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Progastrin maturation during ontogenesis

Accumulation of glycine-extended gastrins in rat antrum at weaning

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The post-translational maturation of antral progastrin was studied in the developing rat. While *N*-terminal proteolysis remained unchanged and tyrosine *O*-sulphation varied only slightly during ontogenesis, major changes were observed in the degree of α-carboxyamidation. In the third week of life the immediate precursor of amidated gastrin, glycine-extended gastrin, accumulated, and at weaning (day 21) the concentrations exceeded those of amidated gastrin. Our results confirm that weaning is accompanied by an increased synthesis of gastrin and imply that α-carboxyamidation is the rate-limiting step during the biosynthetic maturation of gastrin.

INTRODUCTION

α-Carboxyamidation is a modification essential for the bioactivity of a large number of peptide hormones and transmitters [1,2]. The post-translational maturation, however, includes several other modifications as well. Thus maturation of preprogastrin [3–5] involves partial phosphorylation of serine-96, *O*-sulphation of tyrosine-87, *N*-terminal proteolytic cleavage (removal of the signal peptide) and proteolytic cleavage at dibasic sequences in addition to α-carboxyamidation of phenylalanine-92. The many modifications are reflected in the large number of components (Component-I, gastrin-34, gastrin-17, gastrin-14 and gastrin-6 [6–11]) all sharing the active site, the *C*-terminal tetrapeptide-amide -Trp-Met-Asp-Phe-NH_2. The crucial *C*-terminal amidation of gastrin involves cleavage after the dibasic sequence -arginine-94-arginine-95-, successive removal of the basic residues, and, finally, conversion of the glycine-93-extended intermediate into the amide moiety on phenylalanine-92 (Fig. 1).

Previous studies on the ontogenesis of gastrin in rat antrum have shown a correlation between weaning (the shift from liquid to solid food) and increased synthesis of gastrin in its amidated bioactive forms [12,13].

Using an assay system that discriminates between the different biosynthetic intermediates and the amidated end products [14,15], we have now examined the processing of antral progastrin during ontogenesis of the rat.

EXPERIMENTAL

Animals

Female Wistar rats were reared with their mothers in temperature- and light-controlled cages. They were allowed to suckle *ad libitum* and had free access to solid food and water. When 21 days old, the young animals were moved from their mothers to separate cages with free access to solid food and water. Rats aged 14, 21, 30 and 100 days were fasted overnight with free access to drinking water. Pups 7 days old and newborn animals were removed from their mothers on the morning of the dissection day. Fetuses 19 days old were obtained by hysterotomy performed on anaesthetized animals.

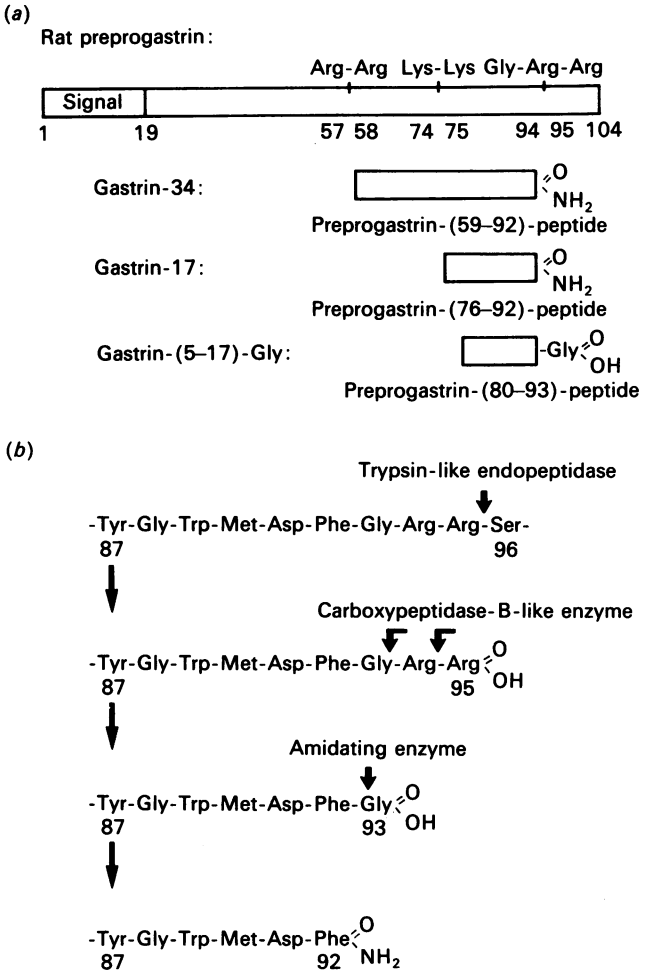

Fig. 1. Block diagram of rat preprogastrin [5], gastrin-34, gastrin-17 and gastrin-(5–17)-Gly (*a*) and the processing at the proteolytic cleavage and amidation site [rat preprogastrin-(93–95)-peptide] leading to bioactivation of gastrin (*b*)

Cleavage after Arg-57-Arg-58 liberates the *N*-terminal sequence of gastrin-34, and cleavage after Lys-74-Lys-75 liberates the *N*-terminal sequence of gastrin-17. Gastrin-(5–17)-Gly was used for raising antisera nos. 3208 and 5284 and used as standard in the assays for gastrin-Gly.

Before dissection, the newborn animals were decapitated, whereas the older animals were anaesthetized with pentobarbital (50–60 mg/kg intraperitoneally). A midline incision was made and the gastric antrum (corresponding to the distal third of the stomach) was excised, opened, and, after removal of the gastric content, the tissue (the whole stomach wall) was immediately frozen in liquid N_2. For each age, 20 animals were dissected, and the tissue divided into five pools of each four antra.

Extraction of tissue

While frozen, tissue was cut into pieces weighing a few mg, immersed in boiling water (5 ml/g of tissue, pH 6.5) for 20 min, homogenized, and centrifuged at 10000 *g* for 30 min. The supernatant was decanted (the neutral extract), and the pellet was re-extracted by addition of 0.5 M-acetic acid (5 ml/g of tissue), rehomogenization and, after 20 min at room temperature, centrifugation as described above.

Radioimmunoassays

The extracts were assayed for glycine-extended and amidated gastrin by using two types of radioimmunoassay. For measurement of glycine-extended gastrins we used antisera nos. 3208 and 5284, which were raised in rabbits against a tetradecapeptide corresponding to sequence 5–17 of human gastrin-17 extended with glycine [gastrin-(5–17)-Gly; Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-Gly] [14]. Gastrin-(5–17)-Gly was monoiodinated by mild chloramine-T treatment and purified on AE (aminoethyl)-cellulose, as previously described [14]. Gastrin-(5–17)-Gly (custom-synthesized by Cambridge Research Biochemicals, Cambridge, U.K.) was used as standard. Both antisera cross-react less than 0.2% with gastrin-17 compared with gastrin-(5–17)-Gly [14]. Whereas antiserum no. 3208 displays enhanced reactivity with sulphated forms (cross-reactivity 133%), antiserum no. 5284 is specific for non-sulphated glycine-extended gastrin, as the reactivity with the sulphated form is less than 1% [15]. The reactivity of antisera nos. 3208 and 5284 towards *N*-terminally extended forms in the rat was determined by measurements before and after tryptic cleavage of gastrin-34-Gly, as previously described [15]. Both antisera recognize gastrin-34-Gly with a potency 80% of that of the shorter forms (gastrin-17-Gly and gastrin-15-Gly). For measurement of amidated gastrins we used antisera nos. 2604 and 2605, raised in rabbits against gastrin-(2–17)-peptide [16]. Gastrin-17 was monoiodinated by our mild chloramine-T technique [17]. For standard we used non-sulphated gastrin-17 (ICI Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, U.K.). Both antisera cross-react less than 0.2% with gastrin-(5–17)-Gly compared with gastrin-17. Antiserum no. 2604 binds sulphated and non-sulphated gastrin with equimolar potency, whereas antiserum no. 2605 recognizes sulphated gastrins poorly ($\leqslant 6\%$) [18]. Antisera nos. 2604 and 2605 recognize rat gastrin-34 with full potency compared with the shorter gastrins. Corrections for reactivity of the antisera with the sulphated forms were included in the calculations of degree of sulphation, i.e. the ratio of sulphated to the sum of sulphated and non-sulphated peptides [s/(s + ns)], as previously described [15]. All other data are given without corrections for enhanced/reduced reactivity of the antisera towards different forms of gastrin.

Enzymic treatment

Determination of gastrins extended beyond the glycine-93 residue [gastrin-Gly-Arg-Arg(-R)] were made by measurement of glycine-extended gastrins before, and after, sequential treatment of the extracts with trypsin (LS 0003741; Worthington, Freehold, NJ, U.S.A.) and carboxypeptidase B (103 233; Boehringer-Mannheim, Mannheim, Germany), as previously described [14,15]. To evaluate the size of these precursors, fractions from the gel chromatography of undigested extract were assayed before, and after, treatment with trypsin and carboxypeptidase B, or carboxypeptidase B only [15].

Chromatography

Extracts were applied to 2.5 cm × 200 cm columns of Sephadex G-50 SF (Pharmacia, Uppsala, Sweden) and eluted with 0.125 M-NH_4HCO_3 at a flow rate of 18 ml/h. Fractions (3.0 ml each) were collected. The columns were calibrated with ^{125}I-albumin and ^{22}NaCl for indication of void and total volume respectively. Subsequent anion-exchange chromatography was performed on 1.0 cm × 15 cm columns of AE-41 cellulose (Whatman), using a linear gradient from 0.05 to 0.16 M-NH_4HCO_3 (pH 8.2, 300 ml). The flow rate was 33 ml/h and fractions (2.2 ml) were collected. The columns were calibrated with synthetic human gastrin-17 and gastrin-34 (Cambridge Research Biochemicals) and with pig natural gastrin-17-Gly and gastrin-34-Gly.

RESULTS

In neutral extracts of foetal-rat antrum, the concentrations of glycine-extended and amidated gastrin, as measured by antisera nos. 3208 and 2604 respectively, were below 5 pmol/g of tissue (Table 1). Amidated gastrin increased to 43 pmol/g in antrum of the newborn animals and increased progressively with age to 564 pmol/g at 21 days and 1.5 nmol/g at 100 days. During the first 2 weeks after birth, glycine-extended gastrin (Table 1) remained at about 40 pmol/g, but in the third week a 20-fold increase was observed, which over the next weeks was succeeded by a gradual decrease to 153 pmol/g in adult rats. The proportion of glycine-extended to amidated gastrin thus changed from 51 to 14% (day 0–14) to 137% (day 21) and to 10% (day 100)

Table 1. Tissue concentrations of glycine-extended and amidated gastrins in the antrum of the developing rat

Results are means (±S.E.M.) for five pools, each pool consisting of four antra.

Age (days)	Gastrin-Gly (pmol/g)	Gastrin-amide (pmol/g)	Gastrin-Gly / Gastrin-amide (%)
−2	<5	<5	–
0	18±4	43±17	51±8
4	47±6	95±13	50±7
7	64±14	195±61	36±6
14	38±5	298±51	14±3
21	773±86	564±33	137±14
30	271±108	831±144	28±8
100	153±43	1455±207	10±2

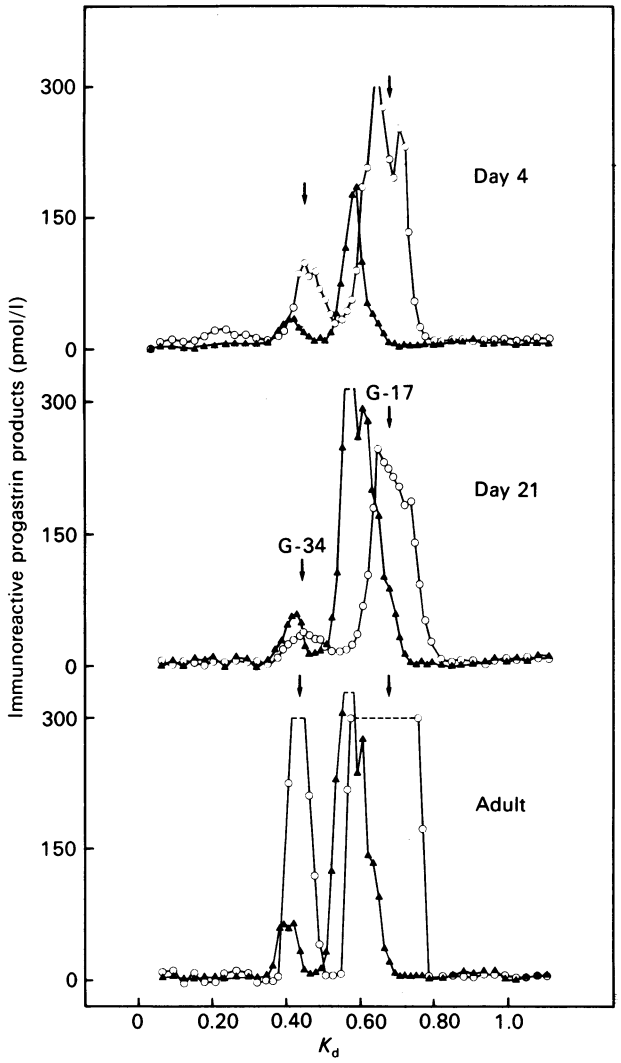

Fig. 2. Gel chromatography on Sephadex G-50 of neutral water extracts of antrum from rats aged 4, 21 and 100 days

The fractions were assayed for glycine-extended (▲, antiserum 3208) and amidated (○, antiserum 2604) gastrin. Arrows indicate elution positions of gastrin-34 and gastrin-17.

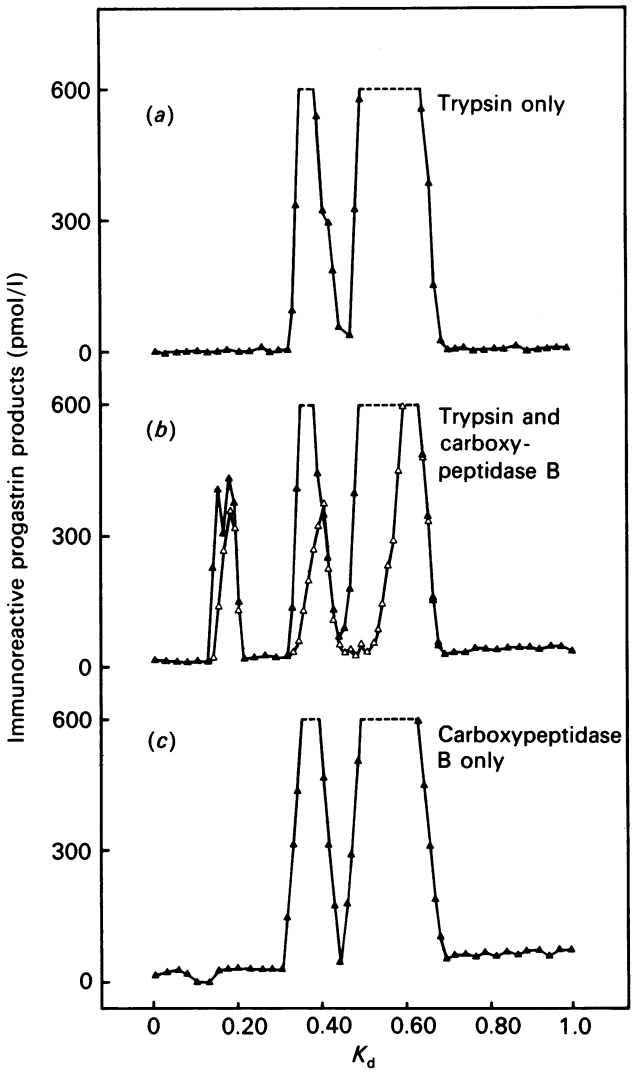

Fig. 3. Identification of gastrin precursors extended beyond glycine-93 using trypsin and/or carboxypeptidase B treatment of the gel-chromatography fractions shown in Fig. 2 (21 days)

The fractions were assayed (in a lower dilution than that shown in Fig. 2) for glycine-extended gastrin (▲, antiserum 3208; △, antiserum 5284) after enzymic treatment with trypsin (*a*), trypsin and carboxypeptidase B (*b*) or carboxypeptidase B (*c*).

(Table 1). The content of gastrins in the acidic extracts was approx. 2% of that found in the neutral extracts, the latter in consequence being used in further characterization.

Gel chromatography (Fig. 2) showed that both amidated and glycine-extended gastrin were eluted in two major peaks that were co-eluted with the amidated forms 'big' gastrin (gastrin-34, K_d 0.42) and 'little' gastrin (gastrin-17, K_d 0.65) and the glycine-extended forms, gastrin-34-Gly (K_d 0.38) and gastrin-17-Gly (K_d 0.56). Small amounts of glycine-extended Component-I-like gastrins were eluted at K_d 0.20.

At all ages (days 4, 7, 14, 21, 30 and 100) gastrin-34 constituted less than 10% of the amidated forms, and 'little' gastrin over 90%. A similar distribution regarding component size was found for the glycine-extended forms.

The elution profile with antisera nos. 2605 (results not shown) and 5284 (Fig. 3) showed that each of the peaks was a mixture of sulphated and non-sulphated forms, the sulphated form being eluted slightly earlier. The degree of sulphation (i.e. the proportion of sulphated to sulphated + non-sulphated peptide), calculated from dilutions of the extracts (Table 2), was slightly higher in the newborn than in the adult animals, 73 and 75% versus 65 and 68% (amidated and glycine-extended gastrin respectively). On day 14, the sulphation was significantly lower for amidated than for glycine-extended gastrin (54 versus 67%). The degree of sulphation was confirmed by using anion-exchange chromatography (Fig. 4), which also showed that 'little' gastrin(-Gly) was a mixture of gastrin-17(-Gly), sulphated and non-sulphated, and yet another pair of sulphated and non-sulphated peptides.

Table 2. Degree of sulphation of glycine-extended and amidated gastrins in the antrum of the developing rat

Results are means ± S.E.M. for five pools. The degree of sulphatation is expressed as 's/(s+ns)', the proportion of sulphated to sulphated + non-sulphated peptide.

	s/(s+ns) (%)	
Age (days)	Gastrin-Gly	Gastrin-amide
−2	–	–
0	75±3	73±2
4	69±3	71±1
7	67±2	69±3
14	67±2	54±1
21	68±2	64±2
30	71±1	70±2
100	68±2	65±2

As rat gastrin-17 has an arginine residue in position 2 [19] and gastrin-15 has previously been demonstrated in rat antrum [20], these forms probably represent sulphated and non-sulphated gastrin-15(-Gly).

By combined trypsin and carboxypeptidase B treatment gastrin precursors extended *C*-terminally beyond glycine-93 were identified. The concentrations varied from less than 1 pmol/g in foetal- and neonatal-rat antra to 154 pmol/g at day 21, and decreased to 53 pmol/g in the adult rat (Table 3). These gastrin precursors were eluted as a single peak at K_d 0.17 and were only partly recognized by antiserum 5284 (Fig. 3*b*). Carboxypeptidase B digestion without previous trypsination induced no increase in glycine-extended gastrin (Fig. 3*c*), thus excluding the presence of the intermediate forms gastrin-Gly-Arg(-Arg). The precursors measured are consequently extended beyond arginine-95.

DISCUSSION

The present study shows that major changes at the crucial step of bioactivation of gastrin (α-carboxyamidation) occur during postnatal development. At about day 21, the glycine-extended gastrins increased 20-fold, the gastrin-Gly concentration temporarily exceeding that of amidated gastrin. No significant differences were observed in the distribution of component sizes for the glycine-extended or the amidated forms during ontogenesis.

Some studies have shown that the antral gastrin concentration increases greatly when weaning starts in the third week of life [12,13,21], but others have demonstrated a progressive increase in gastrin concentrations during the entire neonatal period [22,23]. Our results (Table 1) agree with those of the latter studies. The accumulation of glycine-extended gastrin presumably reflects an increased biosynthesis of gastrin at weaning, as was suggested previously [12,13,21]. Biosynthesis could be stimulated by endogenous corticosterone [21] produced by the stress imposed by weaning [13], since administration of exogenous corticosterone increases the biosynthesis of gastrin in young rats [13,21,23]. Also, the switch from liquid to solid food could enhance gastrin secretion [12], thereby increasing the synthesis of gastrin. In agreement with this concept,

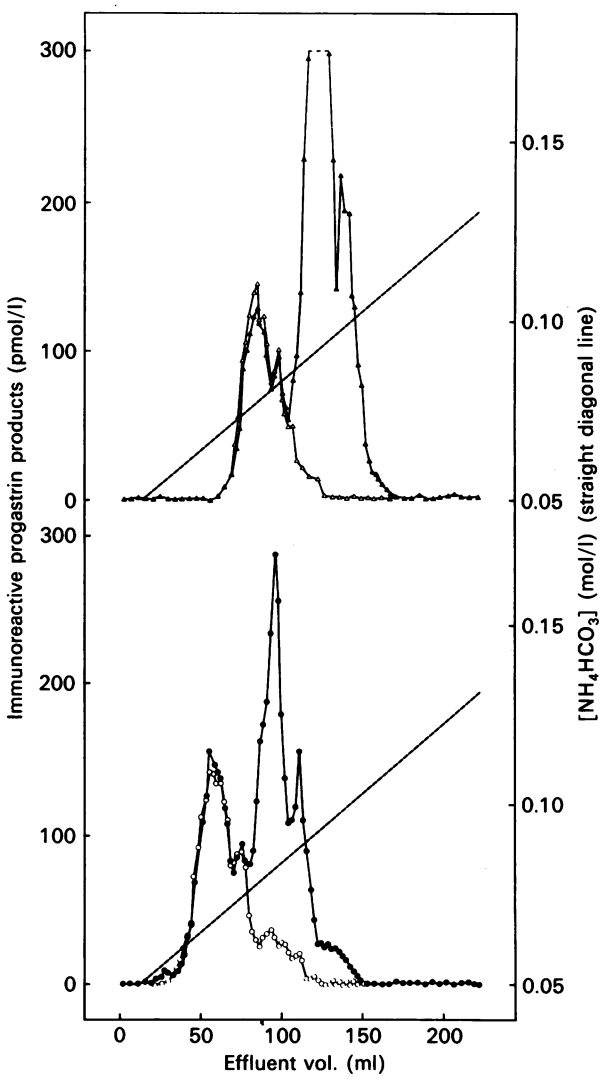

Fig. 4. Anion-exchange chromatography of glycine-extended and amidated gastrins from rat antral mucosa (21 days)

The gel-chromatography fractions containing glycine-extended 'little' gastrin were pooled and applied to AE-41 cellulose. The fractions were assayed for glycine-extended (▲, antiserum 3208; △, antiserum 5284) and amidated gastrin (●, antiserum 2604; ○, antiserum 2605). Antisera 2605 and 5284 recognize only *non*-sulphated forms. As rat 'little' gastrin has been shown to be a mixture of gastrin-17 and gastrin-15 [20], and rat gastrin-17 has an arginine residue in position 2 [19], the peptides which are eluted later from AE-cellulose are probably gastrin-15(-Gly) (sulphated and non-sulphated).

Marino *et al.* [24] found that dietary changes at weaning may stimulate gastrin synthesis and that the surge in corticosteroids may enhance gastrin amidation. In contrast with our results, they could not demonstrate an increase in glycine-extended gastrin relative to other gastrin forms at day 21. The discrepancy is difficult to explain. Like us, Marino *et al.* [24] found it virtually impossible to remove mucosa from muscle and used whole wall containing variable amounts of smooth muscle according to age. However, they did not separate antrum from the fundus and the body of the stomach, and this renders comparison of the data difficult.

Table 3. Tissue concentrations of gastrin precursors extended *C*-terminally beyond glycine-93 [gastrin-Gly-Arg-Arg(-R)] in the antrum of the developing rat

For each age, four antra were treated with trypsin and carboxypeptidase B as described in the Experimental section.

Age (days)	[Gastrin-Gly-Arg-Arg(-R)] (pmol/g)
−2	< 1
0	< 1
4	70
7	62
14	79
21	154
30	35
100	53

The signal recognized by the amidating enzyme(s) is -Xaa-Gly [25,26]. Recently we proposed that the immediate precursor of gastrin-17 is gastrin-17-Gly [15] and not amidated gastrin-34 as previously suggested [27,28]. The identical size distribution of glycine-extended and amidated gastrin observed at all ages lend support to this role of gastrin-17-Gly. Several assumptions would have to be made should gastrin-17 instead be solely derived from gastrin-34. First, gastrin-34-Gly must be a more attractive substrate for the amidating enzyme than gastrin-17-Gly. Second, amidation of gastrin-34-Gly should be followed by proteolytic cleavage to gastrin-17. Although the first assumption may be true, it is unlikely that amidation could function as a signal for proteolysis far away (at 17 amino acids distance) from amidation. Such mechanism is not known for any peptide. However, amidation of gastrin-34-Gly might be followed by proteolytic cleavage to gastrin-17 on equal terms with cleavage of gastrin-34-Gly. This necessitates that gastrin-34-Gly is accessible to the amidating enzyme before the dibasic specific endopeptidase is able to cleave after -lysine-74-lysine-75, and this, in turn, does not agree with dibasic cleavage being regarded as an early intracellular event [29]. Pulse–chase and other kinetic experiments are required to demonstrate which is the major immediate precursor of gastrin-17.

Apparently the rate-limiting step during the increased biosynthesis is the amidation process, since the concentration of gastrin-Gly-Arg-Arg-R was significantly lower than that of gastrin-Gly, and gastrin-Gly-Arg(-Arg) could not be detected.

The similar component size distribution for the glycine-extended and amidated gastrins differs from that of other species. In antrum from man (L. Hilsted & J. F. Rehfeld, unpublished work), guinea pig [30] and pig [15], Component-I-Gly and gastrin-34-Gly comprise more than half of the glycine-extended forms, although Component-I and gastrin-34 constitute less than 10% of the amidated forms. The ratio of gastrin-Gly to gastrin-amide was 10% in the adult rat antrum, which is higher than in the species mentioned.

Recently, Brand *et al.* [20] demonstrated incorporation of [^{35}S]sulphate into Component-I, gastrin-34 and gastrin-17-like peptides in rat antral mucosa. Gel and ion-exchange chromatography (Fig. 4) corroborated that the glycine-extended gastrins were also present in tyrosine-*O*-sulphated and non-sulphated forms. The degree of sulphation of the glycine-extended and the amidated gastrins (Table 2) differed significantly only on day 14 (67 versus 54%). At present we cannot explain this difference.

By gel chromatography combined with trypsin and carboxypeptidase B treatment of the fractions and subsequent assay for gastrin-Gly, precursors extended beyond glycine-93 were identified. Progastrin was eluted at K_d 0.17 (Fig. 3*b*) and was extended beyond arginine-95, as carboxypeptidase B cleavage alone could not expose gastrin-Gly (Fig. 3*c*). The elution position is earlier than that of Component-I (~ 55 or 66 amino acids [5]). By comparison, gastrin-34 extended by the *C*-terminal dodeca sequence of rat preprogastrin [5] would consist of 46 amino acids. Desmond *et al.* [31] have isolated and characterized progastrin from human antral mucosa and a gastrinoma extract, the gel-chromatographic properties of the intact progastrin being found to be similar to those described above. Marino *et al.* [24] could not detect gastrin precursors extended beyond glycine in the developing rat. The fact that the antiserum employed had low reactivity with these extended forms combined with the use of extracts of whole stomachs may explain the discrepancy with our results. Apparently the progastrin was partly sulphated, since antiserum 5284 detected only the later-to-be-eluted part of the peak (Fig. 3). This component could be identical with the early gastrin-like non-immunoprecipitable peak into which Brand *et al.* [20] demonstrated incorporation of [^{35}S]sulphate.

In conclusion the post-translational processing by which progastrin matures seems to be unaltered in rat antrum during postnatal development, except for the period around weaning. At this time a vast increase occurs in the concentration of glycine-extended intermediate, the increased translation apparently exceeding the amidation capacity. Whether changes occur in the recently described serine-96 phosphorylation of progastrin [32] during this period remains to be examined. Such changes might influence the processing of the proteolytic cleavage and amidation site.

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REFERENCES

1. Tatemoto, K. & Mutt, V. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 6603–6607
2. Mains, R. E., Eipper, B. A., Glembotski, C. C. & Dores, R. M. (1983) Trends Neurosci. **6**, 229–235
3. Yoo, O. J., Powell, C. T. & Agarwal, K. L. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 1049–1053
4. Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J. F. & Marcker, K. A. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2866–2869
5. Fuller, P., Stone, D. L. & Brand, S. J. (1987) Mol. Endocrinol. **1**, 306–311
6. Rehfeld, J. F. (1972) Biochim. Biophys. Acta **285**, 364–372
7. Yalow, R. S. & Berson, S. A. (1970) Gastroenterology **58**, 609–615

8. Gregory, R. A. & Tracy, H. J. (1964) Gut **5**, 103–117
9. Gregory, H., Hardy, P. M., Jones, D. S., Kenner, G. W. & Sheppard, R. C. (1964) Nature (London) **204**, 931–933
10. Rehfeld, J. F. & Stadil, F. (1973) Gut **14**, 369–374
11. Gregory, R. A., Dockray, G. J., Reeve, J. R., Shively, J. E. & Miller, C. (1983) Peptides **4**, 319–323
12. Lichtenberger, L. & Johnson, L. R. (1974) Am. J. Physiol. **227**, 390–395
13. Okahata, H., Nishi, Y., Muraki, K., Sumii, K., Tanaka, K., Miyachi, Y. & Usui, T. (1986) Acta Endocrinol. (Copenhagen) **111**, 539–545
14. Hilsted, L. & Rehfeld, J. F. (1986) Anal. Biochem. **152**, 119–126
15. Hilsted, L. & Rehfeld, J. F. (1987) J. Biol. Chem. **262**, 16953–16957
16. Rehfeld, J. F., Stadil, F. & Rubin, B. (1972) Scand. J. Clin. Lab. Invest. **30**, 221–232
17. Stadil, F. & Rehfeld, J. F. (1972) Scand. J. Clin. Lab. Invest. **30**, 361–368
18. Rehfeld, J. F., de Magistris, L. & Andersen, B. N. (1981) Regul. Pept. **2**, 333–342
19. Reeve J. R., Dimaline, R., Shively, J. E., Hawke, D., Chew, P. & Walsh, J. H. (1981) Peptides **2**, 453–458
20. Brand, S. J., Klarlund, J., Schwartz, T. W. & Rehfeld, J. F. (1984) J. Biol. Chem. **259**, 13246–13252
21. Peitsch, W., Takeuchi, K. & Johnson, L. R. (1981) Am. J. Physiol. **240**, G442–449
22. Larsson, L. I., Håkanson, R., Rehfeld, J. F., Stadil, F. & Sundler, F. (1974) Cell Tissue Res. **149**, 275–281
23. Majumdar, A. P. N. & Rehfeld, J. F. (1983) Digestion **27**, 165–173
24. Marino, L. R., Sugano, K. & Yamada, T. (1988) Am. J. Physiol. **254**, G87–92
25. Bradbury, A. F., Finnie, M. D. A. & Smyth, D. G. (1983) Nature (London) **298**, 686–688
26. Eipper, B. A., Mains, R. E. & Glembotski, C. C. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5144–5148
27. Dockray, G. J., Vaillant, C. & Hopkins, C. R. (1978) Nature (London) **273**, 770–772
28. Rehfeld, J. F. & Uvnäs-Wallensten, K. (1978) J. Physiol. (London) **283**, 379–396
29. Steiner, D. F. (1986) Science **234**, 369
30. Hilsted, L., Rehfeld, J. F. & Schwartz, T. W. (1986) FEBS Lett. **196**, 151–154
31. Desmond, H., Pauwels, S., Varro, A., Gregory, H., Young, J. & Dockray, G. J. (1987) FEBS Lett. **210**, 185–188
32. Dockray, G. J., Varro, A., Desmond, H., Young, J., Gregory, H. & Gregory, R. A. (1987) J. Biol. Chem. **262**, 8643–8647

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