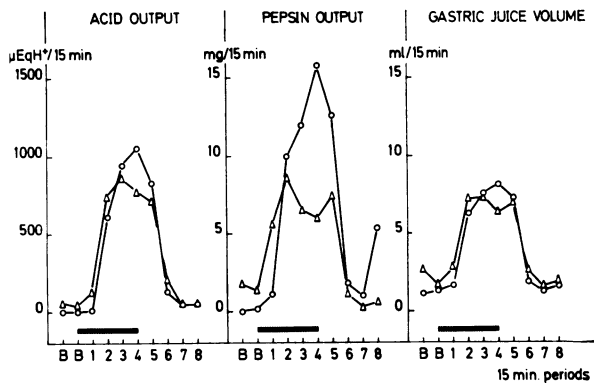


Fig. 5 Acid and pepsin outputs and gastric juice volumes before and after a 1 hour continuous intravenous infusion (bars) of 400 μ g/kg b.w. of fraction V into two cats provided with chronic gastric fistulae. Each curve represents one experiment. See text for details.

cantly alter the peak plasma IRGa response. However, in the post-atropine experiments the raised plasma IRGa values were maintained throughout the infusion period, whereas the plasma IRGa levels in control experiments often fell towards basal levels before cessation of the infusion. In preliminary experiments on two dogs, the infusion of fraction V after β -blockade with propranolol yielded plasma IRGa responses that were not

significantly different from those obtained at control fraction V infusions.

Series D

As seen in Fig. 7, treatment of the active extract with chymotrypsin completely abolished the ability to raise plasma IRGa levels, which was clearly maintained in the control injection. Abolishment of the activity by chymotrypsin treatment suggests that the gastrin releasing factor is peptidal.

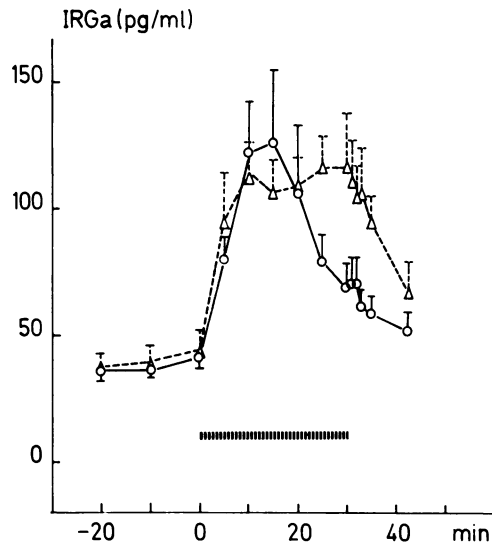


Fig. 6 Plasma IRGa levels (mean \pm SEM) in pg/ml before and after 30 minutes' continuous intravenous infusion (bars) of 200 μ g/kg b.w. of fraction V in eight normal dogs before (\circ — \circ) and after (Δ --- Δ) atropinisation. Atropine at a dose of 0.1 mg/kg b.w. was administered intravenously after the control fraction V infusion. See text for details.

Discussion

In 1906 Edkins obtained extracts from porcine cardiac as well as pyloric gland areas capable of causing gastric acid secretion (Edkins, 1906). Numerous reports followed giving conflicting evidence (for review, see Emås and Fyrö, 1968) that extracts of gastric areas other than the pyloric gland area could yield such activity. This report provides evidence that a peptide is present in porcine non-antral gastric tissue which, on infusion into cats provided with chronic gastric fistulae, causes marked gastric acid and pepsin secretion. Infusion of the same active extract into normal dogs has been shown to cause rapid and substantial rises in plasma IRGa in a dose dependent manner. This gastrin releasing factor has been partially

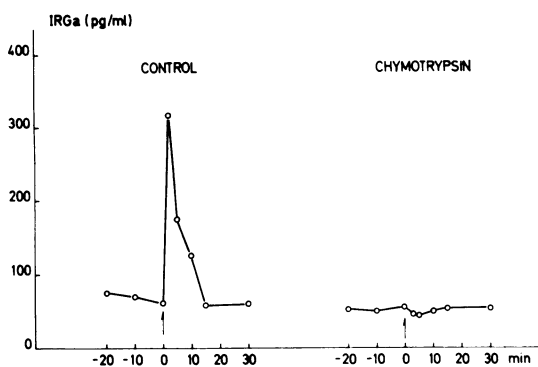


Fig. 7 Plasma IRGa levels in pg/ml before and after a bolus intravenous injection of 200 $\mu\text{g/kg}$ b.w. of the acid and neutral methanol soluble fraction with (chymotrypsin) and without (control) incubation with α -chymotrypsin. See text for details.

purified and the purification technique itself provides evidence that it is basic and of peptidal nature. The dramatic reduction of plasma IRGa response to the active fraction infusion in dogs with antral resections suggests that the antrum is at least the main source of IRGa released into the blood stream.

In certain control infusions of the active extract, the plasma IRGa response faded before the infusion ended; a phenomenon previously noted on bombesin infusion into intact dogs (Bertaccini *et al.*, 1974). This effect was not seen with prior atropinisation in which the raised plasma IRGa levels were well maintained until the infusion was concluded. The explanation of this difference may be that the dose of atropine used inhibited the gastric acid secretion (Nilsson *et al.*, 1972) and thereby eliminated the acid induced inhibition of antral gastrin release. However, the effects of atropinisation are complex (Innes and Nickerson, 1975), and other factors may have been involved.

A number of infusible stimuli for gastrin release have been reported: infusion of adrenaline (Stadil and Rehfeld, 1973; Brandsborg *et al.*, 1975), infusion of calcium in certain species but not in the dog (Reeder *et al.*, 1970, Saik *et al.*, 1975), infusion of parathyroid hormone (Bolman *et al.*, 1977), and infusion of bombesin (Bertaccini *et al.*, 1974). No detectable parathyroid-like immunoreactivity was found in our active extract. The previously well-documented lack of effect of calcium infusion on canine plasma IRGa levels (Reeder *et al.*, 1970; Saik *et al.*, 1975), as well as inhibition of gastric acid secretion after calcium infusion in the dog (Grant, 1941), suggests that the rise in plasma IRGa and increased gastric acid secretion seen with administration of our extract is not mediated by

hypercalcaemia. The lack of effect of prior β -receptor blockade on the rise in plasma IRGa levels on administration of the active extract suggests that the effect is not mediated by adrenaline release (Stadil and Rehfeld, 1973).

Bombesin, a tetradecapeptide isolated from frog skin (Anastasi *et al.*, 1971), has been shown to be a potent stimulant for gastrin release in man, dog and cat (Bertaccini *et al.*, 1974; Erspamer and Melchiorri, 1975). Radioimmunoassay systems based on amphibian bombesin have been used to demonstrate the presence of bombesin-like immunoreactivity in the mammalian gastro-intestinal tract (Erspamer and Melchiorri, 1975; Polak *et al.*, 1976; Walsh and Holmquist, 1976). One of the systems (Polak *et al.*, 1976), has demonstrated IRB in our more purified preparations but the amounts seem quite small in relation to the plasma IRGa raising activity present. Thus in two experiments, 3 and 12 ng/kg of IRB infused over 30 minutes gave an approximately three-fold increase in plasma IRGa. In the same intact dog model, Bertaccini *et al.* (1974) report a two-fold increase in plasma IRGa with 1 $\mu\text{g/kg}$ of synthetic bombesin infused over one hour. It is possible that the factor reported here is the mammalian counterpart to bombesin, but that structural differences cause low reactivity in the immunoassay system used. Alternatively, the gastrin releasing peptide may be distinct from the IRB detected in the impure extract; further purification with both bioassay and IRB testing will help answer this question.

The presence of a gastric factor capable of releasing gastrin has been postulated by Stadil and Rehfeld (Stadil and Rehfeld, 1971; 1974). These authors noted that aspiration of the gastric contents in vagotomised patients was attended by a lowering of basal plasma IRGa levels. This effect, according to these authors, could not be explained as simply due to the relief of distension after aspiration of the gastric contents (Stadil and Rehfeld, 1974). Thompson *et al.* (1976) instilled a liver extract on to the oxyntic gland area and small bowel mucosa via a gastric fistula in dogs provided with innervated antral pouches and gastroduodenostomy and noted resulting significant rises in plasma IRGa. The rise in plasma IRGa was resistant to antral acidification (Thompson *et al.*, 1976) and denervation of the antral pouch (Debas *et al.*, 1975). These authors have suggested the presence of a humoral agent to explain this effect and thought that the most likely source of such an agent would be the small bowel, but could not completely exclude its presence in the oxyntic gland area, which was also bathed by the liver extract. Preliminary experiments in this laboratory have shown that a peptide does exist in small

intestinal extracts which has similar properties to the active gastric extract described in this paper, but seems to be present in smaller amounts (McDonald *et al.*, 1977, unpublished observations). Work is now under way to purify both the gastric and intestinal peptides. These may well prove to be the same peptide and may play a role in the above cited physiological studies.

The mechanism of action of this gastrin releasing peptide is of course unknown and the question of whether the observed effects on gastric secretion are mediated solely or only partially by release of gastrin is unanswered. Investigations into these questions, into other possible actions of the peptide, and into possible structural relationship to bombesin may be undertaken once the peptide is obtained in the pure state. Although the word peptide has been used throughout this report, suggesting a singular nature for the active principle, it is recognised that, until an essentially pure state of the active principle is achieved, the possibility of more than one peptide being involved in the activity cannot be ruled out.

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