# Impaired α-carboxyamidation of gastrin in vitamin C-deficient guinea pigs

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The biosynthetic activation of many hormonal peptides requires enzymatic α-carboxyamidation via glycine-extended intermediate forms. By specific radioimmunochemical measurement on extracts of guinea pig antra, we found that vitamin C deficiency induced a 30-fold increase in glycine-extended (i.e. inactive) gastrins. The results indicate that ascorbic acid is neccessary for α-carboxyamidation in vivo, and thus, that the biosynthetic activation of hormonal peptides can be modified by simple dietary means.

*Post-translational processing* *Carboxy-terminal amidation* *Ascorbic acid* *Glycine-extended gastrin* *Radioimmunoassay*

## 1. INTRODUCTION

Biological activity of many hormonal and neurotransmitter peptides depends on α-amidation of the C-terminal residue [1,2]. Accordingly, precursors of these peptides contain an amidation site, consisting of a glycine residue followed by two basic amino acid residues. Activation includes cleavage by a dibasic specific endopeptidase [3,4], removal of the basic amino acid residues by a carboxypeptidase B-like enzyme [5], and, finally, conversion of the amide donor glycine by a specific amidating enzyme [6,7].

Primary cell cultures lose the amidating activity rapidly and glycine-extended forms of otherwise amidated peptides, such as α-MSH and pancreatic polypeptide, accumulate [8,9]. In vitro studies suggest that the amidating enzyme requires oxygen, copper, and ascorbic acid [7,10,11].

Our purpose was to investigate whether amidation also requires ascorbic acid in vivo. Guinea pigs were studied using our new assay for measurement of glycine-extended gastrin [12]. Guinea pigs were chosen because they lack L-gulonolactone oxidase, which catalyzes the final step in the formation of ascorbic acid from glucuronic acid [13]. Thus, in the guinea pig, as in man, ascorbic acid originates entirely from the diet.

## 2. MATERIALS AND METHODS

### 2.1. *Animals*

Ten guinea pigs (350–385 g) were divided into two groups. Five animals received commercial rabbit pellets (ÖH-rabbit pellets, Brogaarden, Gentofte, Denmark) autoclaved at 120°C for 40 min. The animals had free access to water. Another 5 animals were fed the same diet but had free access to drinking water containing ascorbic acid (DAK, Copenhagen, 1 mg/ml, prepared daily). The groups were kept in separate cages and the animals weighed daily. After 30 days, the animals were killed by injecting 50 mg Mebumal intraperitoneally. Antrum was removed and frozen between two pieces of dry ice. Tissue was stored at −80°C until extraction.

### 2.2. *Laboratory analysis*

#### 2.2.1. Tissue extraction

While frozen, the tissue was cut into small pieces, immersed in boiling water (3–4 ml/g tissue)

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for 20 min, homogenized in a Polytron homogenizer and centrifuged for 30 min at 10000×g. The supernatants were decanted, and the pellets were re-extracted in 0.5 M $CH_3COOH$ for 20 min at room temperature, homogenized and centrifuged as above.

2.2.2. Radioimmunoassay

Antiserum 2604 raised against synthetic human gastrin 2–17 was directed against the $\alpha$-amidated C-terminus [14]. It recognizes neither glycine-extended nor deamidated gastrin [15]. The incubation and separation was performed as described, using mono-iodinated $^{125}$I-gastrin-17 tracer and synthetic human gastrin-17 (ICI, England) standard [12]. Antiserum 7270 was raised against fragment 5–17 of human gastrin-17 extended at the C-terminus with glycine (Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-Gly) [12]. The antiserum reacts negligibly with amidated gastrin ($ID_{50}$ gastrin (5–17)Gly/$ID_{50}$ gastrin-17 being 0.003) and glycine-extended cholecystokinin ($ID_{50}$ gastrin(5–17)Gly/$ID_{50}$ CCK-8-Gly being 0.05) [12]. $^{125}$I-gastrin(5–17)-Gly was used as tracer, and gastrin(5–17)Gly from Cambridge Research Biochemicals (Cambridge, England) as standard [12].

2.2.3. Chromatography

Tissue extracts (1.0–1.5 ml) were applied to calibrated Sephadex G-50 superfine columns (12× 2000 mm) eluted with 0.125 M $NH_4HCO_3$, pH 8.2, at 4°C at a flow rate of 3.6 ml/h. Fractions of 1.2 ml were collected. Subsequent ion-exchange chromatography was performed at room temperature on AE-41 cellulose (Whatman, England) using a linear gradient from 0.05 to 0.20 mol/l $NH_4HCO_3$ (pH 8.2, 300 ml). The flow rate was 33 ml/h, and fractions of 2.2 ml were collected.

## 3. RESULTS

All ascorbic acid-deficient guinea pigs lost weight in the last 7 days (fig.1). The antral concentration of glycine-extended gastrins in the control group was 2.2±0.6 pmol/g, whereas the antrum in the vitamin C-deficient group contained 69±40 pmol/g. As the increase in glycine-extended gastrins was accompanied by a similar decrease in amidated gastrins, the total amount of amidated and glycine-extended gastrin was similar in the two groups

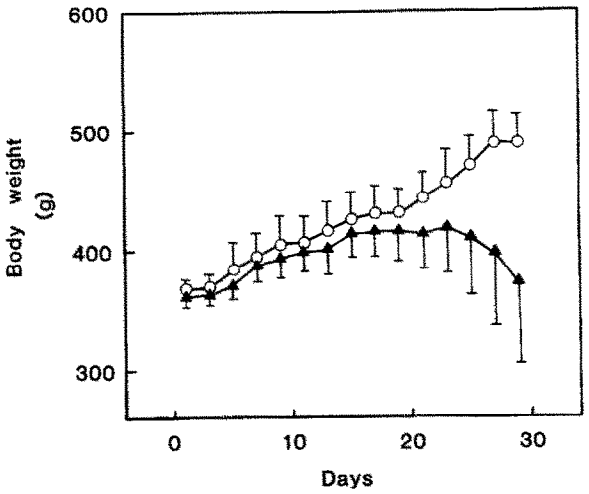


Fig.1. Guinea pig weight curves. Results are presented as mean ± SD for control animals (○—○) ($N$=5) and for those deprived of ascorbic acid (▲—▲) ($N$=5).

(table 1). Glycine-extended gastrins thus constituted 1% of the progastrin fragments measured in the control group vs 30% in the vitamin C-deficient group. To characterize the gastrins further the extracts were examined by gel and ion-exchange chromatography. The gel filtration pattern in the two groups (fig.2) corroborated the differences measured in the unfractionated extracts (table 1). Amidated gastrins eluted in 3 peaks. The first eluted with a $K_d$ of 0.55, corresponding to gastrin-34. (The full amino acid sequence of guinea pig gastrin has not yet been published. The designa-

Table 1

Concentrations of amidated gastrins and glycine-extended gastrins in antral extracts from guinea pigs receiving (+ ascorbic acid) and deprived of vitamin C (− ascorbic acid)

| | + ascorbic acid ($N$ = 5) | − ascorbic acid ($N$ = 5) |
|---|---|---|
| Amidated gastrin (pmol/g) Ab. 2604 | 226 ± 42 | 139 ± 14 |
| Glycine-extended gastrin (pmol/g) Ab. 7270 | 2.2 ± 0.6 | 69 ± 40 |

Results are expressed as mean ± SD

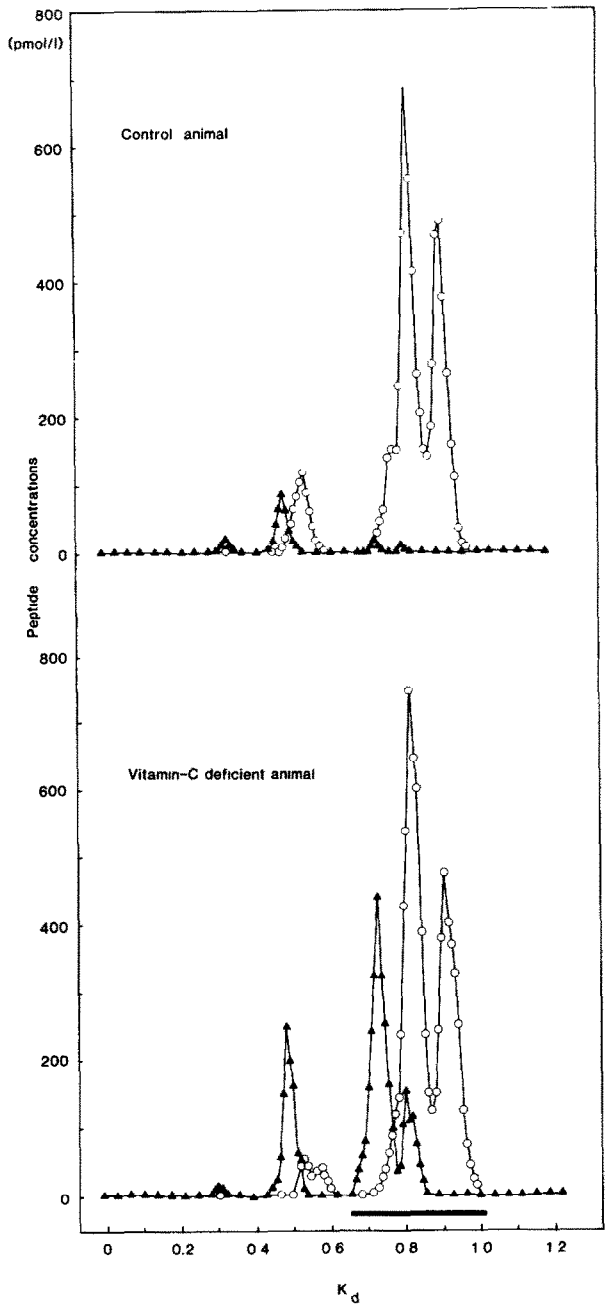


Fig.2. Gel chromatography of antral extracts from a control animal (upper) and a vitamin C-deficient animal (lower). The fractions were assayed using Ab. 2604 (○—○) fractions diluted 1:5, and Ab. 7270 (▲—▲) (fractions undiluted, upper - and diluted 1:5, lower). The solid bar indicates peptides that were further characterized on ion-exchange chromatography (fig.3).

tions 'gastrin-34' and 'gastrin-17' are thus tentative.) The two last peaks with $K_d$ values of 0.88 and 0.92 corresponded to sulfated and non-sulfated gastrin-17, respectively. The peaks detected by the radioimmunoassays specific for glycine-extended gastrins eluted with $K_d$ values of 0.47, 0.72 and 0.80, prior to the corresponding amidated components. The extra glycine residue and negative charge thus had a pronounced effect on the elution from the long Sephadex columns (fig.2). A small peak of glycine-extended gastrin eluted at $K_d$ 0.32 - the corresponding amidated component I [16] was not detectable in these extracts. Ion-exchange chromatography confirmed that the peptides measured by Ab. 7270 were more acidic than the corresponding peptides measured by Ab. 2604 (fig.3). Both gastrin-17 and gastrin-17-Gly were separated in two peaks on AE-cellulose, presumably a sulfated and non-sulfated form. Thus, by arylsulfatase treatment the more negatively

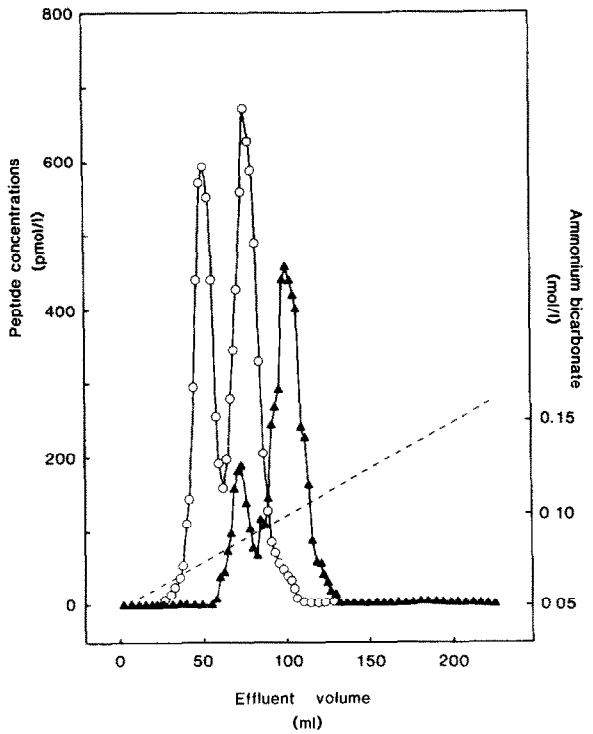


Fig.3. Ion-exchange chromatography of guinea pig antral gastrins. The fractions indicated by a solid bar in fig.2 were pooled and eluted on AE-celulose. The elution pattern was monitored with Ab. 2604 (○—○) and Ab. 7270 (▲—▲).

charged peptide moved to the position of the less charged peptide [12] (not shown).

## 4. DISCUSSION

This study has shown that the amount of glycine-extended gastrin in the antrum increases 30-fold in guinea pigs deprived of ascorbic acid. Glycine-extended peptides are biosynthetic intermediaries of active peptides which are stored and released in $\alpha$-carboxyamidated form. Ascorbic acid has previously been found to be of importance for the in vitro amidation of $\alpha$-MSH [11] as a cofactor for the amidating enzyme [7,10]. It is therefore likely that the effect of vitamin C-deprivation observed here reflects a direct effect on the biosynthesis in the endocrine cell.

Although the amount of glycine-extended peptides increased 30-times, the vitamin C-deficient guinea pigs still amidate two-thirds of the gastrin (table 1). This can be explained in two ways. Either the endocrine cell is more capable than most other cells of concentrating or storing ascorbic acid, or the amidation process can function to a certain degree without ascorbate. In fact, one-third of the joining peptide produced by ATT-20 cell cultures, which contain immeasurable amounts of ascorbic acid, are correctly amidated (Betty Eipper, personal communication). The low concentration of glycine-extended gastrin in normal guinea pig antrum agrees with our results on human antral mucosa (unpublished) and with the very low concentration of glycine-extended $\alpha$-MSH measured in extracts of intermediate pituitaries [8]. Gastrin-17 constitutes approx. 90% of the amidated antral gastrins (fig.2) as found in other species as well. In contrast, about 90% of the glycine-extended gastrins are large components, corresponding to component I-Gly and gastrin-34-Gly ($K_d$ 0.32 and 0.47, fig.2). Similar processing patterns were found in extracts of porcine antral mucose (in preparation). Interpretation of these results awaits further studies on the biosynthesis of gastrin. In conclusion, our results show that it is possible to interfere with the activation of regulatory peptides by simply restricting the access to a vitamin, ascorbic acid.

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