Aminoacid constitution of two gastrins isolated from Zollinger-Ellison tumour tissue

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SUMMARY Two peptides which are potent stimulants of gastric acid secretion are isolated from Zollinger-Ellison tumour tissue. They have aminoacid constitutions identical with those of human gastrin types I and II as isolated from human antral mucosa, and they are present in similar proportions. Their electrophoretic and chromatographic behaviour corresponds to that of human gastrins. Evidence is presented in respect of the one isolated in greater amount that its aminoacid sequence is probably identical with that of human gastrin type I.

It is now well known that in many cases of the Zollinger-Ellison syndrome as characterized by gastric hypersecretion, peptic ulceration, and a non-beta-cell pancreatic tumour, a gastric secretagogue which is neither histamine nor insulin has been extracted from the tissue (primary or metastatic); it shows the same spectrum of physiological actions as do the pairs of peptides which have been isolated from the antral mucosa of several species (including man), and which are generally agreed to represent the hormone gastrin (Gregory and Tracy, 1964; Gregory, 1968). This strongly suggests that the secretagogue present in such tumours is gastrin but decisive evidence on this point can only be provided by isolation of the secretagogue in amounts sufficient for a definitive study of its chemical nature.

Gregory, Grossman, Tracy, and Bentley (1967) isolated from 19·5 g of Zollinger-Ellison tumour tissue 104 μg of a product which stimulated gastric acid secretion in conscious dogs and showed a quantitative aminoacid composition in very good agreement with that of human antral gastrin peptide. The amount of material available was so small that it was impossible to ascertain whether it comprised one or other, or both, of the two peptides present in human antral mucosa; and no evidence could be offered in respect of the aminoacid sequence compared with that of the antral peptide.

In a further study on this problem two biologically active peptides have been isolated from a larger amount of such tumour tissue; they appear to be identical with the human antral gastrins I and II, and are present in similar proportions. An account of the work was presented in July 1968 at a symposium on non-insulin-producing islet cell tumours of the pancreas held at Erlangen, West Germany (to be published).

EXPERIMENTS

PREPARATION OF A CRUDE TUMOUR EXTRACT Pancreatic tumours or metastatic nodules obtained from cases diagnosed clinically as examples of the Zollinger-Ellison syndrome were thinly sliced and the fragments thrown into vigorously boiling water. After five minutes the mixture was cooled, the liquor decanted, and the fragments ground with silver sand. The ground material was washed from the sand back into the liquor with water and the suspension made approximately pH 9 by dropwise addition of strong ammonia solution (sp gr 0·880). After stirring for one hour the suspension was clarified by filtration through cottonwool; the final volume of extract was equivalent to about 100 times the weight of fresh tissue.

Two grams of diethylaminoethylcellulose powder (Whatman DE 11) were precycled with acid and alkali and freed from 'fines' as recommended by the manufacturers and then added to the tumour extract; the pH was brought to 8·5 by dropwise addition of glacial acetic acid and the suspension was stirred for one hour. The DE was collected on a small Buchner funnel using suction and a coarse paper (Green 904); it was then removed and suspended in 20 ml of 0·2 N NaOH for 15 min with occasional stirring. The extract was collected by suction filtration and neutralized to pH 7 by drop-
worse addition of 10 N HCl. An aliquot was taken for
testing by injection into conscious dogs or cats. If
active, the remainder of the extract was lyophilized
and stored at a temperature of -20°C or lower for later
use.

Assays Conscious dogs provided with completely
denervated fundic pouches (Gregory, 1958) and fasted
18 hours were infused subcutaneously with carbachol at
the rate of 2.5 to 5.0 μg every 15 minutes the dose for a
particular animal was determined by trial as that which
did not result in a secretion of acid by the pouch when
continued for a period of six hours. This "background"
of cholinergic stimulation markedly increased the sen-
sitivity of the pouch so that responses to the subcutaneous
injection of as little as 1 μg gastrin or Peptavlon (ICI)
were consistently obtained. Solutions to be tested for
gastrin activity were injected subcutaneously at a site
different from that used for the carbachol infusion.

Some DE eluates were assayed for gastrin activity by
slow intravenous injection in conscious cats provided
with gastric fistulae as described by Uvnäs and Emås
(1961).

Isolation of peptides from the tumour extract The
isolation procedure described below was carried out
using lyophilized DE extract equivalent to a total of
about 50 g fresh tumour tissue. The material was pros-
cessed in three separate portions to minimize loss in the
event of an accident. Further small amounts of material
were also used during the development of the method;
for various reasons the products of these trials were not
included in the material used for the final analyses.

An amount of powder equivalent to 10 to 20 g of fresh
tumour was dissolved in 25 ml of water; to the solution
was added 0.5 ml of strong ammonia solution (sp gr
0.880). In the solution 12.5 g of powdered anhydrous di-
phosphonium hydrogen phosphate (K₄HPO₄) was dissolved,
with constant stirring, in a water bath at room tem-
perture. Then 17 ml of peroxide-free isopropanol was dripped
in with vigorous stirring. The mixture was briefly
centrifuged and the upper layer removed. To the lower
phase was added 15 ml of the upper phase of a mixture
of 50 ml water, 1 ml strong ammonia solution,
25 g K₂HPO₄, and 37 ml isopropanol. After vigorous
shaking for one minute and then briefly centrifuging
the upper phase was removed. This extraction was re-
peated once more and the combined isopropanol
extracts were then shaken with 3 volumes of peroxide-free
ethyl ether after the addition of 5 ml water. After brief
centrifuging the aqueous phase was collected. Five
ml of water was added to the ethereal phase; the mixture
was shaken and then briefly centrifuged. The aqueous
layer was collected and the ethereal layer discarded.
The combined aqueous fractions were extracted twice
with 2 volumes of ether, briefly centrifuging each time
and discarding the ethereal phase. The aqueous residue
was freed from ether by a current of air in partial vacuum
and then lyophilized.

The residue was dissolved in 1 ml H₂O and applied to
a column of Sephadex G 25 superfine bead form 1-5 cm
× 50 cm packed in 0.04 M ammonium bicarbonate
solution. The effluent was collected in fractions of 1-3 ml
every 15 minutes and these read at 280 mμ. Non-volatile
salt (phosphate from the isopropanol extraction) was
located by conductivity determinations on each fraction.
Gastrin activity was located by testing pooled one-drop
aliquots from selected groups of fractions.

A typical result is shown in Fig. 1; regions A and B
contained secretagogue activity, C was inactive. The
fractions corresponding to A and B were pooled separ-
ately, lyophilized, and dissolved in buffer for refrac-
tionation.

Fraction A was applied in a volume of 0.5 ml to a
column of Sephadex G 50 superfine bead form 1 cm ×
100 cm packed in 0.04 M ammonium bicarbonate
solution. The effluent was collected in fractions of 0.4 ml
every 15 minutes and these read at 280 mμ. A typical
result is shown in Fig. 2; secretagogue activity was found
in the shaded region, well in advance of the position of
the 'salt zone' on this column. The appropriate fractions
were pooled, lyophilized, and redried in vacuo over phos-
phoric oxide until free from ammonium bicarbonate.
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Fraction B was applied in a volume of 1 ml to a column of Sephadex G 10, 1 cm × 100 cm, packed in 0.04 M ammonium bicarbonate solution. The effluent was collected in fractions of approximately 0.3 ml every 15 minutes and these were read at 280 mμ. A typical result is shown in Fig. 3; secretagogue activity was found in peaks I, II, and III but not in peak IV. In view of the small amount of total activity present and the incomplete separation of the peaks no attempt was made to deal with them separately; all the active salt-free fractions were pooled, lyophilized, and redried in vacuo over phosphoric oxide until free from ammonium bicarbonate.

Fractionation on Aminoethylcellulose In preliminary trials it was found that the products obtained from the above fractionations on Sephadex G 50 and G 10 both gave the same pattern of peaks when subjected to gradient elution chromatography on aminoethylcellulose despite the obvious difference in molecular size of the material with which the activity was associated in the two cases, as indicated by the separation on Sephadex G 25 (Fig. 1); the explanation seems to be that at this stage some of the activity is electrostatically bound to denatured protein from which it dissociates on the AE column, emerging in the free form. For this reason and in view of the very small amount of active material being dealt with, the products from the G 50 and G 10 columns were combined for the final stage of isolation on aminoethylcellulose. The salt-free residues were dissolved in 4 ml of 0.02 M ammonium bicarbonate buffer and applied to a column of aminoethylcellulose (Whatman AE 41 1 cm × 5 cm) packed in 0.04 M ammonium bicarbonate buffer. The aminoethylcellulose had been precycled in acid and alkali and freed from 'fines' as recommended by the manufacturers and then washed thoroughly with starting buffer.

After application of the sample, the column was washed with 0.04 M buffer until the absorption at 280 mμ of the

FIG. 2. Fractionation on Sephadex G 50 of fraction A from Sephadex G 25 (Fig. 1).

FIG. 3. Fractionation on Sephadex G 10 of fraction B from Sephadex G 25 (Fig. 1).
Quantitative aminoacid composition of the four fractions obtained by chromatography on amionoethylcellulose are shown in the Table. These and other data in respect of each fraction may be summarized as follows:

FRACTION IA This could not be shown to stimulate gastric secretion. The aminoacid analysis indicated that it was not homogeneous; its composition bore some resemblance to a gastrin peptide, but valine and serine were present in significant amounts. Thin-layer chromatography showed two major components and a third in trace amount; the former had Rt values of 0 and 0.23, the latter 0.53; this is similar to that of human synthetic gastrin I in the same system.

FRACTION I This was highly active in stimulating gastric acid secretion. Its aminoacid composition

<table>
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<tr>
<th>Residue</th>
<th>Human Gastrin</th>
<th>Fraction (umoles)</th>
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<tr>
<td></td>
<td>IA</td>
<td>I</td>
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<tr>
<td>Aspartic</td>
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<tr>
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</tr>
<tr>
<td>Tryptophan</td>
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<td>Present</td>
</tr>
</tbody>
</table>

1Present as tyrosine sulphate.
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FIG. 5. High-voltage electrophoresis of the peptide fragments resulting from digestion by thermolysin of (a) synthetic human gastrin type I and (b) peak I material from fractionation on aminoethylcellulose of Zollinger-Ellison tumour extract (Fig. 4). Samples of unhydrolyzed material from fractions I and II are also included. Glu = glutamic acid markers.

FIG. 6. High-voltage electrophoresis of the peptide fragments resulting from digestion by subtilisin of (a) synthetic human gastrin type I and (b) peak I material from fractionation of Zollinger-Ellison tumour extract (Fig. 4) on aminoethylcellulose. Glu = glutamic acid markers.

was in excellent agreement with that of human gastrin peptide and its position in the elution gradient from the aminoethylcellulose column corresponded to that anticipated for a gastrin type I (unsulphated) on the basis of previous experience. The amount present (calculated from the aminoacid analysis) was approximately 550 µg. On paper electrophoresis it ran as a single spot with a trace contaminant; the mobility of the former in comparison with that of glutamic acid corresponded with that expected for a gastrin type I (Fig. 5). On thin-layer chromatography it ran as a single spot with a trace contaminant; the mobility of the former corresponded to that of synthetic human gastrin type I. The trace contaminant can be identified as gastrin in which the methionine residues have become oxidized; it is commonly present to this extent in preparations of natural or synthetic gastrin. Direct electrophoretic comparison of the peptide fragments resulting from digestion by the enzymes subtilisin and thermolysin of fraction I and synthetic human gastrin type I (Figs. 5 and 6) showed a perfect correspondence between the two substances, both in regard to the number and mobilities of the fragments.

FRACTION II This was highly active in stimulating gastric acid secretion. Its aminoacid composition showed excellent agreement with that of a human gastrin peptide; the use of alkaline, rather than acid, hydrolysis in preparing the sample for aminoacid analysis established that the single tyrosine residue present was in the sulphated form which is characteristic of human gastrin type II. Its position in the aminoethylcellulose elution diagram corresponded to that expected for gastrin type II. The calculated amount present was approximately 250 µg. On paper electrophoresis it ran as a single spot with a trace contaminant; the mobility of the former relative to that of glutamic acid corresponded to that expected for gastrin type II. Thin-layer chromatography showed a single spot with a trace contaminant. As mentioned in respect of fraction I, the trace contaminant can be identified as oxidized gastrin. Unfortunately no enzymatic degradation studies could be made on fraction II owing to loss of most of the material when the aminoacid analyser broke down on two occasions while analyses of it were in progress.

FRACTION III This was a weak stimulant of gastric acid secretion. Its aminoacid composition was in good agreement with that of a human gastrin peptide apart from rather low values for methionine and phenylalanine. Thin-layer chromatography showed one major component (Rf = 0·40) closely followed
by two minor ones, and a fast-moving trace ($R_f = 0.71$). The amount present, calculated as gastrin, was approximately 200 $\mu$g.

**DISCUSSION**

Two peptides (fractions I and II) have been isolated from pooled Zollinger-Ellison tumour tissue which are potent stimulants of gastric acid secretion when injected subcutaneously into conscious pouch dogs. They have the same quantitative aminoacid constitution as the gastrins isolated from human antral mucosa, including the presence of the sulphated form of tyrosine in fraction II as in human gastrin type II. Their electrophoretic and chromatographic behaviour corresponds to that of the human gastrins and they have been found in similar proportions (Gregory, Tracy, and Grossman, 1966).

Electrophoretic comparison of the peptide fragments resulting from enzymatic degradation by subtilisin and thermolysin of fraction I and synthetic human gastrin type I showed a perfect correspondence in their number and mobility. Unpublished studies by one of us (K.L.A.) on the structure of dog gastrin have shown that this method is a powerful one for revealing variations in the position of a single residue, and hence it appears highly probable that the aminoacid sequences of fraction I and human gastrin type I are identical. Proof of this by complete analysis, as carried out for hog gastrin (Gregory, Hardy, Jones, Kenner, and Sheppard, 1964), would require far larger amounts of material than were available here. However, an improved method of peptide sequence analysis using mass spectrometry which has been evolved in this university will, it is hoped, be applicable to future preparations, and this may be expected to provide decisive evidence.

The nature and significance of the components identified in fractions IA and II remains for the present uncertain. If larger preparations can be obtained in the future it may be possible to separate and characterize them more precisely. It seems probable that fraction III consisted largely of gastrin which had become oxidized, perhaps during the process of extraction. This would account for its aminoacid composition, its greater acidity than fraction II (as indicated by its position in the aminothylecellulose elution diagram) and also its low activity, for Morley, Tracy, and Gregory (1965) found that oxidation of the methionine residue in the C-terminal tetrapeptide amide of gastrin resulted in severe but not complete loss of the power to stimulate gastric acid secretion.

Since the studies reported here were completed, McGuigan and Trudeau (1968) and Strempel and Meade (1968) have described the detection of circulating gastrin in patients with the Zollinger-Ellison syndrome using methods of radioimmunoassay. McGuigan and Trudeau used an antibody raised in rabbits with a conjugate of residues 2-17 of synthetic human gastrin I and bovine serum albumin; Strempel and Mead used an antibody raised in chickens with synthetic human gastrin I adsorbed on polymethylmethacrylate particles. McGuigan and Trudeau found levels of 2,550 to 62,000 $\mu$g per ml in four patients, compared with values of 245 to 668 $\mu$g per ml in 24 control subjects, and they also assayed the gastrin content of tumour tissue from one patient using the same method. It is not yet known whether one or other, or both, of the gastrins which we have shown to be present in the tumour tissue are released into the circulation.

We would like to express our gratitude to the many friends who made this work possible by providing us with tumour tissue from their patients.

**REFERENCES**


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