The elution profile also shows that the molecular size of the native γ-MSH-like peptides is larger than that of the iodinated synthetic γ_3-MSH with apparent molecular weights (MWs) of 13,000, 8,800 and 4,500 for the peaks at $K_d = 0.14$, 0.27 and 0.55, respectively. A possible explanation for the larger MW may be that native γ-MSH-like peptides contain more than the 27 amino acids of γ_3-MSH as derived from Nakanishi *et al.*[1]. Another explanation is that native γ-MSH-like peptides may contain carbohydrate; indeed, it has been reported that in mouse ACTH-secreting pituitary tumours, the 16 K fragment of the 31 K ACTH/β-LPH precursor protein, which should contain the γ-MSH region, is glycosylated[5]. Both possibilities may account for the higher molecular form of native γ-MSHs.

The fractions corresponding to the two immunoreactive γ-MSH peaks in the anterior lobe extract and the three peaks in the intermediate lobe extract of bovine pituitary were examined with affinity chromatography on concanavalin A (Con A) coupled to Sepharose 4-B. As shown in Fig. 2, all the immunoreactive (IR-) γ-MSHs from both the anterior and intermediate pituitary are glycopeptides; all were retarded on the Con A column and could only be displaced with α-methyl-D-mannopyranoside (α-MM).

In the post-translational processing of the murine ACTH/β-LPH precursor[6], glycosylation occurs in the ACTH fragment and the resulting glycopeptide is called 13 K ACTH; glycosylation also occurs in the non-ACTH/β-LPH portion of the precursor to give the 16 K fragment. In general, the asparagine residue within the amino acid sequence Asn-X-Thr (or Ser) is the site to which a carbohydrate chain is linked through an *N*-acetylglucosamine[7]. However, it was proposed[6] that the aspartic acid residue at the 29th amino acid position of $ACTH_{1-39}$ is the most likely site for attachment of a carbohydrate chain in the murine 13 K ACTH fragment. In the case of the γ-MSH-like peptides, the amino acid sequence Asn-X-Ser corresponds to residues 15–17 of γ_3-MSH, and could be a likely site for glycosylation. However, native γ-MSHs may contain more than one carbohydrate chain as the Asn 15-X-Ser 17 sequence is followed by Ser 18-Ser-Ser in γ_3-MSH. This series of serine residues could also be candidates to which oligosaccharides are linked through an *O*-glycosidic linkage[8].

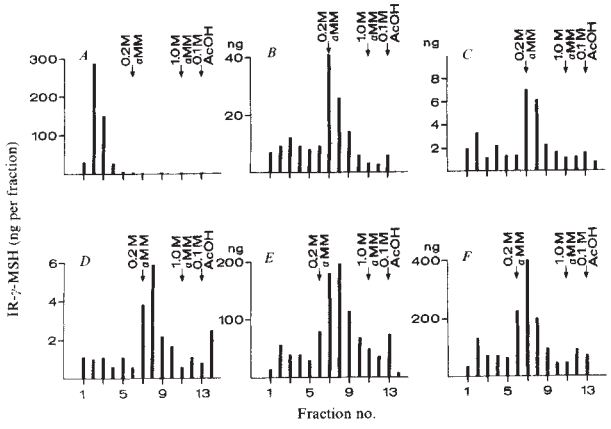

Fig. 2 Affinity chromatography of γ-MSH-like peptides on Con A-coupled Sepharose 4-B. A 1-ml siliconised glass syringe was packed with 0.4 ml Con A coupled to Sepharose 4-B. After being washed with 3 ml 0.1 m acetic acid, the column was equilibrated with Con A buffer composed of 0.01 M Tris-HCl, 0.7 mM $MgCl_2$, 0.1% bovine serum albumin, 1.0 M NaCl and 0.1% Triton X-100 (*p*H 7.4)[11]. γ-MSH-like peptides for affinity chromatography were obtained by applying the anterior and intermediate lobe extracts of bovine pituitary, respectively, to a Sephadex G-75 column in 1.0 M acetic acid. The elution profiles of the two extracts were almost the same as that in the Biogel P-100 column chromatography in 4 M guanidine-HCl. The peak tubes corresponding to the elution volume at $K_d = 0.27$ and 0.55 in the anterior lobe extract and at $K_d = 0.14$, 0.27 and 0.55 in the intermediate lobe extract were lyophilised. The residue reconstituted in 0.5 ml of Con A buffer was applied to the column. After washing the column with 4.5 ml of the same buffer, 5 ml of 0.2 M α-methyl-D-mannopyranoside (α-MM) in Con A buffer was used to elute the retarded material, followed by 1.0 M α-MM and 0.1 M AcOH. Fractions (1 ml) were collected, lyophilised and the lyophilised fractions were reconstituted in buffer for RIA of γ-MSH. Recovery following elution with 0.2 M α-MM was 51–65% of applied IR-γ-MSH. *A*, Synthetic γ_3-MSH; *B*, *C*, native γ-MSHs corresponding to $K_d = 0.27$ and 0.55, respectively, of the anterior lobe extract; *D*, *E*, *F*, native γ-MSHs corresponding to $K_d = 0.14$, 0.27 and 0.55, respectively, of the intermediate lobe extract.

Post-translational glycosylation of the ACTH/β-LPH precursor may protect it from intracellular degradation by providing it with specific conformational stability[9]. Glycosylation of native γ-MSHs may similarly have a role in extending their biological half life. Further studies are in progress to determine the amino acid sequence of the γ-MSHs and to characterise their carbohydrate moieties.

After submission of this report, Hakanson *et al.*[10] described the isolation from extracts of porcine pituitaries of a large glycopeptide (103 amino acids), a partial sequence of which showed it to be the whole *N*-terminal region of the 31 K precursor molecule down to the starting point of ACTH. The present report shows that this large fragment is further processed to the smaller γ_3-MSH. Furthermore, we have recent evidence that an even smaller fragment corresponding to γ_1-MSH is also present in pituitary extracts and released with other fragments of the 31 K precursor.

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Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides

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Naturally occurring peptides with biological actions have in most cases been detected by observing their biological activities in crude extracts and their isolation has been followed using bioassays. As a complement to the classical biological detection systems, we have proposed a chemical detection system based on fragmentation of peptides in tissue extracts followed by identification of certain of these peptide fragments having distinct chemical features[1,2]. One such chemical feature is the C-terminal amide structure which is characteristic of many

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biologically active peptides[3,4]. We have devised a chemical assay method for peptides having such a structure and have found several previously unknown peptide amides in porcine upper small intestinal tissues[1]. We report here the isolation and characterization of two of them, designated PHI and PYY. PHI is related to secretin, vasoactive intestinal polypeptide (VIP, glucagon and gastric inhibitory polypeptide (GIP); PYY is related to the pancreatic polypeptide and to neurotensin. Both peptides exhibit biological activities and appear to be present not only in the intestine but also in brain.

The presence of peptides with the amidated C-terminal structure can be detected in crude extracts using a chemical assay in which the amidated C-terminal portion of the peptide chain is cleaved off enzymatically, converted into the fluorescent dansyl derivative and then selectively isolated and identified by TLC[1]. In the present work, the levels of PHI or PYY in the tissue samples were estimated by measuring the concentration of isoleucine amide or tyrosine amide which was cleaved off from PHI or PYY, respectively, by degradation of the samples with thermolysin.

The starting material for the isolation work was a side fraction obtained during the purification of porcine secretin[5]. Boiled pig intestines (the uppermost 1 m of the small intestine) were extracted with 0.5 M acetic acid and the extracted peptides were adsorbed to alginic acid. They were then eluted from the alginic acid with 0.2 M HCl and precipitated with NaCl at saturation. The peptide concentrates were further purified by extraction with 66% ethanol, gel filtration (Sephadex G-25) and extraction with methanol[5]. The methanol-soluble fraction (after removal of the methanol) was subjected to ion-exchange chromatography on CM-cellulose (CMC) and PHI and PYY were found in a fraction eluting before secretin. This fraction was rechromatographed on CMC to separate PHI from PYY. The fractions containing PHI were further purified by gel filtration (Sephadex G-25), chromatography on DEAE-cellulose, isoelectric precipitation at *p*H 7.0 and finally by HPLC on the reversed phase, Bondapak C-18. The fractions containing PYY were further purified by gel filtration (Sephadex G-25), chromatography on CMC and finally by HPLC on Bondapak C-18. The final products were found to be homogenous by analysis with TLC, gel electrophoresis, HPLC, and by amino acid determinations.

The N-terminal analysis revealed the presence of histidine for PHI and tyrosine for PYY. Since the biological functions of these newly-isolated peptides are not yet established, we decided to give chemical names to them based on their N- and C-terminal amino acids (one-letter system). PHI thus designates 'the peptide (P) having N-terminal histidine (H) and C-terminal isoleucine (I) amide' and PYY designates 'the peptide (P) having N-terminal tyrosine (Y) and C-terminal tyrosine (Y) amide'.

PHI consists of 27 amino acids: 2 Ala, 1 Arg, 2 Asx, 2 Glx, 2 Gly, 1 His, 1 Ile, 5 Leu, 2 Lys, 2 Phe, 4 Ser, 1 Thr, 1 Tyr, 1 Val. A preliminary sequence analysis revealed the N-terminal sequence of His-Ala-Asp-Gly-Val-Phe-Thr-. The C-terminal sequence, -Leu-Ile-NH_2 was determined by the amide identification method[1]. The terminal sequences were compared with those of the secretin-glucagon family (Fig. 1) and remarkable sequence similarities were seen between PHI and secretin, VIP, glucagon and GIP, indicating that PHI probably belongs to this peptide group. PHI activated adenylate cyclase in various rat membrane systems and inhibited VIP binding to its receptors[6]. It was also found that PHI induced insulin release from an isolated rat pancreas at basal and elevated glucose levels, while it enhanced glucagon release in the presence of arginine[7].

Fig. 1 The partial sequence of PHI and the comparison with peptides of the secretin-glucagon family. a, The carboxyl end is amidated. b, The peptide (P) with N-terminal histidine (H) and C-terminal isoleucine (I). c, The vasoactive intestinal peptide (porcine). d, The gastric inhibitory peptide (porcine).

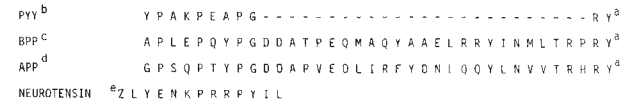

Fig. 2 The partial sequence of PYY and the comparison with the pancreatic polypeptide and neurotensin. a, The carboxyl end is amidated. b, The peptide (P) with N-terminal tyrosine (Y) and C-terminal tyrosine (Y). c, The porcine pancreatic polypeptide. d, The avian pancreatic polypeptide. e, The N-terminal amino acid is pyroglutamic acid.

PYY consists of 36 amino acids: 4 Ala, 4 Arg, 2 Asx, 5 Glx, 1 Gly, 1 His, 4 Leu, 1 Lys, 4 Pro, 3 Ser, 1 Thr, 5 Tyr, 1 Val. The N-terminal sequence was found to be Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly. Treatment of PYY with trypsin yielded tyrosine amide. Since the only lysine residue in the molecule is found near the N-terminus, the C-terminal sequence should be -Arg-Tyr-NH_2, the same as that of the pancreatic polypeptide[8,9]. The partial sequence of PYY was therefore compared with the sequence of the porcine and avian pancreatic polypeptides (Fig. 2). In addition to the distinct sequence homology to the pancreatic polypeptide, PYY possesses sequence similarities to neurotensin[10] (Fig. 2). PYY was found to inhibit the action of secretin on the pancreas of the anaesthetized cat, the bicarbonate secretion induced by secretin in a single intravenous dose (0.2 μg per kg) being reduced to about 30% in the presence of PYY (5 μg per kg) (data not shown). In a dose range of 0.5–5 μg per kg, PYY contracted the gallbladder of the anaesthetized guinea pig in a dose-responsive fashion. Further studies on the biological properties of PYY are under way.

Preliminary studies indicate that there are unknown peptide amides in brain as well as in intestine. Judging from the chromatographic patterns, solubility properties, and the C-terminal amide determinations, it seems highly probable that both PHI and PYY are present and that PYY may constitute one of the major peptide amides of the brain.

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