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Adrenocorticotropin-like and α-melanotropin-like peptides in a subpopulation of human gastrin cell granules: Bioassay, immunoassay, and immunocytochemical evidence

(immunosorbent–bioassay/radioimmunoassay/electron microscopy/chromatography/isoelectric focusing)

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ABSTRACT **Adrenocorticotropin (ACTH)-like and α-melanotropin (α-MSH)-like peptides have been localized to a subpopulation of cytoplasmic (secretory) granules of human antropyloric gastrin cells and of fetal and neoplastic gastrin cells. These granules also store gastrin and belong to the electron-dense variety of gastrin cell granules. Gastrin cells also contain granules of low to medium electron density; these store only gastrin and do not react with ACTH or α-MSH antisera. The α-MSH immunoreactive peptide was shown also to display α-MSH bioactivity by a combined immunosorbent–bioassay technique. This peptide cochromatographs with synthetic α-MSH in several systems and is not detected in oxyntic mucosa or in gastric muscle wall. As in the pituitary intermediate lobe, the α-MSH-like peptide may be formed by cleavage of ACTH-like peptides also in gastrin cells. These data provide additional evidence for local formation of ACTH/α-MSH-related peptides in gastrin cells and suggest a heterogenous peptide make-up of endocrine cell granules.**

Exocrine cells are known to simultaneously produce numerous secretory molecules. Only recently, however, has the possible production of multiple messenger molecules by endocrine cells been considered. Among cell types suspected to secrete multiple biologically active peptides are antropyloric gastrin cells, which have been shown also to contain adrenocorticotropin (ACTH) immunoreactive peptides (1–4). Upon Sephadex G-50 gel chromatography of antropyloric mucosal extracts, a predominant immunoreactive component elutes in the position of pituitary ACTH-(1–39) component (4), whereas smaller amounts of immunoreactivity elute in the void volume (4). Recent studies have suggested that the void volume immunoreactivity represents a glycosylated peptide, possibly similar to the ACTH-β-endorphin precursor of the pituitary (5).

In the pituitary, ACTH and β-lipotropin (β-LPH) are derived from a common precursor (6). β-LPH-(61–91) corresponds to the opioid peptide β-endorphin of which the NH_2-terminal part [= β-LPH-(61–65)] corresponds to [Met]enkephalin. The previously described [Met]enkephalin immunoreactivity of gastrin cells (7) seems to be due not to the pentapeptide per se but to a COOH-terminally extended [Met]enkephalin congener (unpublished data). Along with this, recent studies suggest that β-LPH/β-endorphin may occur in gastrin cells (8).

These data indicate that the gastrin cell peptides are similar to the corresponding pituitary peptides. However, the possibility remains that the peptides detected may differ from true ACTH or that, as in the pituitary intermediate lobe, the ACTH-like peptides may be processed to corticosteroidogenically inactive fragments such as α-melanotropin [α-MSH; *N*-acetylated and amidated ACTH-(1–13)] (9, 10). We have therefore further analyzed these peptides and obtained evidence for the presence of both ACTH-like and α-MSH-like peptides in a distinct subpopulation of gastrin cell granules.

MATERIAL AND METHODS

Tissue Material. Human oxyntic and antropyloric mucosa and gastric muscle wall was collected at surgery for gastric carcinoma ($n = 5$) or duodenal ulcer ($n = 7$) or at necropsy ($n = 4$). For chemical analyses, tissues were frozen in liquid nitrogen and for cytochemistry and electron microscopy they were: (*i*) fixed in 4% (wt/vol) paraformaldehyde at 4°C and processed for cryostat sectioning (3); (*ii*) frozen in melting Freon-22, freeze-dried, fixed in formaldehyde vapor, and embedded in paraffin (11); or (*iii*) fixed in a 3% formaldehyde/2% glutaraldehyde (wt/vol) mixture, postfixed in 1% OsO_4, and embedded in Epon 812 (11).

Material for cytochemistry/electron microscopy also included duodenal specimens from human fetuses (14–21 weeks, cf. ref. 11) and from six patients with gastrin-producing (Zollinger-Ellison) tumors of the pancreas or duodenum (one case) (12, 13).

Immunocytochemistry. Sections pretreated as described (3, 11), were processed for indirect immunocytochemistry, using α-MSH antisera Albert, Asger, Axel, and Arnold raised in rabbits against synthetic α-MSH (Peninsula Laboratories, San Carlos, CA), coupled to bovine serum albumin by carbodiimide. Optimal dilutions (from 1:500 to 1:10,000) were identical in pituitary intermediate lobes and antropyloric mucosa. In addition, NH_2-terminal-specific ACTH-(1–24) antisera (no. 94, Ab. Sigurth) and antisera recognizing the COOH-terminal (14–17) (nos. 4562 and 2717) and middle (6–13) regions (no. 4710) of synthetic human gastrin-17 were used (3, 14, 15).

Sections were immunocytochemically stained by the peroxidase–antiperoxidase (PAP) method (16) or by indirect immunofluorescence (16), using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (SBL, Stockholm). Immunocytochemical double stainings involved primary staining for one antigen with the PAP method and diaminobenzidine as peroxidase substrate (16). Subsequently, antibodies, but not the peroxidase reaction product, were eluted (17) and the second antigen was stained by indirect immunofluorescence (18). Completeness of antibody elution was tested by fluorescein-labeled anti-rabbit IgG and by staining and specificity controls.

Immunocytochemical Controls. Conventional staining controls (3, 16) included the sequential deletion of the various antibody layers, and substitution of the primary antiserum for normal rabbit serum, preimmune serum, or control antisera, containing high IgG concentrations. Specificity controls con-

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Abbreviations: MSH, melanotropin; ACTH, adrenocorticotropin; β-LPH, β-lipotropin; PAP, peroxidase–antiperoxidase.

sisted of antisera absorptions with various concentrations of synthetic peptides, including human ACTH-(1–39), α-MSH, porcine and human β-MSH, γ-MSH, ACTH-(1–10), ACTH-(4–10), ACTH-(4–11), β-endorphin, [Met]- and [Leu]enkephalin (Peninsula), ACTH-(1–24), ACTH-(1–16) (CIBA Pharmaceutical), human gastrin I (ICI, Macclesfield, England), and somatostatin (UCB, Brussels, Belgium). In addition, exogenous antigen–antibody complexes (19) or heating at 56°C for 30 min (16) were used for removing complement activity.

Bioassay and Immune Affinity Chromatography. Tissues were homogenized (1 g/10 ml) in ethanol/HCl (96% ethanol/concentrated HCl/H_2O; 75:2:23, vol/vol), allowed to stand for 20 min, and centrifuged; the residue was reextracted in fresh ethanol/HCl, and recentrifuged, and the supernatants were combined, dried, and redissolved in 0.04 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 15 mM sodium azide, 0.5% bovine serum albumin, 0.06 M mercaptoethanol, and 2000 units of Trasylol (aprotinin) per ml (buffer A). Supernatants were mixed with 300 μl of a prewashed immunosorbent, consisting of the highly specific α-MSH antiserum Axel coupled to CNBr-activated Sepharose 4B beads (Pharmacia). The immunosorbent was prewashed in three 5-ml cycles of 15% (wt/vol) formic acid, followed by four cycles of buffer A, which removed all endogenously bound α-MSH. After rotating with extracts for 16 hr at 4°C, the immunosorbent was washed in four cycles of buffer A and stripped by three 5-ml cycles of 15% formic acid. Formic acid eluates were pooled, lyophilized, and redissolved in *Anolis* buffer (20). Recovery (at 20 ng of synthetic α-MSH) averaged 36–41%.

Tissues were also extracted in boiling water (1 g/10 ml), homogenized, centrifuged, and lyophilized. Extracts and immunosorbent eluates were assayed by the *Anolis* skin method (20, 21). Sensitivity was 42–50 pM and specificity was tested by using synthetic human ACTH-(1–39), somatostatin, cholecystokinin-8, human gastrin I, and [Met]enkephalin. Synthetic α-MSH served as standard.

Chromatography. Extracts were applied to: (*i*) Sephadex G-25 SF or G-50 SF columns (10 × 1000 mm), eluted with *Anolis* buffer, 0.25 M ammonium bicarbonate, pH 8.2, or 0.1 M acetic acid containing 0.5% bovine serum albumin; (*ii*) Amberlite XAD-2 columns, precycled, washed, and eluted as described (22); (*iii*) precoated cellulose thin-layer chromatography plates (Merck), developed with 1-butanol/pyridine/acetic acid/H_2O (4:1:1:2, vol/vol) containing 5 mM dithiotreitol; (*iv*) isoelectric focusing in 4.5% polyacrylamide gels using Ampholines (LKB) to produce a pH gradient from 4.5 to 10.0 (23).

Radioimmunoassays. (*i*) An ACTH radioimmunoassay employing iodinated synthetic human ACTH-(1–39) as tracer and primarily recognizing ACTH-(7–18) and devoid of crossreactivity with up to 1 μM α-MSH, β-MSH, vasoactive intestinal peptide, gastrin-17, [Met]- and [Leu]enkephalin, and β-endorphin, as described (4); (*ii*) a specific α-MSH assay, employing antiserum Albert (final dilution, 1:16,000) and synthetic α-MSH, iodinated by the chloramine-T method (24) and purified by Quso glass absorption (24), and chromatography on Sephadex G-25 SF, having a specific activity of 450–500 μCi/μg (1 Ci = 3.7×10^{10} becquerels). Samples were assayed in dilutions in duplicates and sample volume (150 μl) was 10% of incubation volume. Incubation was for 24 hr at 4°C and separation by dextran-coated charcoal. Sensitivity was at 1.2 fmol per tube (12 pM) and incubation damage was below 4%. Specificity was tested by using synthetic peptides, including human ACTH-(1–39), ACTH-(1–24), ACTH-(18–39), ACTH-(1–16), ACTH-(1–10), ACTH-(4–10), ACTH-(4–11), porcine β-MSH, human β-MSH, γ-MSH, [Met]enkephalin, [Leu]enkephalin, β-endorphin, somatostatin, human gastrin I, and cholecystokinin-8, none of which displaced tracer at concentrations up to 1 μM.

RESULTS

Immunocytochemistry. All α-MSH and ACTH-(1–24) antisera stained endocrine cells of the antropyloric mucosa. Staining with antisera Albert and Axel was not inactivated by absorptions with the various ACTH congeners, but it was abolished by absorptions with synthetic α-MSH (5 μg/ml). Thus, antisera Albert and Axel contain antibodies specifically recognizing α-MSH without reacting with ACTH. In contrast, the ACTH-(1–24) antisera were not inactivated by α-MSH but were inactivated by synthetic ACTH-(1–24). Thus, the antropyloric cells contain both α-MSH-like and ACTH-like antigenic sites. These cells were identical with gastrin cells (Fig. 1) and were also stained by α-MSH and ACTH-(1–24) antisera preabsorbed with gastrin, somatostatin, β-endorphin, enkephalins, β-MSH, or γ-MSH. The immunoreactive cells failed to react with control sera and the staining was complement independent.

The α-MSH-like and ACTH-like immunoreactivity occurred in cytoplasmic granules, the number of which varied between cells (Fig. 2). Double staining for ACTH or α-MSH and gastrin showed that the cells contained many gastrin-storing granules,

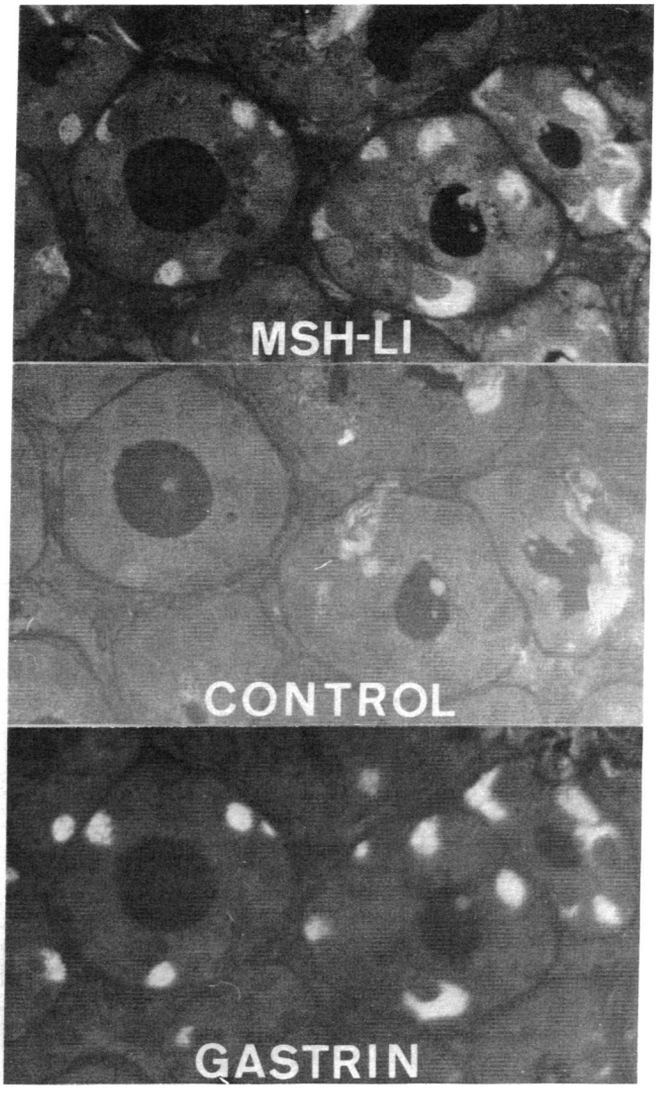

FIG. 1. Human antropyloric mucosa. Three consecutive sections stained with α-MSH antiserum Albert (MSH-LI), with α-MSH-preabsorbed antiserum Albert (control), and with gastrin antiserum no. 4562 (gastrin), using indirect immunofluorescence. The sections all contain parts of the same cells. Note that the α-MSH-immunoreactive cells correspond to gastrin cells and that α-MSH preabsorption abolishes staining. (×750.)

which were not ACTH or α-MSH immunoreactive. The PAP stain for α-MSH or ACTH masked the coexisting gastrin immunofluorescence of the "α-MSH/ACTH granules" but not of granules storing only gastrin. That the former granules also contained gastrin was shown by reversing the staining sequence and by electron microscopy (see below).

Scattered α-MSH and ACTH immunoreactive cells also occurred in human fetal duodenum. These cells were also gastrin immunoreactive and corresponded to two types of cells previously identified (11): (*i*) typical antropyloric-type G cells, containing granules of variable electron density; and (*ii*) "type 3" cells containing only electron-dense, slightly angular, granules, which also occurred in G cells.

All six gastrin-producing tumors contained various numbers of α-MSH- and ACTH-immunoreactive cells, which also were gastrin immunoreactive. In one tumor, two types of granulated cells occurred: (*i*) cells structurally identical to G cells and (*ii*) cells containing only electron-dense granules similar to the fetal type 3 gastrin cells (11, 13). The latter cells reacted strongly with ACTH-(1–24), α-MSH, and gastrin antisera (Fig. 3). The G cells reacted strongly with gastrin antisera, whereas their reactivity to α-MSH or ACTH-(1–24) antisera varied with their content of electron-dense (type 3) granules. Thus, G cells containing almost exclusively electron-lucent granules showed no or weak reactivity to α-MSH antisera (Fig. 3). All tumors were from patients with the Zollinger–Ellison syndrome. The ectopic ACTH syndrome and hyperpigmentation had occurred in two of the six cases.

We conclude that the electron-dense granules found in both typical G cells and in type 3 cells represent the storage sites of α-MSH-like and ACTH-like immunoreactivity.

Chemical Characterization. Acid/ethanol extracts of all gastric regions contained α-MSH-like bioactivity. This activity was chromatographically heterogenous, and extracts were therefore prepurified by the α-MSH immunosorbent procedure. After such purification, only antropyloric mucosal extracts contained α-MSH-like bioactivity. This activity diluted in parallel with the α-MSH standard (skin rate method; ref. 21) and chromatographed as synthetic α-MSH on cellulose thin layers and on Sephadex G-25 columns. Concentrations measured averaged 3–13 pmol of α-MSH equivalents per g (wet weight) of antropyloric mucosa ($n = 5$).

The α-MSH-specific radioimmunoassay revealed 12–63 pmol of α-MSH equivalents per g (wet weight) of antropyloric mucosa ($n = 5$). When the around 40% recovery of the immunosorbent-bioassay procedure is taken into account, the concentrations measured are in close agreement. Immunoreactive and bioactive α-MSH were quantitatively retrieved in the methanol fraction of Amberlite XAD-2 columns, which were used for purifying and concentrating the activity. Radioimmunoassayable α-MSH cochromatographed with synthetic α-MSH on cellulose thin layers and on Sephadex G-25 columns (Fig. 4). Parallel monitoring of the Sephadex G-25 columns with the ACTH radioimmunoassay showed ACTH immunoreactivity to be absent from fractions containing α-MSH immunoreactivity and bioactivity. On Sephadex G-50 SF columns, a main ACTH-immunoreactive component eluted in the position of ACTH-(1–39). Smaller amounts of ACTH-like immunoreactivity were detected in the void volume.

Isoelectric focusing of antropyloric extracts revealed a main ACTH-immunoreactive peak with an isoelectric point identical to that of ACTH-(1–39) (pI = 7.5–8.0) and a second major and more acidic component (pI = 7.0–7.2) (Fig. 5). Two more components, one very acidic (pI = 5.5) and one more basic (pI ≈ 9.0) were poorly resolved at the edges of the pH gradient. A similar pattern was detected in extracts of cat antropyloric mucosa and cat pituitary. The isoelectric focusing experiments were not monitored for α-MSH due to its highly basic character.

DISCUSSION

Our results indicate that human gastrin cells contain a peptide resembling α-MSH in its chromatographic, immunological, and biological properties. These cells also contain ACTH-like peptides, which by isoelectric focusing and gel chromatography cannot be distinguished from pituitary ACTH components. Possibly, the α-MSH-like peptide may be derived from cleavage of such ACTH-related peptides.

These peptides probably do not stem from the pituitary; the adult human pituitary lacks a functioning intermediate lobe and

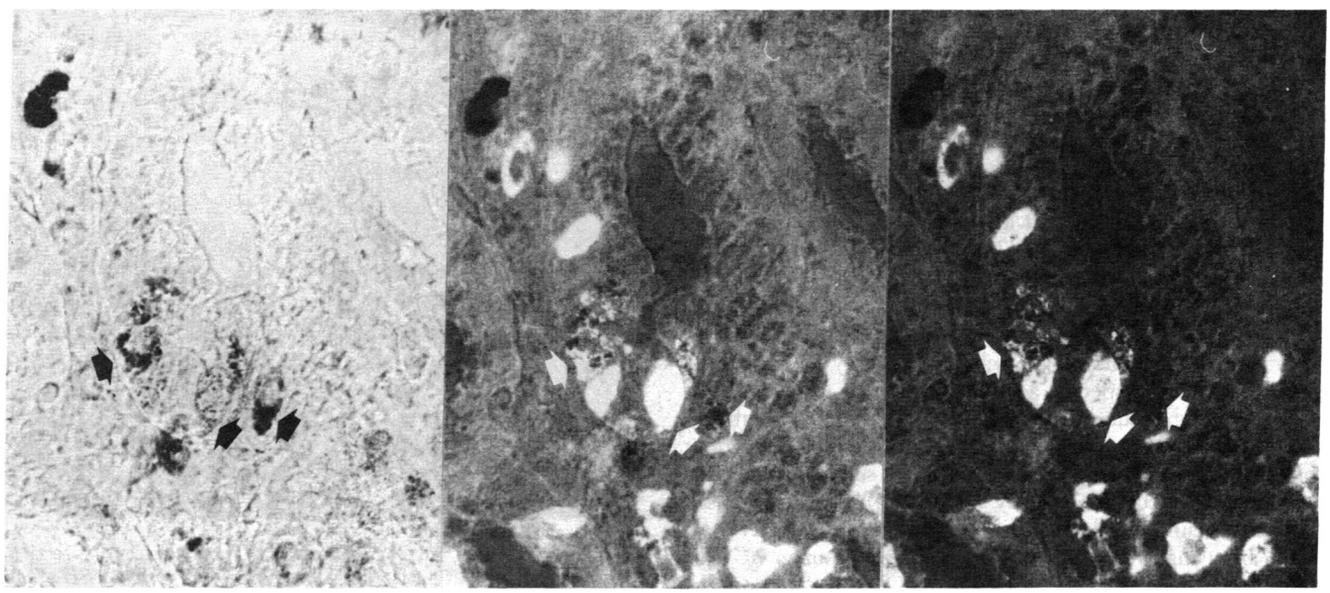

FIG. 2. Human antropyloric mucosa. The section was first stained with α-MSH antiserum (Albert) by using the PAP method and, after antibody elution, restained with gastrin antiserum (no. 4562), using indirect immunofluorescence, and examined in ordinary light (*Left*), combined phase contrast and fluorescence (*Center*), and in fluorescence alone (*Right*). Note that the gastrin cells contain variable numbers of α-MSH-positive granules (black) and, in addition, contain many immunofluorescent gastrin granules, which do not react with the α-MSH antiserum. (×700.)

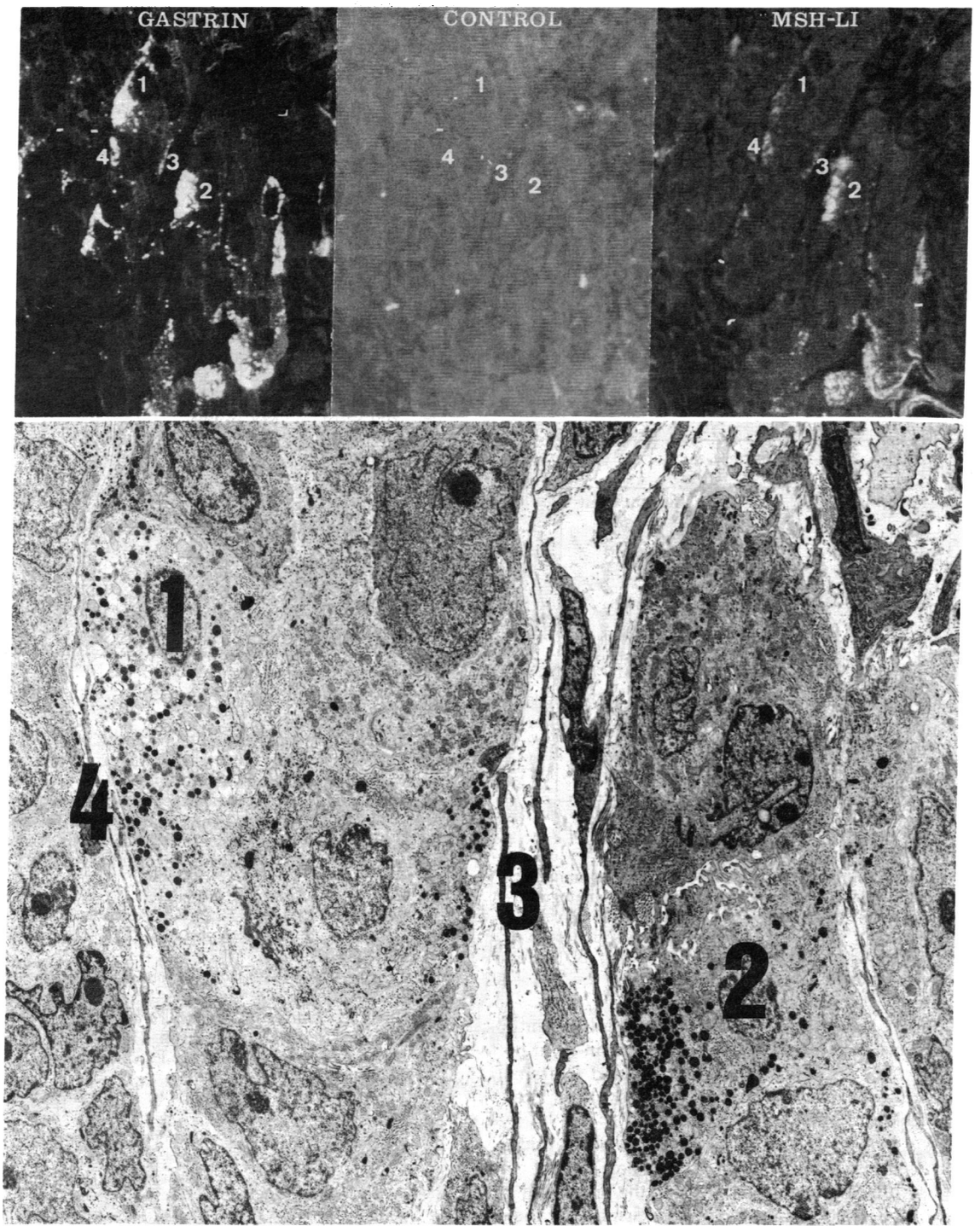

FIG. 3. Human gastrin-producing tumor. Four serial sections containing parts of the same cells have been cut for immunocytochemical staining with gastrin antiserum 4562 (gastrin), with α-MSH-absorbed antiserum Albert (control), and with α-MSH antiserum Albert (MSH-LI). (×400.) The fourth, ultrathin, section was examined in the electron microscope. (×3440.) Note that whereas cells 2, 3, and 4 react strongly with both α-MSH and gastrin antisera and contain large angular type 3 granules, cell 1 reacts strongly with the gastrin antiserum but very weakly with the α-MSH antiserum and contains a preponderance of electron-lucent granules.

does not contain detectable quantities of α-MSH (25). Additionally, hypophysectomy does not reduce the ACTH-like immunoreactivity of gastrin cells (1, 2). Recent unpublished data, moreover, indicate that antropyloric mucosae in organ culture incorporate radioactive amino acids into specifically ACTH-immunoprecipitable peptides.

Demonstration of peptides with specific chromatographical properties and with measurable bioactivity argues against the possibility that the results obtained are due to damage or unspecific interference in the immunoassay procedures. This possibility is also disproven by testing for damage to the ^{125}I-labeled-ACTH-(1–39) and α-MSH tracers, by the immunosorbent experiments, and by the immunocytochemical results. Addition of ACTH-(1–39) to gastric extracts was not associated with measurable conversion of this peptide into α-MSH-immunoreactive peptides.

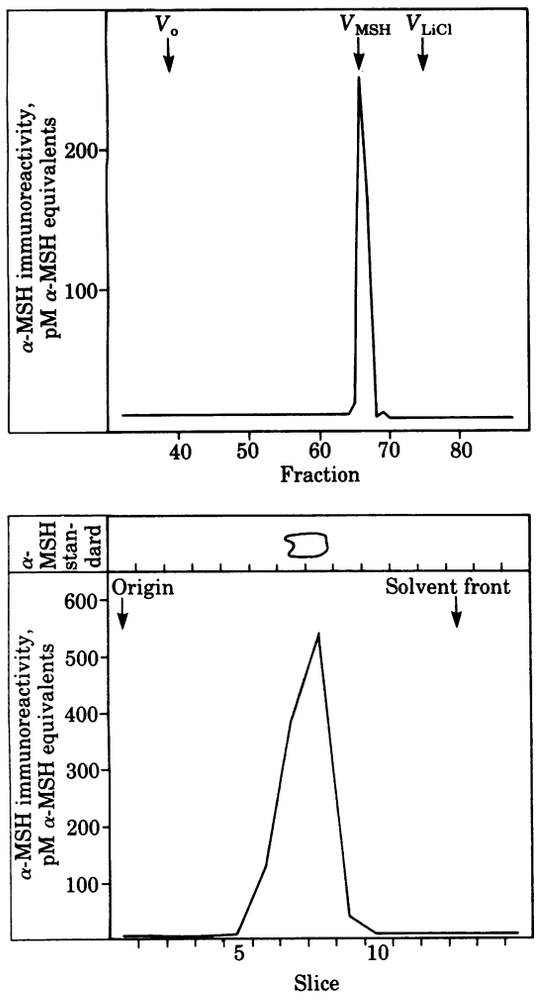

FIG. 4. Chromatography of acid/ethanol extracts from human antropyloric mucosa. The extracts were prepurified by chromatography on Amberlite XAD-2 and then applied to a Sephadex G-25 SF column eluted with acetic acid (*Upper*) or to cellulose thin-layer plates developed with butanol/acetic acid/pyridine/H_2O (4:1:1:2, vol/vol) (*Lower*) as monitored by the α-MSH radioimmunoassay.

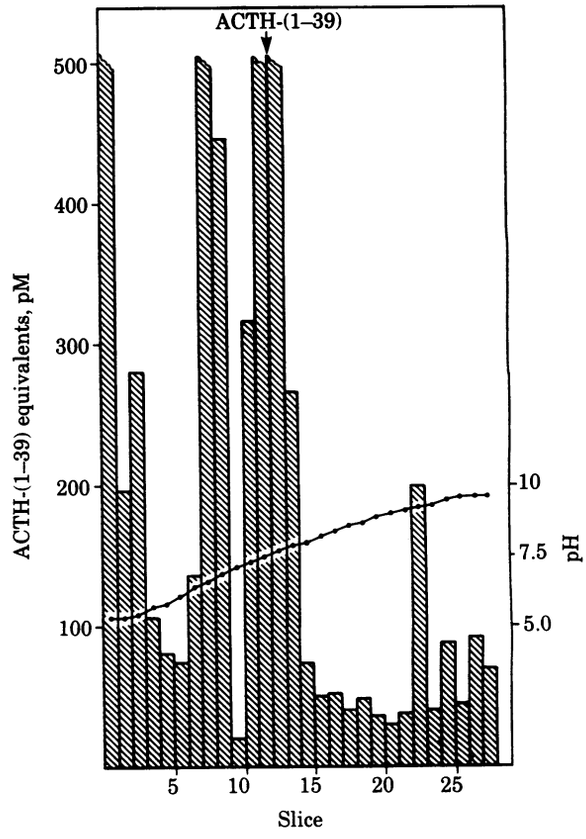

FIG. 5. Boiling water extract of human antropyloric mucosa after isoelectric focusing in a 4.5% polyacrylamide gel. Peptides in the sliced gel were eluted overnight in assay buffer and assayed with the ACTH radioimmunoassay. Note the presence of a main component focusing at pH 7.5–7.9. This position corresponds to the isoelectric point of ACTH-(1–39), as determined by calibrations in this system and with the theoretically calculated isoelectric point of ACTH-(1–39).

A relationship between the pituitary and gut was recently suggested by the detection of gastrin-17 and gastrin-34 in pituitary extracts (26). By immunocytochemistry and assays on isolated pituitary cells, pituitary gastrins have now been localized to the ACTH and MSH cells (27).

In the gastrin cells, the α-MSH-like and ACTH-like peptides are stored in a subpopulation of secretory granules. It is not yet known whether this finding reflects stages in the processing of these peptides or reflects an independent mechanism for their release (cf. refs. 28 and 29). Isolation and sequencing work is necessary to exactly define the structures of these peptides.

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