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FURTHER PURIFICATION OF A POLYPEPTIDE DEMONSTRATING ENTEROGASTRONE ACTIVITY

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SUMMARY

1. The further purification of a polypeptide having potent enterogastrone activity, without CCK–PZ effects, is described.

2. The material was inhibitory when doses of 1·0 μg/kg.hr were administered intravenously. Amino acid analyses demonstrated the absence of proline, a high content of glutamine and a preponderance of lysine over arginine. Tryptic degradation destroys the inhibitory effect of the polypeptide. Further studies must be performed before the physiological status of the polypeptide can be ascertained.

INTRODUCTION

Brown, Pederson, Jorpes & Mutt (1969) previously described the isolation of a material with enterogastrone (EG) activity from a partially purified preparation of the gastrointestinal hormone cholecystokinin–pancreozymin (CCK–PZ, GIH research laboratory, Karolinska Institutet, Stockholm, Sweden). The isolated material was highly inhibitory for H^+ secretion, antral and fundic motor activity and pepsin secretion, and did not possess any significant cholecystokinin or secretin activity.

This report deals with the further purification of this material.

METHODS

Fractions were tested for enterogastrone activity (EG) in three conscious dogs, prepared several months previously with a pouch of the fundus of the stomach, which was vagally and sympathetically denervated. A plateau of H^+ secretion was obtained using gastrin pentapeptide (1·5–4·0 μg/kg.hr) infused intravenously. These doses gave approximately 70 % of maximal acid secretion which varied in these preparations from 500 to 900 μ-equiv/15 min approximately. The infusion of EG samples was commenced only after three consecutive periods of H^+ secretion were within

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± 10 % of each other. All fractions were infused intravenously in 0·9 % (w/v) saline over either a 10 min or 60 min period.

Cholecystokinin (CCK) activity was tested for, by the method of Ljungberg (1962), employing a sensitive technique which measured the shortening of the exposed gall bladder of the anaesthetized guinea-pig.

Trypsin was 'TRTPCK' from Worthington, and made up to a concentration of 2 mg/ml. in 1 % aqueous NH_4HCO_3. The solution was stored frozen at −20° C until immediately before use. Tryptic digestion of EG stage II was for 6 hr, with addition of fresh trypsin every 2 hr.

Amino acids were determined qualitatively by two-dimensional paper chromatography, according to Redfield (1953) and quantitatively according to Spackman, Stein & Moore (1958) with a Spinco 120B amino-acid analyser.

All stages of purification were checked by electrophoresis at low pH on polyacrylamide gels, essentially according to the method of Johns (1967).

RESULTS

The starting material for this study was a partially purified cholecystokinin–pancreozymin (CCK–PZ), which contained approximately 275 Ivy dog units (I.D.U.) of CCK/mg. It was prepared by the method of Jorpes & Mutt (1961). In a typical experiment, 1·0 g of starting material was subjected to gel filtration using Sephadex G-50 fine. The column size was 5 × 90 cm and the material was eluted with 0·25 M phosphate buffer, pH 8·0. Fractions of 50 ml. were collected. Absorbance was measured at 280 mμ through a 1·0 cm light path. The results are plotted in Fig. 1. A small aliquot of each fraction (diluted 1:250 with physiological saline) was prepared for CCK assay using the anaesthetized guinea-pig method of Ljungberg (1964). All fractions not demonstrating CCK activity were pooled. About 150 ml. of solution was obtained which possessed strong EG activity.

Adsorption to alginic acid. The solution containing EG activity was diluted tenfold with distilled water; the pH adjusted to 2·5 with 2·0 M-HCl and the absorbance at 215 mμ, through a 1·0 cm light path, was measured and found to be 2·0. Alginic acid (7·0 g wet weight), which had been previously washed with acid ethanol followed by dilute aqueous HCl and water, was added to the diluted solution and stirred for 15 min. The absorbance at 215 mμ was read and adsorption was considered to be complete when this was less than 0·10. The alginic acid was collected by vacuum filtration on two layers of Whatman 541 filter paper and washed several times with ice-cold 0·005 M-HCl. The adsorbed material was eluted from the alginic acid with approximately 35–40 ml. ice-cold 0·2 M-HCl. The eluate was passed through a 1·5 × 10 cm column of DEAE Sephadex which had been equilibrated with 0·2 M acetic acid. The effluent, about 40 ml. and free of Cl^-, was lyophilized for 24 hr. The average yield was 152 mg. This material was referred to as EG stage I.

Fractionation of EG stage I on CM-cellulose. In a typical experiment 50 mg EG stage I was dissolved in 5·0 ml. 0·01 M ammonium bicarbonate and the pH adjusted to 7·8 with 0·01 M ammonia. A slight precipitate formed, which was removed by centrifugation and discarded. The clear solution was allowed to sink into a column (1·5 × 19 cm) of CM-cellulose

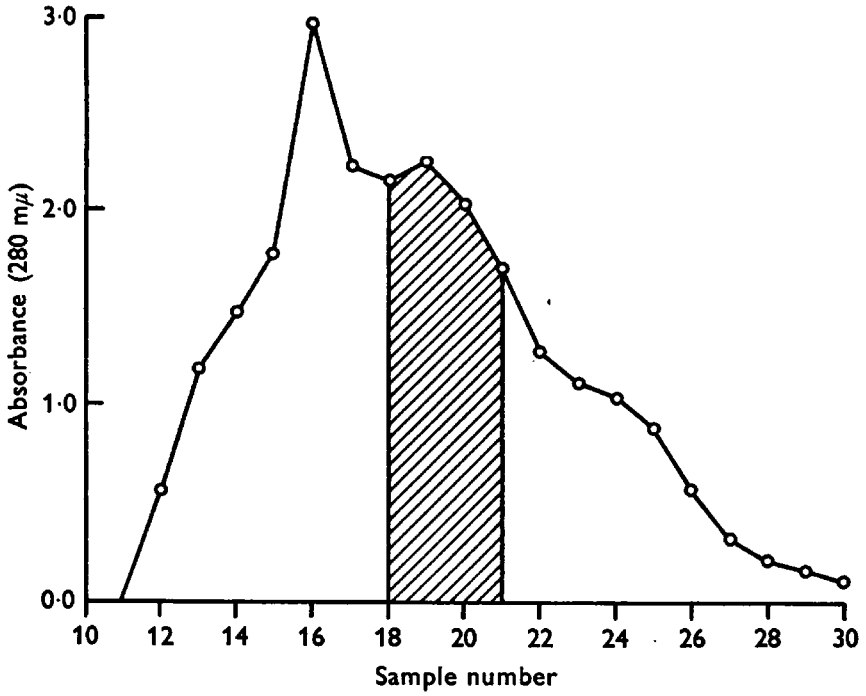

Fig. 1. Fractionation of starting material on Sephadex G-50 fine. 1·0 g of starting material applied to a column 5 × 90 cm and eluted with 0·25 M phosphate buffer, pH 8·0. Fraction size 50 ml. Absorbance measured at 280 mμ through a 1·0 cm light path. Shaded area demonstrated EG activity, and fractions were pooled for subsequent adsorption to alginic acid.

(Whatman CM 11) equilibrated and developed with 0·01 M-NH_4HCO_3 buffer, pH 7·8. The eluate was collected in fractions of 5·0 ml. at a flow rate of 130 ml./hr.

The absorbance of the fractions was measured at 280 mμ through a 1·0 cm light path. The results are shown in Fig. 2. The material in fractions 11 to 42 contained the EG activity. Fractions 12 to 33 inclusive were pooled and lyophilized. This procedure yielded 16·7 mg of material which possessed highly potent EG activity. This stage is referred to as EG stage II. The remainder of the material, approximately 30 mg, was eluted from the column with 0·2 M-NH_4HCO_3 buffer, pH 8·0.

Fractionation of EG stage II on Sephadex G-25. A further stage of purification involved the use of Sephadex G-25 fine. In a typical experiment 12·0 mg EG stage II was dissolved in 1·0 ml. 0·2 M acetic acid and applied to a column of Sephadex G-25 fine (0·6 × 120 cm) and eluted with 0·2 M acetic

acid. The eluate was collected in 2·0 ml. fractions at a flow rate of 8·0 ml./hr. The absorbance of the fractions was measured at 280 mμ through a 1·0 cm light path. The chromatogram is shown in Fig. 3. Fractions 12, 13 and 14 were pooled and lyophilized. This fractionation yielded 4·1 mg of highly potent material and is referred to as EG stage III.

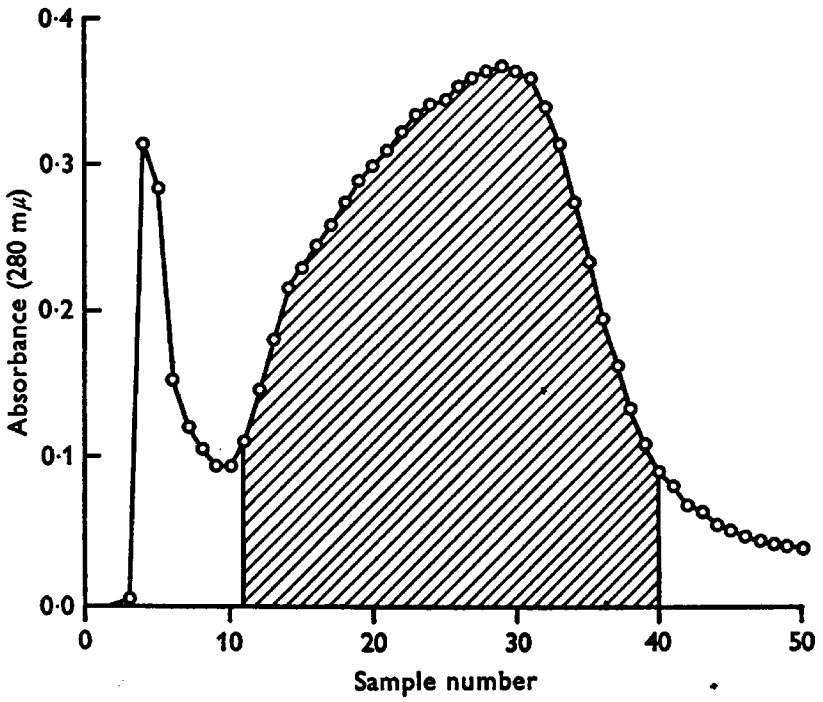

Fig. 2. Fractionation of EG stage I on CM-cellulose. 50 mg EG stage I was applied to a column 1·5 × 19 cm and eluted with 0·01 NH_4HCO_3 buffer, pH 7·8. Fraction size 5·0 ml. Absorbance measured at 280 mμ through a 1·0 cm light path. Shaded area demonstrated EG activity and fractions were pooled and lyophilized for further chromatography on Sephadex G-25.

Amino acid analysis. Qualitative amino acid analysis (Redfield, 1953) of EG stage III demonstrated the absence of the amino acid proline, high concentrations of aspartic, glutamic acid and lysin. These observations have been subsequently confirmed by two independent laboratories with quantitative amino acid analyses. The qualitative amino acid analysis also revealed the presence of tryptophan.

Inhibitory action of EG stage III on gastrin pentapeptide-stimulated H^+ secretion. Fig. 4 shows the effect of the intravenous infusion of EG (1·0 μg/kg. stage III in a dog receiving gastrin-pentapeptide to stimulate H^+ secretion from a vagally denervated pouch of the fundic region of the stomach. When this material was assayed for cholecystokinin, it was found to contain 2·0 I.D.U./mg.

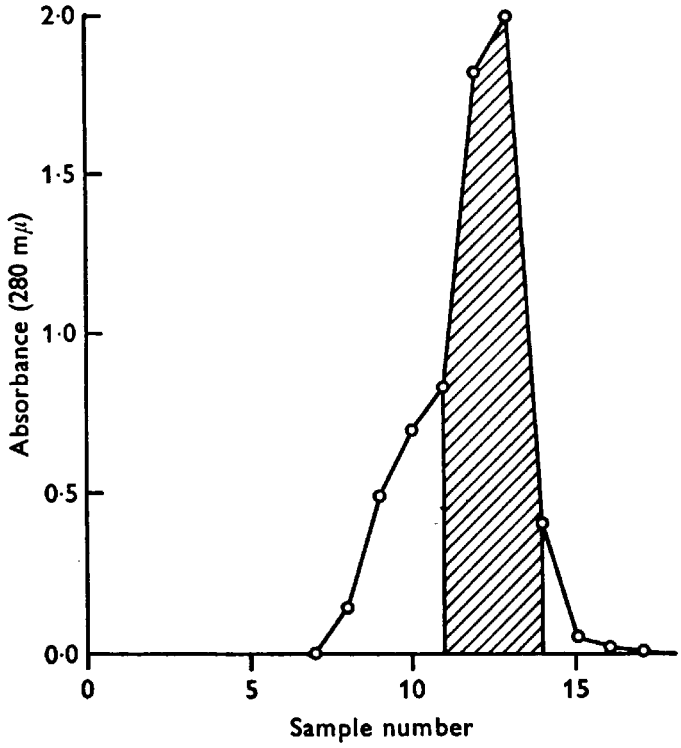

Fig. 3. Fractionation of EG stage II on Sephadex G-25 fine. 12·0 mg EG stage II was applied to a column 0·6 × 120 cm and eluted with 0·2 M acetic acid. Fraction size 2·0 ml. Absorbance measured at 280 mμ through a 1·0 cm light path. Shaded area demonstrated EG activity. This material was EG stage III.

TABLE 1. Experimental results with EG stage III at intravenous doses of 0·25, 0·5 and 1·0 μg/kg.hr. Periods were of 15 min duration. Only three control periods are included; range of H^+ secretion was approximately 500–900 μ-equiv/15 min, i.e. about 70% of maximum secretory capacity. Acid outputs varied from animal to animal and on different occasions, but are expressed as a ratio of the mean of the three control periods

		Control periods			Test periods				Post-test periods		
Dog	μg/kg.hr EG III	1	2	3	4	5	6	7	8	9	10
A	1·00	0·99	0·99	1·02	0·82	0·44	0·37	0·37	0·58	0·75	0·87
A	1·00	1·00	0·92	1·08	0·89	0·55	0·45	0·39	0·59	0·72	0·78
B	1·00	0·89	1·02	1·07	0·79	0·38	0·30	0·32	0·40	0·42	0·42
B	1·00	1·04	1·01	0·95	1·08	0·77	0·58	0·48	0·54	0·97	—
C	1·00	1·04	0·98	0·98	0·86	0·63	0·32	0·11	0·35	0·72	1·04
C	1·00	0·99	0·98	1·01	0·84	0·54	0·37	0·20	0·41	0·62	0·91
A	0·50	1·03	1·01	0·95	0·81	0·54	0·44	0·41	0·59	0·69	0·81
A	0·50	1·08	0·94	0·97	1·02	0·60	0·48	0·45	0·64	0·78	0·90
B	0·50	0·96	1·04	1·01	0·93	0·64	0·51	0·48	0·48	0·59	0·68
C	0·50	1·01	1·03	0·97	0·95	0·58	0·45	0·40	0·51	0·67	0·84
A	0·25	0·93	1·04	1·04	0·89	0·69	0·64	0·59	0·63	0·73	0·79
A	0·25	0·99	1·01	0·99	0·94	0·72	0·71	0·76	1·04	1·13	—
C	0·25	0·96	0·98	1·05	0·93	0·74	0·67	0·57	0·70	0·81	0·94

To date, three different doses of EG stage III have been employed: 0·25, 0·5 and 1·0 μg/kg.hr. These results are presented in Table 1.

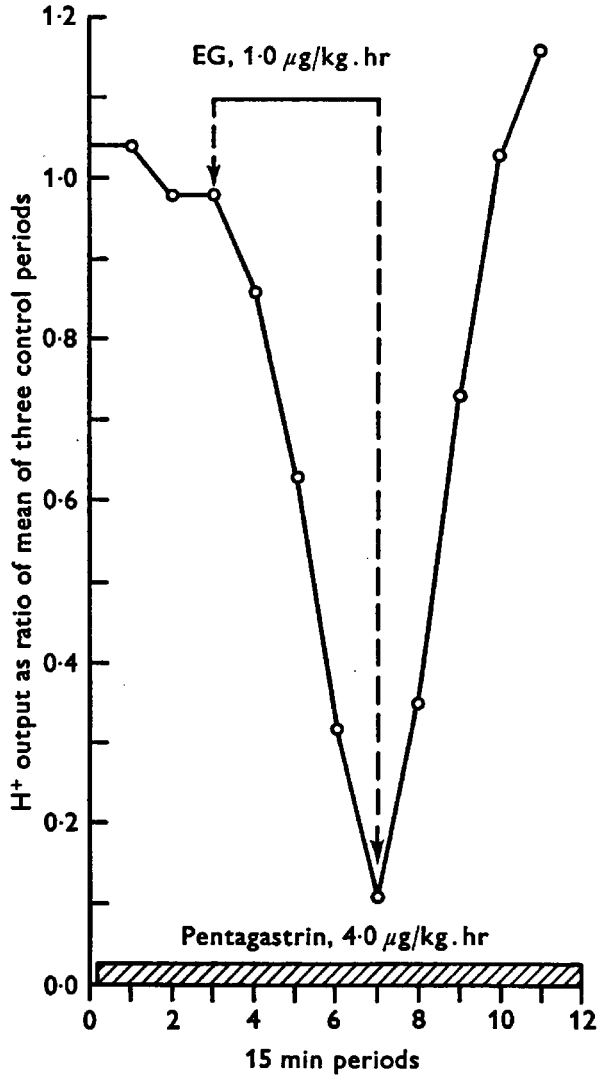

Fig. 4. Effect of EG stage III on H^+ secretion in the dog. H^+ secretion stimulated by pentagastrin, 4·0 μg/kg.hr, given intravenously. EG stage III, 1·0 μg/kg.hr, infused intravenously after a plateau of H^+ secretion (three periods ± 10 % of each other) had been achieved. Inhibition of H^+ secretion was almost complete with this dose.

DISCUSSION

The inhibitory effects on gastric acid secretion of the gastrointestinal hormone secretin and of partially purified preparations of the hormone CCK–PZ have been well documented. Brown *et al.* (1969) described the preparation of a material with inhibitory activity for gastric secretion and motor activity in which they were unable to demonstrate significant secretin or CCK–PZ activity. The starting material in this study and in the

previous one was the most common CCK–PZ preparation used in gastric inhibitory studies, i.e. the 10 % pure material of Jorpes & Mutt (1961) commercially available from the GIH research laboratory, Karolinska Institutet, Stockholm, Sweden. In the earlier study (Brown *et al.* 1969) the non-CCK–PZ-containing material was precipitated from the phosphate buffer, used to develop the column, by saturation with NaCl. This technique has been replaced by adsorption on to alginic acid as described, followed by further purification using CM-cellulose and Sephadex G-25.

The preliminary amino-acid analyses have been important in distinguishing this material from CCK–PZ by demonstrating the absence of the amino acid proline. In addition, the analyses show a high glutamic acid content and a preponderance of lysine over arginine.

The intravenous infusion of the material in dogs has revealed it to produce inhibition of H^+ secretion in excess of 70 % in doses as low as 1·0 μg/kg.hr. A few experiments at lower doses, 0·25 and 0·5 μg/kg.hr were performed. At the 0·25 μg/kg.hr level, approximately 36 % inhibition was observed and with 0·5 μg/kg.hr, 57 % inhibition was seen. These results are shown in Table 1.

The cholecystokinin content as determined by assay in the guinea-pig reveals it to be less than 2·0 u./mg. The cholecystokinin activity was in that fraction eluted from CM-cellulose by 0·2 M-NH_4HCO_3 buffer, quite separate from the gastric inhibitory activity.

The purest material was found not to stimulate pancreatic enzyme secretion in the anaesthetized cat preparation in a dose as high as 10 μg/kg. However, there does appear to be a slight inhibitory effect on the volume of secretin-stimulated pancreatic secretion which is being investigated.

The starting material used in this study did demonstrate vasodepressor activity in the anaesthetized rabbit, an effect which the purest EG does not have. No other side effects with this material at the doses employed have been observed.

Degradation of EG stage II with trypsin completely destroyed its biological activity.

Despite the fact that the polypeptide described here has been demonstrated to possess potent gastric secretory inhibitory activity (EG activity) and has been extracted from the duodenal mucosa of hogs by techniques similar to those used for CCK–PZ and secretin, reservations must be held as to its physiological status. It must, at the moment, be considered to be primarily of biochemical interest, until several physiological criteria are satisfied. These should include identification of the material in the blood stream, and a more complete analysis of the spectrum of the physiological effects.

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