Identification and characterization of variant forms of the gastrin-releasing peptide (GRP)

T.J. McDonald, H. Jörnvall*, K. Tatemoto+ and V. Mutt+

*Department of Medicine, University of Western Ontario, London, Canada, *Department of Chemistry I and +Department of Biochemistry II, Karolinska Institutet, Stockholm, Sweden*

Received 14 April 1983

Porcine intestinal gastrin-releasing peptide (GRP) has been demonstrated to be structurally identical to the previously characterized gastric GRP. Ion-exchange and high-performance liquid chromatography of porcine intestinal extracts have identified two variant GRP forms. Studies on one of these variant forms suggest that a β-aspartyl shift has occurred in the Asn–His structure of GRP; such a modification in an Asn–His structure occurring in a natural peptide or protein has not been previously reported. This variant GRP, although retaining bioactivity, appears to have reduced potency in elevating canine plasma gastrin levels.

Gastrin-releasing peptide (GRP) *Bombesin* *Gastrin release* *HPLC* *Ion-exchange chromatography* *β-Aspartyl shift*

1. INTRODUCTION

Gastrin-releasing peptide (GRP) was originally isolated from porcine upper gastric tissue [1] and its structure was found to contain a C-terminal region with striking homology towards bombesin [2]. During purification of a similar peptide from porcine intestinal extracts [1,3], carboxymethylcellulose (CMC) chromatography revealed the presence of a gall bladder contracting factor in a fraction which elutes before the identified form of GRP. Considering the numerous reports of heterogeneity of peptides in gastrointestinal tissue [4–9] and of multiple forms of bombesin-like immunoreactivity in avian and mammalian gastrointestinal tissue [10–12], this early eluting fraction was thought to contain a variant form(s) of GRP. Here, the isolation and chemical characterization of one such GRP variant is described, a second variant form identified and the previously identified form of porcine intestinal GRP demonstrated to have the same amino acid sequence as the gastric peptide.

2. EXPERIMENTAL

2.1. *Purification procedures*

Chemicals, materials and details of chromatographic procedures have been described [1,13]. GRP-like bioactivity was determined by an in vivo guinea pig gall bladder contracting assay [14] and the criterion for an increase in purity during isolation of the GRP and its variants was an increase in potency of the gall bladder contracting activity. Fractions generated by reverse-phase high performance liquid chromatography (HPLC) were assayed [15,16] for bombesin-like immunoreactivity (using an antiserum generously supplied by Drs M.A. Ghatei and S.R. Bloom). For purification, the starting material was the post-secretin fraction obtained during purification of porcine secretin [17]. This material was dissolved in a 25.5 mM sodium phosphate (pH 6.4) buffer, applied to a CMC column (5 × 19 cm) and eluted with a linear gradient from 0–0.3 M NaCl in the same buffer. Two bioactive fractions (variant and GRP fraction, fig.1) in distinctly separated peaks were col-

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

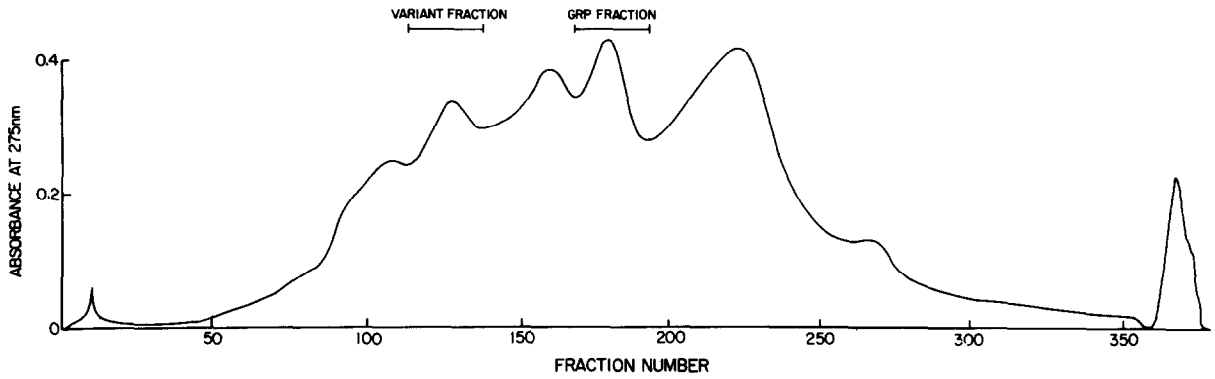

Fig.1. CMC chromatography of 3.79 g post-secretin fraction [17]. Elution with a linear gradient of 0–0.3 M NaCl in 0.0225 M sodium phosphate buffer (pH 6.4); column, 5 × 19 cm; fraction vol., 14 ml. The elution pattern was monitored by measuring absorbancy at 275 nm. Fractions containing GRP-like activity were pooled as indicated by the bars. After recovery, the peptide material in the GRP and variant fractions weighed 363 mg and 268 mg, respectively.

lected and desalted on a Sephadex G-25 column eluted with 0.2 M acetic acid.

Pooled fractions, from such initial CMC chromatographies, containing GRP (1.1 g) and proposed variant GRP (1.51 g) were dissolved in 0.02 M ammonium bicarbonate (pH 8.0) and separately subjected to further CMC chromatographies. The GRP containing sample was applied to a CMC column (5 × 19 cm), eluted with 600 ml 0.02 M ammonium bicarbonate and then with 0.05 M ammonium bicarbonate during which the GRP-like bioactivity eluted between 2890–3180 ml. The proposed variant GRP containing sample was applied to a CMC column (5 × 19 cm) and during elution with 0.02 M ammonium bicarbonate, GRP-like bioactivity emerged between 3865–4550 ml. After recovery by lyophilization, the peptide material in the GRP and proposed variant GRP containing fractions weighed 23.1 mg and 34.7 mg, respectively. Final purification was by reverse-phase HPLC using a Waters instrument consisting of a micro-Bondapack C18 column (7.8 × 300 mm), a U6K injector, two M 6000 A pumps, a 660 solvent programmer and a 450 detector. Peptide peaks were detected by monitoring absorbancy at 215 nm, solvent systems were 0.1% trifluoroacetic acid in water (A) and acetonitrile (B), respectively and the flow rate was 2 ml/min. The GRP fraction was purified by a linear gradient from 25–40% B solvent in 1 h, the variant fraction was purified by an isocratic elution with 25% B for 24 min followed by a linear gradient from 25–40% B solvent over 1.5 h. Tryptic fragments were purified by linear gradient elution from 0–50% B solvent in 45 min.

The HPLC purified preparations were bioassayed for gastrin-releasing properties in dogs as in [1,13]. Plasma gastrin levels were estimated by radioimmunoassay [18] using the antiserum 2604 (a generous gift of Dr Jens Rehfeld).

2.2. *Structural analysis*

C-Terminal amide structures were determined by enzymatic hydrolysis, dansylation, and subsequent identification of amides [19]. N-Terminal amino acids were determined by the dansyl technique and identified on polyamide sheets using 4 chromatographic systems [20]. Peptides were digested with trypsin [20] and the resulting fragments were separated by reverse-phase HPLC. Amino acid analyses were performed on a Beckman 121M analyzer after hydrolysis at 110°C for 24 h in evacuated tubes containing 6 M HCl. Liquid-phase sequencer degradation in a Beckman 890D instrument using a 0.1 M Quadrol peptide program was performed in the presence of polybrene added together with glycine and degraded for 2 cycles before peptide application [21]. Phenylthiohydantoin amino acids were determined on a Hewlett-Packard 1084B HPLC instrument using an acetonitrile gradient system [22]. Samples were also analyzed by silica gel thin-layer chromatography with fluorescent indicator and subsequent staining with collidine–ninhydrin [23].

Manual sequence analysis was by the dimethylaminoazobenzene isothiocyanate technique [24] using by-products as an aid in identification [25].

3. RESULTS

3.1. *Isolation*

Reverse phase HPLC of the GRP fraction demonstrated one major area of immunoreactivity (fig.2) while that of the variant fraction demonstrated two distinct peaks of immunoreactivity (fig.3) which were designated V-1 and V-2. The GRP preparation (1.41 mg final yield), as with previous preparations (26), co-eluted on HPLC with a synthetic replicate of GRP. Final yields of V-1 and V-2 were 0.92 and 0.723 mg respectively, and both had different HPLC retention times from that of GRP (fig.3). Silica gel thin-layer chromatographies of GRP and V-1 preparations were consistent with both having a high degree of

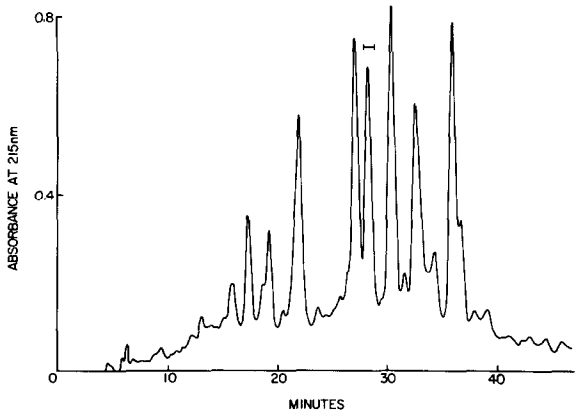

Fig.2. Reverse-phase HPLC of 1 mg GRP fraction from CMC chromatographies. The active material was collected as shown by the bar.

Table 1

Amino acid compositions of the tryptic fragments of GRP and V-1

Residue	GRP			V-1		
	T1	T2	T3	T1a	T2a	T3a
Asx			1.0 (1)			1.0
Thr		1.0 (1)			0.9 (1)	
Ser		1.0 (1)	0.2 (0)		0.9 (1)	0.3
Glx			0.2 (0)			
Pro	1.0 (1)	1.1 (1)		1.1 (1)	1.2 (1)	
Gly		3.0 (3)	2.1 (2)		3.0 (3)	2.0
Ala		2.0 (2)	1.1 (1)	0.4 (0)	2.0 (2)	1.0
Val		3.1 (3)	1.0 (1)		2.9 (3)	1.0
Met	0.8 (1)		0.8 (1)	1.0 (1)		0.6
Ile						
Leu		0.9 (1)	0.9 (1)		0.9 (1)	0.8
Tyr	0.9 (1)			0.9 (1)		
Phe						
Trp			– (1)			–
Lys		1.2 (1)			1.0 (1)	
His			1.8 (2)			1.9
Arg	1.0 (1)			1.0 (1)		

The first values show molar ratios from acid hydrolysates (values below 0.1 omitted), values in parentheses the sum from sequence analysis

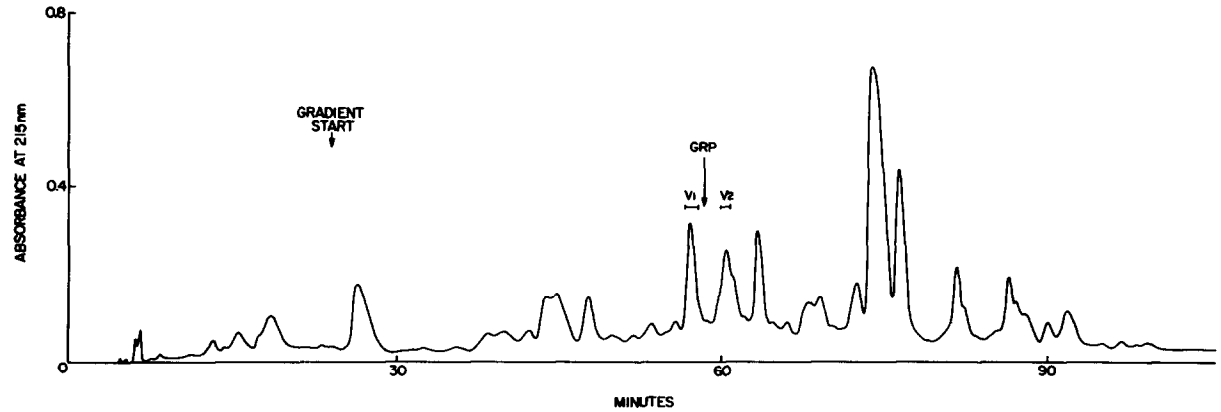

Fig.3. Reverse-phase HPLC of 1 mg variant fraction from CMC chromatographies. Activity was found in two peaks, designated V-1 and V-2, which were collected as shown by the bars. (↓) Retention time of GRP.

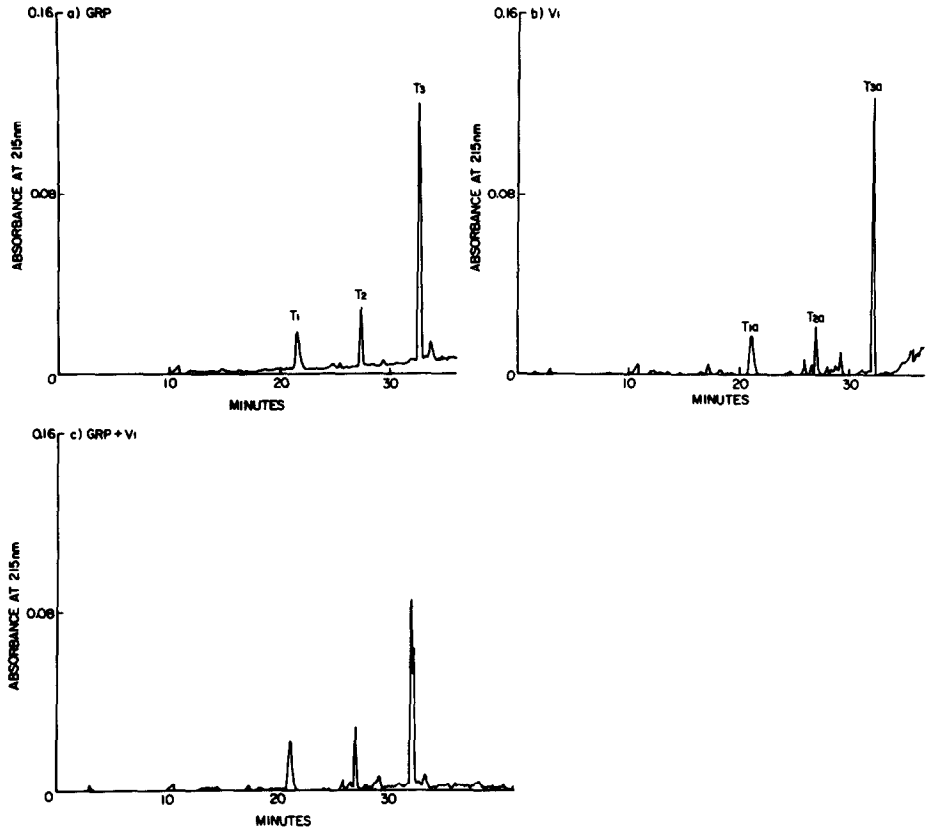

Fig.4. Reverse-phase HPLC of 6 μg tryptic digests of GRP (4a) and V-1 (4b), and a mixture of 3.5 μg of each of the tryptic digests of GRP and V-1 (4c).

homogeneity. The mobilities of GRP and its synthetic replicate were identical (R_F 0.59) while that of V-1 was slightly less (R_F 0.57). Thin-layer chromatography of preparation V-2 demonstrated the presence of contaminating peptides and hence, this fraction has not been fully characterized.

3.2. *End groups, tryptic fragments*

Analysis by the dansyl method revealed that both GRP and V-1 had an N-terminal alanine residue and that lysine and tyrosine were present in the peptides (as α-dansyl-alanine, ϵ-dansyl-lysine and *O*-dansyl-tyrosine were demonstrated with virtually no contamination by other derivatives). Analysis of the V-2 preparation demonstrated the presence of the same main derivatives, suggesting that the N-terminus of V-2 is alanine, but contamination with other derivatives was present. C-terminal amide analysis [1,19], demonstrated that all 3 preparations contained the C-terminal leucyl-methionine amide structure.

N-Terminal analysis of the peptide fragments (nomenclature as in fig.4) isolated from tryptic digests of GRP and V-1 suggested that T1 and T1a (both having N-terminal methionine), T2 and T2a (both having N-terminal alanine), and T3 and T3a (both having N-terminal glycine) were, respectively, the middle, N-terminal and C-terminal fragments of the parent peptides. The amino acid compositions of the corresponding GRP and V-1 tryptic fragments were identical (table 1) and in agreement with the compositions predicted from the previous sequence analysis of the gastric GRP [1]. Reverse-phase HPLC of mixtures of the corresponding GRP and V-1 tryptic fragments (fig.4) showed co-elution of T1 and T1a and of T2 and T2a but not of the C-terminal decapeptides T3 and T3a. As T3 and T3a have identical compositions

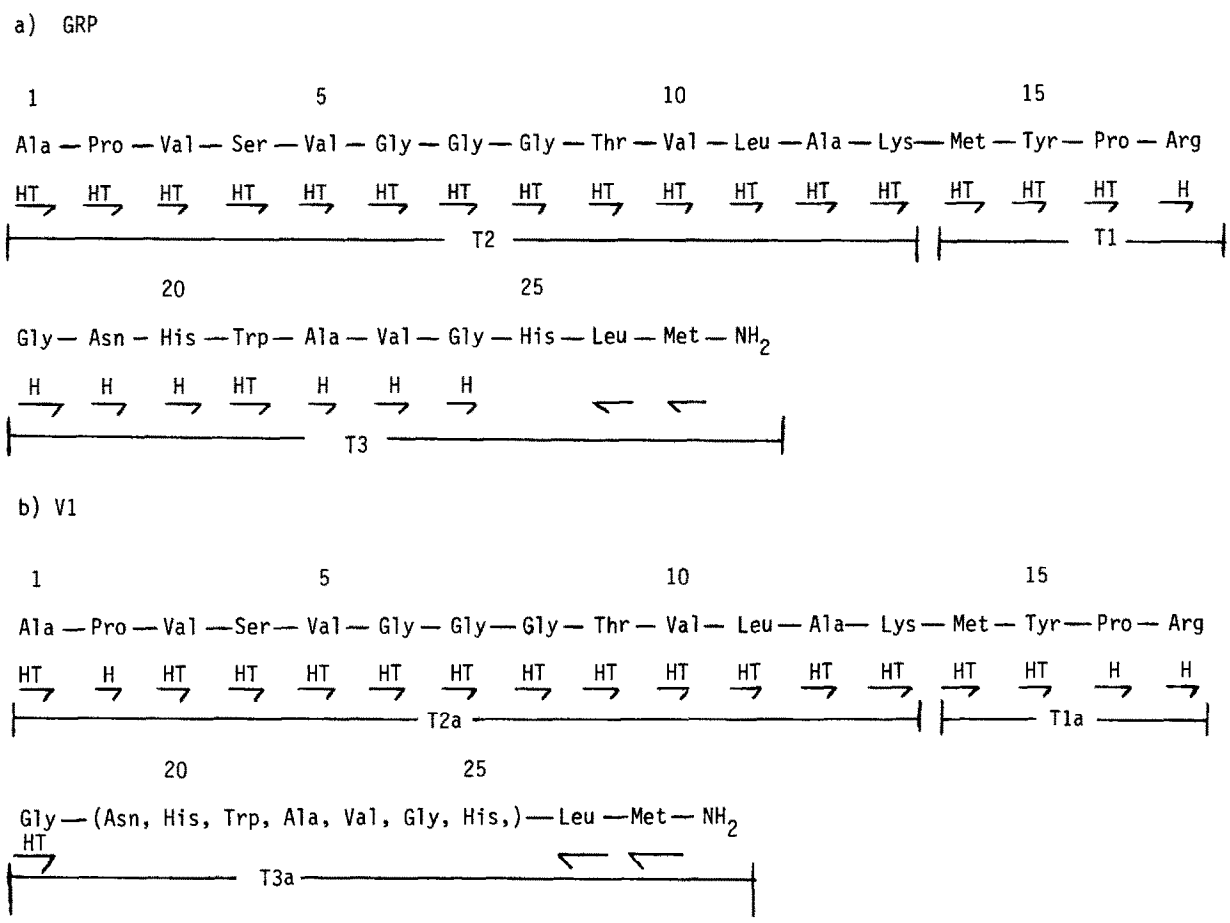

Fig.5. Summaries of the sequence analysis of 25 nmol GRP (repetitive yield 95%) and V-1 (repetitive yield 96%) are given in 5a and 5b, respectively. The positions of the 3 tryptic fragments in GRP (T_1, T_2, T_3) and V-1 (T_{1a}, T_{2a}, T_{3a}) are also given. Residues identified by sequencer degradation are given by —; HPLC PTH-analysis by H; TLC PTH-analysis by T; and residues identified by C-terminal amide analysis by ⌐—.

and N- and C-termini, the different chromatographic behaviour must be explained by an internal rearrangement.

3.3. *Sequence determination*

Liquid phase sequencer degradation of GRP identified residues up to position 24. With the histidine residue in position 25 assigned from the amino acid composition of T3, the resulting structure (fig.5a) is identical to that determined for the gastric peptide [1]. Liquid-phase sequence degradation demonstrated that V-1 had structural identity with GRP up to position 18 (fig.5b) but degradation of the peptide abruptly halted after this position. Manual sequence analysis of the C-terminal tryptic fragment of GRP confirmed the sequencer degradation results but a similar manual sequence analysis of the C-terminal tryptic fragment of V-1 failed after identification of the initial glycine residue (position 18 in the intact molecule, fig.5a,b). Hence, the demonstrated differences between the GRP and V-1 appear to involve the asparagine residue in position 19 (Asn-19). A possible explanation for the observed phenomena is that a rearrangement has occurred in V-1 whereby the peptide chain after Asn-19 has migrated from the α-carboxyl of Asn-19 to the β-carboxyl. Such a β-aspartyl shift would explain the block in the Edman degradation at residue 19 [27,28] and result in the formation of a now free

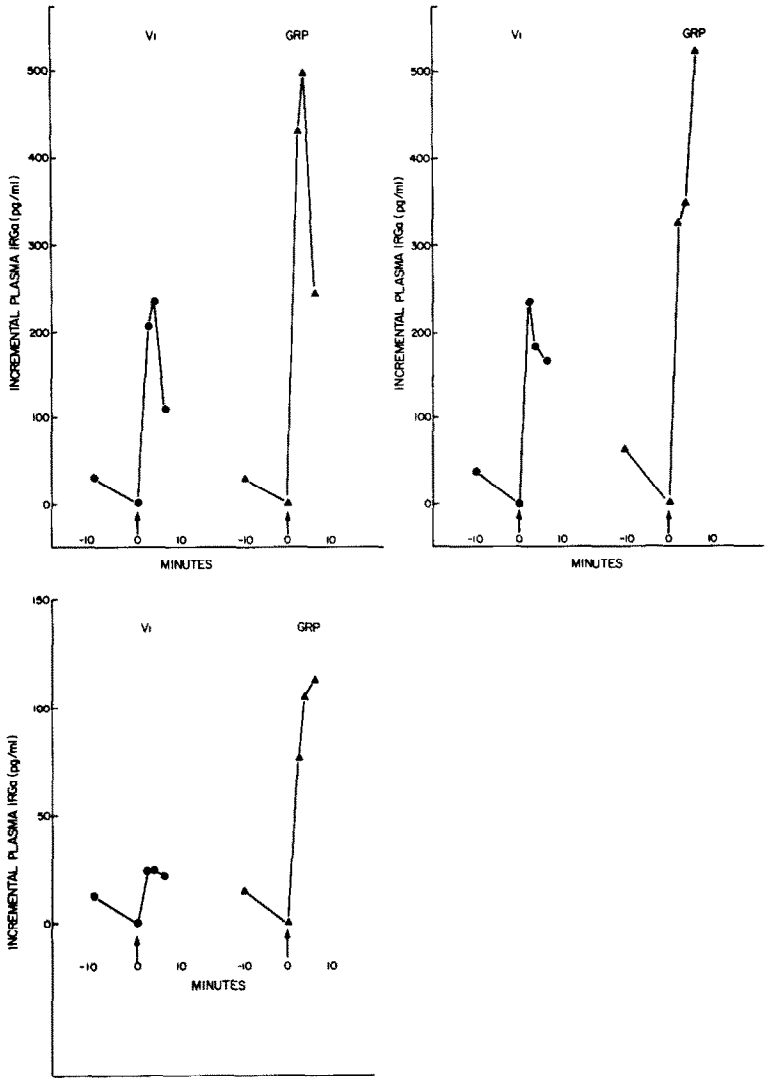

Fig.6. Plasma immunoreactive gastrin levels (pg/ml) before and after intravenous bolus injection (↑) of 300 pmol/kg of GRP and V-1 into 3 dogs.

carboxyl group which may result in the different ion-exchange chromatographic properties of GRP and V-1. The differences observed during the purifications and sequence degradations and the unchanged total amino acid compositions of the two peptides are compatible with this proposed β-aspartyl shift.

3.4. *Biological activity*

V-1 has gastrin-releasing properties but appears to be less potent than GRP (fig.6). Although complete dose–response studies are necessary to determine the precise degree of relative potencies of V-1 and GRP, the suggested β-aspartyl shift is not associated with complete inactivation.

4. DISCUSSION

This study confirms the original structure proposed for the porcine GRP by demonstrating identity between the intestinal and gastric peptides. A recent study [29] reports that a canine intestinal bombesin-like peptide is identical to the porcine GRP except for 4 residue differences occurring in

the N-terminal 12 residues. Hence, an originally proposed His/Gln change between porcine GRP and bombesin occurring at the eighth position from their C-termini [1], is also present in related peptides isolated from the chicken proventriculus [30] and the canine intestine [29].

The differences between GRP and V-1 observed during reverse-phase HPLC, ion-exchange chromatography and sequence degradations are consistent with a β-aspartyl shift occurring in the Asn–His (position 19–20) structure. β-Aspartyl shifts have been observed during analytical studies on naturally occurring proteins [27,28,31], and the Asp–Gly structure in the peptides secretin [32,33] and PHI [34] have been noted to undergo such an internal rearrangement. Studies on synthetic peptides suggest that a β-aspartyl shift may occur via formation of a cyclic imide which, on opening, partly gives rise to the β-aspartyl peptide [35,36]. This rearrangement occurs particularly easily if an asparaginyl residue is linked to a subsequent glycine, serine, or threonine residue [36] and the conversion of asparaginylglycine into the β-isomer occurs more rapidly than a similar conversion of aspartylglycine [37]. Other than the reported presence of a β-aspartylhistidine dipeptide in human urine [38], there appears to have been no previous demonstration of the occurrence of a β-aspartyl shift in the Asn–His structure in a natural protein or peptide. However, a study using the synthetic peptide *N*-benzyloxycarbonyl-β-benzylaspartyl histidine methyl ester has demonstrated that such a β-aspartyl shift occurs readily, even at −25°C, on treatment of the synthetic peptide with anhydrous HF and anisole and subsequently with 1% ammonium bicarbonate [39]. During the purification of GRP [1,13], the peptide extracts are briefly exposed to 0.2 M HCl and subsequently to ammonium bicarbonate; these procedures may provide conditions under which such a β-aspartyl shift could occur in GRP.

Heterogeneity of bioactive gastrointestinal and neuropeptides has usually been described in terms of differences in molecular size, with either precursors or fragments of a parent molecule being recognized. Such precursor or fragment molecules may exhibit heterogeneity on ion-exchange chromatography due to deletions or additions of charged residues. Heterogeneity of a peptide entity on ion-exchange chromatography in the absence of demonstrable size heterogeneity, would suggest modification or substitution of an internal residue(s); one such possible alteration is the β-aspartyl shift which, if involving a residue such as asparaginyl, might result in a less positively charged peptide. The GRP variant, V-1, very likely results from a β-aspartyl shift and it is possible that some of the previous ion-exchange heterogeneity reported for canine intestinal bombesin-like peptides [11] may be similarly explained. Of practical import, V-1 was found to be less potent than GRP. Consequently, GRP preparations which show less than the expected potency [40] should probably be investigated (by ion-exchange chromatography) for the presence of this variant form.

Finally, the present study also identifies a second variant of GRP, V-2, which has not yet been obtained completely free of contaminants. Structural studies on V-2 may provide additional insights into the heterogeneity of these bioactive gastrointestinal tract peptides.

ACKNOWLEDGEMENTS

Excellent technical assistance by Mrs L. Melin and Ms C. Palmberg is gratefully acknowledged. This study was supported by grants from the Medical Research Councils of Canada and Sweden. T.J.McD. is supported by a Career Scientist Award from the Ontario Ministry of Health.

REFERENCES

[1] McDonald, T.J., Jörnvall, H., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S.R. and Mutt, V. (1979) Biochem. Biophys. Res. Commun. 90, 227–233.
[2] Anastasi, A., Erspamer, V. and Bucci, M. (1971) Experientia 27, 166–167.
[3] McDonald, T.J., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S.R. and Mutt, V. (1978) Scand. J. Gastroent. 13, suppl.49, 119 abst.
[4] Rehfeld, J.F., Holst, J.J. and Jensen, S.L. (1982) Reg. Pept. 3, 15–28.
[5] Rehfeld, J.F. and Larsson, L.I. (1977) R.-C. Gastroenterol. 9, 15.
[6] Dimaline, R. and Dockray, G.J. (1979) Life Sci. 25, 1893–1900.
[7] Pradayrol, L., Jörnvall, H., Mutt, V. and Ribet, A. (1980) FEBS Lett. 109, 55–58.

[8] Dockray, G.J. (1981) in: Gut Hormones, 2nd edn (Bloom, S.R. and Polak, J.M. eds) pp.43–48, Churchill Livingstone, Edinburgh.
[9] Jörnvall, H., Carlquist, M., Kwauk, S., Otte, S.C., McIntosh, C.H.S., Brown, J.C. and Mutt, V. (1981) FEBS Lett. 123, 205–210.
[10] Erspamer, V., Falconieri Erspamer, G., Melchiorri, P. and Negri, L. (1979) Gut 20, 1047–1056.
[11] Walsh, J.H., Reeve, J.R. and Vigna, S.R. (1981) in: Gut Hormones 2nd edn (Bloom, S.R. and Polak, J.M. eds) pp.413–418, Churchill Livingstone, Edinburgh.
[12] Hutchinson, J.B., Dimaline, R. and Dockray, G.J. (1981) Peptides 2, 23–30.
[13] McDonald, T.J., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S.R. and Mutt, V. (1978) Gut 19, 767–774.
[14] Ljungberg, S. (1964) Svensk Farm. Tidskr. 68, 351–354.
[15] Ghatei, M.A., Jung, R.T., Stevenson, J.C., Hillyard, C.J., Adrian, T.E., Lee, Y.C., Christofides, N.D., Sarson, D.L., Mashiter, K., MacIntyre, I. and Bloom, S.R. (1982) J. Clin. Endo. Metab. 54, 980–985.
[16] McDonald, T.J., Ghatei, M.A., Bloom, S.R., Track, N.S., Radziuk, J. and Dupre, J. (1981) Reg. Pept. 2, 293–304.
[17] Said, S.I. and Mutt, V. (1972) Eur. J. Biochem. 28, 199–204.
[18] Rehfeld, J.F., Stadil, F. and Rubin, B. (1972) Scand. J. Clin. Lab. Invest. 30, 221–232.
[19] Tatemoto, K. and Mutt, V. (1978) Proc. Natl. Acad. Sci. USA 75, 4115–4119.
[20] Jörnvall, H. (1970) Eur. J. Biochem. 14, 521–534.
[21] Jörnvall, H. and Philipson, L. (1980) Eur. J. Biochem. 104, 237–247.
[22] Zimmerman, C.L., Appella, E. and Pisano, J.H. (1977) Anal. Biochem. 77, 569–573.
[23] Inagami, T. and Murakami, K. (1972) Anal. Biochem. 47, 501–504.
[24] Chang, J.Y. (1977) Biochem. J. 163, 517–520.
[25] Von Bahr-Lindström, H., Hempel, J. and Jörnvall, H. (1982) J. Prot. Chem. 1, 257–262.
[26] Yanaihara, N., Yanaihara, C., Sakagami, M., Mochizuka, T. and Kubota, M. (1981) in: Gut Hormones, 2nd edn (Bloom, S.R. and Polak, J.M. eds) pp.25–31, Churchill Livingstone, Edinburgh.
[27] Smyth, D.G., Stein, W.H. and Moore, S. (1962) J. Biol. Chem. 237, 1845–1850.
[28] Jörnvall, H. (1974) FEBS Lett. 38, 329–333.
[29] Reeve, J.R., Chew, P., Shively, J.E. and Walsh, J.H. (1982) in: Program 4th Int. G.I. Hormone Symp., p.3, Stockholm.
[30] McDonald, T.J., Jörnvall, H., Ghatei, M., Bloom, S.R. and Mutt, V. (1980) FEBS Lett. 122, 45–48.
[31] Haley, E.E. and Corcoran, B.J. (1967) Biochemistry 6, 2668–2672.
[32] Bodanszky, M., Ondetti, M.A., Levine, S.D. and Williams, N.J. (1967) J. Am. Chem. Soc. 89, 6753–6757.
[33] Jaeger, E., Knof, S., Scharf, R., Lehnert, P., Schultz, I. and Wünsch, E. (1978) Scan. J. Gast. 13, suppl.49, 93.
[34] Tatemoto, K. and Mutt, V. (1981) Proc. Natl. Acad. Sci. USA 78, 6603–6607.
[35] Ondetti, M.A., Deer, A., Sheehan, J.T., Pluscec, T. and Kocy, O. (1968) Biochem. J. 7, 4069–4075.
[36] Bodansky, M. and Kwei, J.Z. (1978) Int. J. Pept. Prot. Res. 12, 69–74.
[37] Haley, E.E., Corcoran, B.J., Dorer, F.E. and Buchanan, D.L. (1966) Biochemistry 5, 3229–3235.
[38] Kakimoto, Y. and Armstrong, M.D. (1961) J. Biol. Chem. 236, 3280–3282.
[39] Baba, T., Sugiyama, H. and Seto, S. (1973) Chem. Pharm. Bull. 21, 207–209.
[40] McDonald, T.J., Ghatei, M.A., Bloom, S.R., Adrian, T.E., Mochizuki, T., Yanaihara, C. and Yanaihara, N. (1983) Reg. Pept. 5, 125–137.