0270-6474/82/0207-0861$02.00/0

The Journal of Neuroscience
Vol. 2, No. 7, pp. 861-878
July 1982

IMMUNOCYTOCHEMICAL AND ULTRASTRUCTURAL DIFFERENTIATION BETWEEN MET-ENKEPHALIN-, LEU-ENKEPHALIN-, AND MET/LEU-ENKEPHALIN-IMMUNOREACTIVE NEURONS OF FELINE GUT[1]

LARS-INGE LARSSON[2] AND KRISTIAN STENGAARD-PEDERSEN

Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Received November 18, 1981; Accepted January 15, 1982

Abstract

Selective immunocytochemical procedures, applied at the light and electron microscopical level, have resulted in the identification of three types of enkephalin-immunoreactive nerves in the feline gastrointestinal tract, including neurons containing either Met- or Leu-enkephalin immunoreactivity or those simultaneously storing both Met- and Leu-enkephalin immunoreactants. The three different types of enkephalin nerves show a similar distribution in the gut with the exception of the small arteries which frequently appear to be innervated only by Met-enkephalin-immunoreactive terminals. Electron microscopical identification using optimally fixed, osmicated, and contrasted tissue revealed ultrastructural differences between the three types of nerve terminals. Thus, the terminals contained variable proportions of large granular and large opaque p-type vesicles. Large granular vesicles were associated predominantly with Met-enkephalin immunoreactants, whereas large opaque vesicles were associated predominantly with Leu-enkephalin immunoreactants. It is presently impossible to decide whether the different p-type vesicles reflect a continuous processing of a precursor contained within them or whether they are truly different in peptide content. Thus, granular vesicles had cores of either high or medium electron density. Medium electron-dense granular vesicles were sometimes difficult to distinguish from large opaque vesicles, suggesting the existence of possible transitional forms. All types of enkephalin terminals also contained small clear ("cholinergic-like") vesicles which were not immunoreactive to enkephalins. These data indicate that multiple enkephalin precursors/enkephalin-containing peptides occur in the gut or, alternatively or additionally, that a common Met/Leu-enkephalin precursor is processed differently in different neurons.

Leu- and Met-enkephalin are a pair of opioid pentapeptides first isolated from the brain and subsequently shown to be present in many other organs, including the gastrointestinal tract and adrenal glands. Immunological differentiation between the two pentapeptides has been troublesome since they differ only in that their COOH-terminal amino acid is either leucine or methionine. Moreover, many other molecules are known to contain the enkephalin sequence. Thus, β-lipotropin (β-LPH), β-endorphin (β-LPH-(61-91)), peptides E, F, and I, BAM 22P, and several others are known to contain the sequence of Met-enkephalin, whereas α-neoendorphin, peptides E and I, and dynorphin contain the Leu-enkephalin sequence (for review, see Rossier, 1981). Thus, peptides E and I contain both Met- and Leu-enkephalin (peptide E = peptide I-(15-39)) (Rossier, 1981). Peptide I is currently believed to form part of an even larger (>50,000) precursor molecule, containing six or seven copies of the Met-enkephalin sequence and one copy of the Leu-enkephalin sequence (Jones et al., 1980, Kimura et al., 1980). Since the sequences of dynorphin (Goldstein et al., 1979) and α-neoendorphin (Kangawa et al., 1979) differ, at most, one of them may occur in the above-mentioned precursor. This indicates that at least one more Leu-enkephalin precursor exists.

The available evidence indicates that β-endorphin does not function as a precursor to Met-enkephalin (Rossier, 1981). It is quite possible that some of the other peptides to which we referred above are not simple enkephalin precursors but represent messenger molecules in their own right.

[1] We thank the Danish Medical Research Council for grant support and Mrs. B. Blankenberg, J. B. Lauridsen, and Lillian Laursen for technical assistance.

[2] To whom correspondence should be addressed.

So far, only one report has appeared where immunocytochemical differentiation between Leu- and Met-enkephalin immunoreactants has been achieved (Larsson et al., 1979). Using improved immunocytochemical procedures at the light and electron microscopical level, together with model-staining techniques, we now report on the occurrence of three types of enkephalin-immunoreactive nerves in the feline gut, including nerves containing either Leu- or Met-enkephalin immunoreactants as well as nerves containing both types of immunoreactants. The ultrastructural properties of the nerves have been identified in optimally fixed, osmicated, and contrasted ultrathin sections. Our results may suggest either that the proteolytic processing of a common large Leu/Met-enkephalin precursor may differ between individual neurons or that multiple enkephalin precursors or enkephalin-containing peptides occur and show a differentiated distribution in the body.

Materials and Methods

Tissue material

Young and adult cats (body weight, 500 gm to 3 kg) of either sex were anesthetized with chloralose/Mebumal Sodium and were perfused intracardially with saline, followed by fixative. For light microscopy, 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3, was employed and for electron microscopy, a mixture of 2% paraformaldehyde and 3% glutaraldehyde in the same buffer was used. Specimens from the gastrointestinal tract were postfixed in the same fixative for 24 hr (light microscopy) or 30 min (electron microscopy) at 4°C. Specimens for light microscopy then were rinsed in 20% sucrose in 0.1 M sodium phosphate buffer, pH 7.3, overnight and frozen in melting Freon 22 for cryostat sectioning. Specimens for electron microscopy were rinsed in 0.1 M sodium cacodylate buffer, pH 7.3, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812 over propylene oxide (polymerization at 45°C). Epon sections were cut on an LKB Ultrotome III. Adjacent ultrathin sections were cut so that sections 1 and 3 were mounted on glass slides for immunocytochemical staining for either Met- or Leu-enkephalin immunoreactivity, whereas section 2 was mounted on a copper grid and conventionally contrasted (uranyl acetate/lead citrate) for electron microscopical identification of the immunoreactive structures detected in sections 1 and 3. Electron microscopy was done with a JEOL 100C electron microscope. In addition, whole mount preparations from cat gastric muscle wall were prepared. Stomach strips from formaldehyde-perfused cats were postfixed in 4% paraformaldehyde for 24 to 48 hr, dehydrated in graded ethanols and xylene, and rehydrated. The mucosa, submucosa, inner circular muscle layer, and outer longitudinal muscle layer (the latter together with the myenteric plexus) were separated with forceps (Costa et al., 1979). The sheets were stained with enkephalin antisera by procedures described below.

Antisera

The antisera included rabbit antisera against Leu-enkephalin (L_2 and SM) coupled with carbodi-imide or glutaraldehyde to bovine serum albumin or keyhole limpet hemocyanin as well as rabbit antisera to Met-enkephalin (M12, M14, and KA_3) similarly conjugated. The production and evaluation of these antisera have been detailed elsewhere (Childers et al., 1977; Larsson et al., 1979; Larsson and Stengaard-Pedersen, 1981). All antisera were pretreated with an excess of the conjugating protein prior to use. Antisera L_2, M12, and M14 were kindly donated by Drs. S. Childers and S. H. Snyder (Department of Pharmacology and Experimental Therapeutics, Johns Hopkins Medical School, Baltimore, MD).

Antisera characterization

The specificities of the antisera were analyzed in two ways.

Model-staining experiments. Model-staining experiments, including the immobilization of various concentrations of different Met- and Leu-enkephalin congeners on strips of Whatman No. 1 filter papers, were performed as described previously (Larsson, 1981a). The paper models were fixed with paraformaldehyde vapors and were stained immunocytochemically by procedures identical to those used for tissue sections. The peptides immobilized included: synthetic Met- and Leu-enkephalin, β-endorphin, γ-endorphin, β-LPH-(61–69), dynorphin-(1–13), α-neoendorphin, Met-enkephalin-Arg^6-Phe^7, gastrin-17, ACTH-(1–39), ACTH-(1–24), ACTH-(18–39), α-melanocyte-stimulating hormone (α-MSH), and highly purified (99% pure) porcine cholecystokinin-33 (CCK-33). All peptides were obtained from Peninsula Laboratories, San Carlos, CA, except CCK-33, which was a kind gift from Professor V. Mutt (Department of Biochemistry, Karolinska Institutet, Stockholm, Sweden) and synthetic human gastrin-17, which was kindly donated by Dr. J. Morley (ICI, Alderley Park, England).

Absorption controls. Absorption controls, including the pretreatment of the antisera with the above peptides, covalently coupled to Sepharose 4B beads (Larsson et al., 1979), also were used. Such solid phase absorptions were carried out with peptide concentrations up to 200 μg/ml of diluted antiserum.

Immunocytochemical staining

Epon sections were deplasticized in saturated KOH in ethanol, post-treated with 1% periodic acid in distilled water for 7 min, and rinsed in distilled water (details summarized in Larsson, 1981b). Cryostat sections cut at 4 μm, the deplasticized and deosmicated Epon sections, and the paper models were all soaked in Tris-buffered saline (TBS), pH 7.4, containing 1% Triton X-100 for 30 min, exposed to a 1% solution of human serum albumin in TBS for 30 min, and after a brief rinse, exposed to varying dilutions of the antisera for 20 hr at 4°C, followed by 2 hr of re-equilibration at room temperature. The site of the antigen-antibody reaction was revealed either by the peroxidase-antiperoxidase (PAP) method of Sternberger (1979) (in which case, the optimal dilutions of the primary antibodies were: L_2, 1:800; KA_3, 1:1000; M14, 1:800; and SM, 1:600) or by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (SBL, Stockholm, Sweden; optimal dilutions: L_2, 1:100; KA_3, 1:200; M14, 1:200; and SM, 1:200).

Immunofluorescence preparations were examined in a Zeiss standard 18 microscope employing epi-illumination with a xenon XBO 75 lamp and selective interference filters to produce exciting light of 490 nm. PAP preparations were developed either with the diaminobenzidine (DAB) technique (Sternberger, 1979) or, when restaining was desired, with 3-amino-9-ethylcarbazole (Graham et al., 1965).

Immunocytochemical double staining

Two variations of double staining were employed.

Mixed PAP/indirect immunofluorescence (Larsson et al., 1979). The preparations were first stained with one of the antisera using the PAP procedure and development with DAB. Subsequently, antibodies, but not the DAB reaction product, were removed by the elution procedure of Tramu et al. (1978). Following rinsing, the sections were checked for remaining immunoglobulins by application of FITC-labeled anti-rabbit IgG and, if negative, were restained with another primary antiserum and indirect immunofluorescence. This method has been detailed previously (Larsson et al., 1979; Larsson, 1981b). If two immunoreactive peptides are present in different nerves, it produces a very clear picture of the interrelationships of the immunoreactive structures. If, however, there is a total or partial overlap between the two immunoreactive structures, it is essential also to use sequential staining.

Sequential PAP staining. Sections were first stained with one of the primary antisera using the PAP method and development with 3-amino-9-ethylcarbazole (Graham et al., 1965). After photography, antibodies *and* peroxidase reaction product were eluted using the method of Tramu et al. (1978), followed by a dehydration-rehydration sequence in graded ethanols to assure complete removal of all reaction product. Subsequently, the specimens were checked for remaining immunoglobulins by exposure to anti-rabbit IgG and PAP complexes and 3-amino-9-ethylcarbazole development. If negative, they then were restained with another primary antiserum, exposed to the PAP sequence, and developed in 3-amino-9-ethylcarbazole, and the previously photographed areas were rephotographed.

Controls

Controls included both specificity and staining controls (Sternberger, 1979; Larsson, 1981b). The specificity controls were carried out using the peptides listed in Table I covalently coupled to cyanogen bromide-activated Sepharose 4B beads as immunosorbents. Staining controls included the use of sequential deletions of the various antibody layers as well as the substitution of the primary antisera for other unrelated hyperimmune sera (gastrin antiserum 4562 and somatostatin antiserum R213/3) as well as nonimmune and preimmune sera.

Ultrastructural immunocytochemistry

Ultrathin sections were collected on unsupported 200 mesh nickel grids, etched for 5 to 25 min in 10% H_2O_2, pretreated as described for the light microscopical specimens, and exposed to the different antibodies (in dilutions of 1:500 to 1:50,000) for 24 hr at 4°C. The site of the antigen-antibody reaction was revealed either by (*1*) the PAP method of Sternberger (1979), including postosmication but no contrasting; (*2*) the gold-labeled antigen detection (GLAD) method (Larsson, 1979, 1981b), using 12- or 18-nm colloidal gold granules coated with Leu- or Met-enkephalin conjugated to bovine serum albumin by glutaraldehyde or carbodi-imide as described previously (Larsson, 1979, 1981b); or (*3*) the protein A-colloidal gold technique, using protein A-coated 18-nm colloidal gold granules prepared as described by Roth et al. (1978). With the methods employing colloidal gold granules, sections were contrasted in lead citrate and uranyl acetate after immunocytochemical staining.

Results

Susceptibility of enkephalins to oxidation and degradation

Antisera that had been inactivated by the addition of surplus amounts of enkephalins did not stay inactivated when stored at 4°C. Presumably this was caused by slow proteolytic degradation of the peptide by the serum since the "reactivation" of the antisera could be stopped by the addition of the protease inhibitor bacitracin (100 μg/ml of diluted antiserum). Bacitracin alone did not inactivate the antisera even when added in large quantities. We speculated whether the proteases present in the antisera also could degrade enkephalins present in tissue sections and, as previously reported (Larsson and Stengaard-Pedersen, 1981), found that the addition of bacitracin to the antisera used for staining improved the titer of the antisera without in any way qualitatively changing the outcome of the staining. As a consequence, antisera fortified with 100 μg/ml of bacitracin were used. Moreover, absorption (inactivation) controls were always performed with the antigen coupled to a solid support (Sepharose 4B beads) and using bacitracin-pretreated antisera.

The methionine residue of Met-enkephalin is susceptible to oxidation and this has been exploited previously for differentiating between Met- and Leu-enkephalin in radioreceptor assays, radioimmunoassays, and immunocytochemistry (cf., Larsson et al., 1979). Many antisera, like the L_2 antibody used by us, are unable to detect Met-enkephalin when oxidized. Thus, if no oxidation is undertaken, antibody L_2 reacts with both Leu- and Met-enkephalin. However, if Met-enkephalin is preoxidized, antibody L_2 is unable to react with it. We have used the oxidation-L_2 sequence previously for selectively demonstrating Leu-enkephalin-like immunoreactivity (Larsson et al., 1979). The availability of antibody SM, which, when preabsorbed against an excess of Met-enkephalin, detects only Leu-enkephalin immunoreactivity (*vide infra*), has corroborated the applicability of the oxidation-L_2 sequence.

Due attention must be paid to oxidation when tissue sections are stained. Thus, both the elution method employed for removing antibodies, when using double staining techniques, and the hydrogen peroxide or periodic acid treatments of Epon sections may oxidize the methionine residue of Met-enkephalin. Interestingly, in our model-staining experiments, we found that, although both of these pretreatments make Met-enkephalin undetectable by antibody L_2, other enkephalin antisera are

less dependent upon the presence of an unoxidized methionine. Thus, antisera M14 and KA_3 reacted also with oxidized Met-enkephalin. This may be related to the fact that no specific precautions were taken against spontaneous oxidation of Met-enkephalin during the immunization schedule. The animals may, therefore, have been immunized with a mixture of oxidized and unoxidized Met-enkephalin and may have produced antibodies against both derivatives. Alternatively, although these latter antibodies evidently require the presence of a COOH-terminal methionine, they may be unable to differentiate between its oxidized and reduced form.

The susceptibility of Met-enkephalin to oxidation has two important implications for enkephalin immunocytochemistry: (*1*) spontaneous oxidation of Met-enkephalin in tissue sections and cytochemical models must be avoided (e.g., by always staining fresh specimens) and (*2*) the effects of "intentional" oxidation (induced, e.g., by H_2O_2 or by $KMnO_4/H_2SO_4$ pretreatment) on the ability of different antisera to react with different Met-enkephalin congeners must be tested in cytochemical models. Moreover, when double staining and sequential staining of enkephalins are attempted, it is of course essential to use a sequence of antisera so that the necessary oxidative removal of antibodies does not interfere with subsequent enkephalin immunocytochemistry. Although alternative, presumably nonoxidative, procedures for antibody removal exist, these are, according to our experiences, not efficient enough to remove high avidity antibodies (cf., Larsson, 1981b).

Antibody characterization

The results are illustrated in Table I and show that Leu-enkephalin-preabsorbed antiserum M14 detected only Met-enkephalin and not Leu-enkephalin, dynorphin-(1–13), α-neoendorphin, or the COOH-terminally elongated Met-enkephalin congeners, Met-enkephalin-Arg^6-Phe^7, γ-endorphin, β-endorphin, β-LPH-(61–69), or other unrelated peptides. When unabsorbed, the antiserum reacted with both Met- and Leu-enkephalin, dynorphin-(1–13), α-neoendorphin, and Met-enkephalin-Arg^6-Phe^7. Antiserum KA_3, when used unabsorbed, reacted with the same peptides and, in addition, also reacted with α-, β-, and γ-endorphins. When preabsorbed against Leu-enkephalin, this antibody only reacted with Met-enkephalin and the α-, β-, and γ-endorphins. When preabsorbed against dynorphin-(1–13), both antisera M14 and KA_3 were still able to react with Leu-enkephalin and Met-enkephalin but not with the other peptides tested. Antibody SM reacted with both Met- and Leu-enkephalin, dynorphin-(1–13), and α-neoendorphin. After preabsorption against Met-enkephalin, antibody SM reacted exclusively with Leu-enkephalin, dynorphin-(1–13), and α-neoendorphin. Antibody L_2 displayed the same reactivity pattern as antibody SM when used unabsorbed. However, when used after absorption against either Leu-enkephalin, Met-enkephalin, or dynorphin-(1–13), antibody L_2 failed to react with any of the peptides tested. After oxidative pretreatment, as already reported, antibody L_2 failed to react with Met-enkephalin but still reacted with the Leu-enkephalin congeners tested.

These results were reproduced exactly at the level of tissue sections using preoxidation and/or antisera preabsorbed with all peptides listed in Table I. For these reasons, we used Leu-enkephalin-preabsorbed antisera M14 and KA_3 for the detection of Met-enkephalin-like immunoreactivity, dynorphin-preabsorbed antiserum KA_3 for detecting Met- *and* Leu-enkephalin immunoreactivity, without detecting COOH-terminally elongated Met/Leu-enkephalin congeners, and Met-enkephalin-preabsorbed antiserum SM, as well as the oxidation-L_2 sequence for detecting Leu-enkephalin immunoreactivity (including dynorphin- and α-neoendorphin-like immunoreactivity).

Distribution of enkephalin immunoreactants in feline gut

The Met-enkephalin-directed antibodies (Leu-enkephalin-preabsorbed antisera KA_3 and M14) detected more nerve profiles than did the Leu-enkephalin-directed anti-

TABLE I

Immunocytochemical specificities of enkephalin antisera as determined by model-staining experiments and by absorption controls

Peptides	Antisera																
	KA_3[a]	KA_3 + 1[b]	KA_3 + 2	KA_3 + 4	KA_3 + 9	M14[a]	M14 + 1	M14 + 2	M14 + 4	L_2[a]	ox. + L_2[c]	L_2 + 1	L_2 + 2	L_2 + 9	SM[a]	SM + 1	SM + 2
1. Met-enkephalin	+[d]	−	+	+	+	+	−	+	+	+	−	−	−	−	+	−	−
2. Leu-enkephalin	+	−	−	+	+	+	−	−	+	+	+	−	−	−	+	+	−
3. Met-enkephalin-Arg^6-Phe^7	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−
4. β-Endorphin	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5. β-Endorphin-(61–69)	(+)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6. α-Endorphin	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7. γ-Endorphin	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8. α-Neoendorphin	+	−	−	n.t.	−	+	−	−	+	+	+	−	−	−	+	+	−
9. Dynorphin-(1–13)	+	−	−	n.t.	−	+	−	−	+	+	+	−	−	−	+	+	−
10. Others[e]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Unpretreated antisera.

[b] Antiserum designation followed by a number *x* designates antiserum preabsorbed with peptide number *x*. Thus, KA_3 + 1 designates antiserum KA_3 preabsorbed with Met-enkephalin, KA_3 + 2, preabsorbed with Leu-enkephalin, etc.

[c] Oxidation-L_2 sequence.

[d] + denotes a positive reaction, (+) indicates a weak reaction, and − shows an absence of reaction with the peptide tested in the cytochemical model systems. Absorption controls performed on tissue gave equivalent results. n.t., not tested.

[e] Others include ACTH-(1–39), ACTH-(1–24), ACTH-(18–39), α-MSH, CCK-33, and gastrin-17.

bodies (Met-enkephalin-preabsorbed antiserum SM and the oxidation-L_2 sequence, *vide supra*). Although differing in frequency, the Leu- and Met-enkephalin-immunoreactive nerves were distributed very similarly in the cat gut and only one major anatomical discrepancy was noted. This discrepancy involves blood vessels (mainly small sized arteries), which were moderately well supplied with Met-enkephalin-immunoreactive nerve terminals but which contained very few or no Leu-enkephalin-immunoreactive terminals (Fig. 1), indicating an innervation of the blood vessels. Additionally, small transected bundles of both Leu- and Met-enkephalin-immunoreactive nerves were detected in the vascular adventitia, clearly separated from the tunica media. The latter bundles probably represent nerve fibers entering (or leaving) the gut wall. The bundles also contained variable numbers of nonimmunoreactive axons.

Apart from the vascular nerves, the Leu- and Met-enkephalin-immunoreactive nerves showed similar distributions, as they were most numerous in the external muscle coat of all gut regions (Fig. 2). Immunoreactive nerve cell bodies were detected with all antisera in both the myenteric and submucous plexuses. They were much more numerous in the former location and seemed particularly frequent in the upper part of the gut (Fig. 2). Both nervous plexuses also received numerous, beaded enkephalin-immunoreactive terminals. In the small intestines, the inner, circular muscle layer also contained numerous bundles and individual fibers displaying Leu- or Met-enkephalin immunoreactivity, whereas the outer, longitudinal muscle layer contained few such nerves. Also, in all divisions of the stomach and the colon, the external muscle coat and nervous plexuses were richly endowed with enkephalin-immunoreactive nerves (Fig. 3).

Occasional blood vessels traversing the muscle coat carried with them enkephalin-immunoreactive nerves (Figs. 1 and 3). Again, nerves occurring at the zone between the media and adventitia were mainly of the Met-enkephalin-immunoreactive variety, whereas nerves occurring within the adventitia or more loosely associated with the vessels belonged to either variety. At the juxtasubmucosal face of the inner circular muscle layer, enkephalin nerves of both varieties were often very numerous and made up a small plexus one to three muscle cells away from the submucosa.

In the submucosa, both Leu- and Met-enkephalin nerves and cell bodies were less numerous and were associated mainly with the plexus of Meissner. The lamina muscularis mucosae received a moderate number of Leu- and Met-enkephalin nerves. The nerves were very sparse or absent from the lamina propria mucosae. Scattered endocrine-like cells of the gastrointestinal epithelium displayed enkephalin immunoreactivity. These have been described and analyzed in detail previously (Larsson and Stengaard-Pedersen, 1981).

Immunocytochemical double staining

Sections were stained for both the simultaneous and sequential detection of Leu- and Met-enkephalin-immunoreactive nerves. For *simultaneous demonstration*, we stained first for either Leu- or Met-enkephalin using the PAP method and, after antibody elution, for the remaining enkephalin using indirect immunofluorescence (cf., "Materials and Methods"). When staining for Met-enkephalin with the PAP method (using Leu-enkephalin-preabsorbed antisera KA_3 or M14) was followed by immunofluorescence for Leu-enkephalin (using Met-enkephalin-preabsorbed antiserum SM or the oxidation-L_2 sequence), it was possible to detect numerous immunofluorescent nerves (and cell bodies) that were devoid of PAP reaction product. Thus, these nerves were Leu-enkephalin-immunoreactive but had not reacted for Met-enkephalin (in the preceding PAP sequence) (Fig. 4). This result leads us to conclude, in agreement with a previous report (Larsson et al., 1979), that the feline gut contains Leu-enkephalin-immunoreactive nerves that are devoid of detectable Met-enkephalin immunoreactivity. Parallel sections were first stained for Leu-enkephalin (Met-enkephalin-preabsorbed antiserum SM), using the PAP method and subsequently for Met-enkephalin immunoreactivity, using indirect immunofluorescence (Leu-enkephalin-preabsorbed antisera M14 or KA_3). Numerous Met-enkephalin immunofluorescent nerves, devoid of PAP reaction product, were detected (Fig. 5).

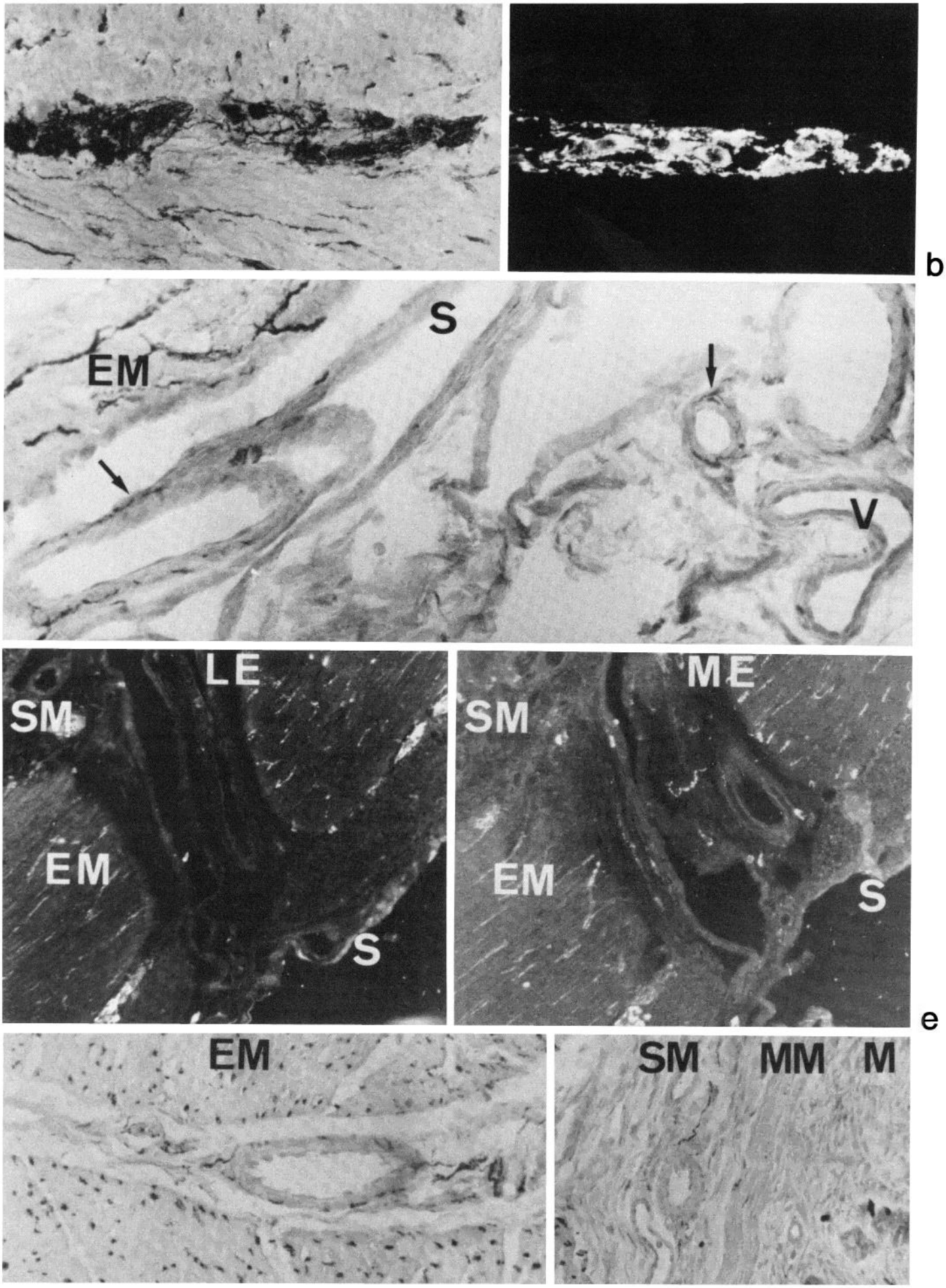
These experiments lead us to conclude that the feline gut contains (*1*) Leu-enkephalin-immunoreactive nerves that are devoid of Met-enkephalin immunoreactivity and (*2*) Met-enkephalin-immunoreactive nerves devoid of Leu-enkephalin immunoreactivity. This staining pattern was detected in both nerve cell bodies and in beaded, terminal-like portions of the axons.

Since the PAP reaction product effectively quenches subsequent immunofluorescence of the same structures (but not of neighboring structures as demonstrated in Figs. 4 and 5), it was quite possible that some nerves that contained both Met- and Leu-enkephalin would escape detection since they would already be PAP positive in the first step of the double staining procedure. Careful quantitative comparisons indicated, in fact, that this could be the case since the total number of exclusively Leu-enkephalin-immunoreactive nerves plus exclusively Met-enkephalin-immunoreactive nerves was lower than the total number of enkephalin-immunoreactive nerves.

We therefore supplemented our double staining data with the use of *sequential staining*. In this procedure, one antigen is first visualized by the PAP procedure and the peroxidase activity is demonstrated by a reagent (3-amino-9-ethylcarbazole), giving an ethanol-soluble reaction product. After photography, both antibodies and reaction product are removed (cf., "Materials and Methods") and the second antigen can be demonstrated without the interference of the pre-existing stain (Fig. 6). Use of this procedure confirmed our above suspicions and documented that three types of enkephalin-immunoreactive nerves occurred in the feline gut: (*1*) exclusively Leu-enkephalin-immunoreactive nerves, (*2*) exclusively Met-enkephalin-immunoreactive nerves, and (*3*) nerves displaying both Leu- and Met-enkephalin immunoreactivity.

Ultrastructural identification of enkephalinergic nerve terminals

In order to be useful for subsequent and independent ultramorphological studies, ultrastructural identification of peptidergic nerve terminals requires the use of opti-


a
b
S
EM
V
c
LE
SM
EM
S
d
ME
SM
EM
S
e
EM
f
SM
MM
M

mally fixed, osmicated, and contrasted specimens. Enkephalin-immunoreactive nerve terminals were identified using several independent techniques.

Adjacent ultrathin section technique. A series of three ultrathin sections were cut. Since the diameter of the enkephalin terminals averaged 0.5 to 2 μm, these sections all contained parts of the same terminals cut in series. The first and last sections were mounted on glass slides and immunocytochemically stained for Leu-enkephalin (using Met-enkephalin-preabsorbed antiserum SM or the oxidation-L_2 sequence) and for Met-enkephalin immunoreactivity (using Leu-enkephalin-preabsorbed antisera M14 or KA_3), respectively. The middle ultrathin section was mounted on a copper grid and conventionally contrasted. The immunoreactive structures in the first and last section were photographed and were all identified in the intervening section in the electron microscope. Figure 7 shows an example of such an experiment. Two of the serially cut nerve terminals show immunofluorescence for both Leu- (section 1) and Met-enkephalin (section 3), whereas the remaining terminals are either Leu- or Met-enkephalin immunoreactive. All terminals can be identified in the electron micrograph of the middle section.

Nerve terminals displaying exclusively Met-enkephalin immunoreactivity contain small agranular ("cholinergic-like") vesicles as well as larger granular vesicles (diameter, 100 to 183 nm). The larger vesicles contain an electron-dense core surrounded by a well defined clear halo and a membrane (Figs. 7 and 8). Nerve terminals displaying exclusively Leu-enkephalin immunoreactivity contain similar small agranular vesicles as well as larger opaque vesicles, with a granular/filamentous content of medium electron density (diameter, 140 to 230 nm). The latter granules are devoid of a wide halo and the mem-

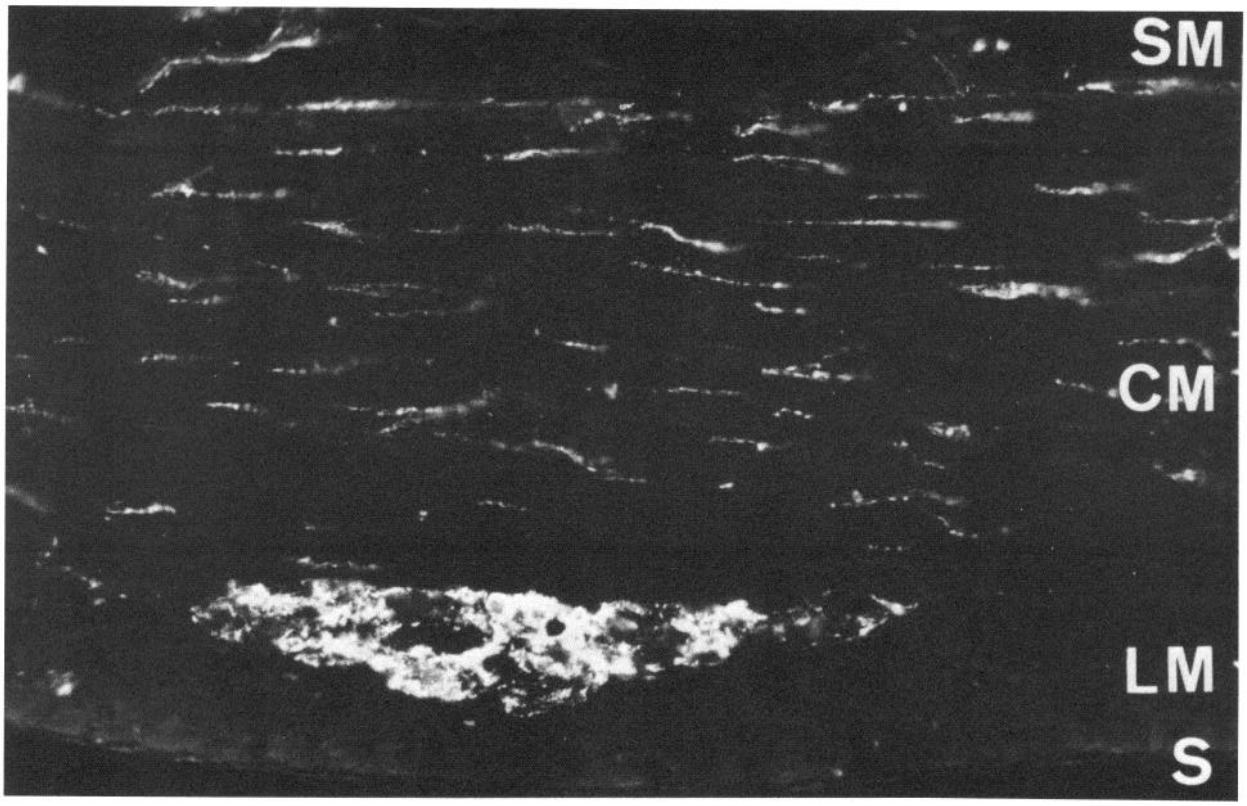


Figure 2. Cat duodenum, immunocytochemically stained for Met- and Leu-enkephalin (unpretreated antiserum KA_3, indirect immunofluorescence). Note the occurrence of numerous terminals and cell bodies in the myenteric plexus and of numerous single fibers and bundles in the circular muscle layer (*CM*). The submucosa (*SM*), longitudinal muscle layer (*LM*), and serosal layer (*S*) contain much fewer enkephalin nerves. Magnification × 310.

Figure 1. Cat antrum (*a*, *c*, and *f*) and duodenum (*b*, *d*, and *e*). Immunocytochemical staining for Met- and Leu-enkephalin immunoreactivity reveals numerous cell bodies and fibers in and around the myenteric plexus of the antrum (*a*; antibody KA_3, PAP technique) and duodenum (*b*; antibody KA_3, indirect immunofluorescence). The vessels in the serosal (*S*) lining of the stomach (*c*) and penetrating the external muscle (*EM*) wall of the stomach (*f*) or duodenum (*d* and *e*) on their way to the submucosa (*SM*) are moderately well supplied with Met-enkephalin-immunoreactive fibers (Leu-enkephalin-preabsorbed antiserum KA_3, PAP technique in *c* and *f*; same antiserum, indirect immunofluorescence in *e*). Leu-enkephalin (*LE*)-immunoreactive fibers are rarely found associated with such vessels (*d*; Met-enkephalin-preabsorbed antiserum SM, indirect immunofluorescence) but elsewhere seem to follow the pattern of the Met-enkephalin (*ME*)-immunoreactive fibers (compare *d* and *e*). Note that the Met-enkephalin-immunoreactive fibers occur at the zone of junction between the vascular adventitia and media (*arrows* in *c*) and that veins (*V*) generally are not innervated. Leu- and Met-enkephalin fibers are more scarce in the submucosa (*SM*) and around the lamina muscularis mucosa (*MM* in *f*). As discussed in the text, scattered endocrine (gastrin) cells, immunoreactive with the KA_3 antiserum occur in the mucosa (*M* in *f*). Magnification: *a* and *b*, × 360; *c*, × 540; *d* and *e*, × 80; *f*, × 200.

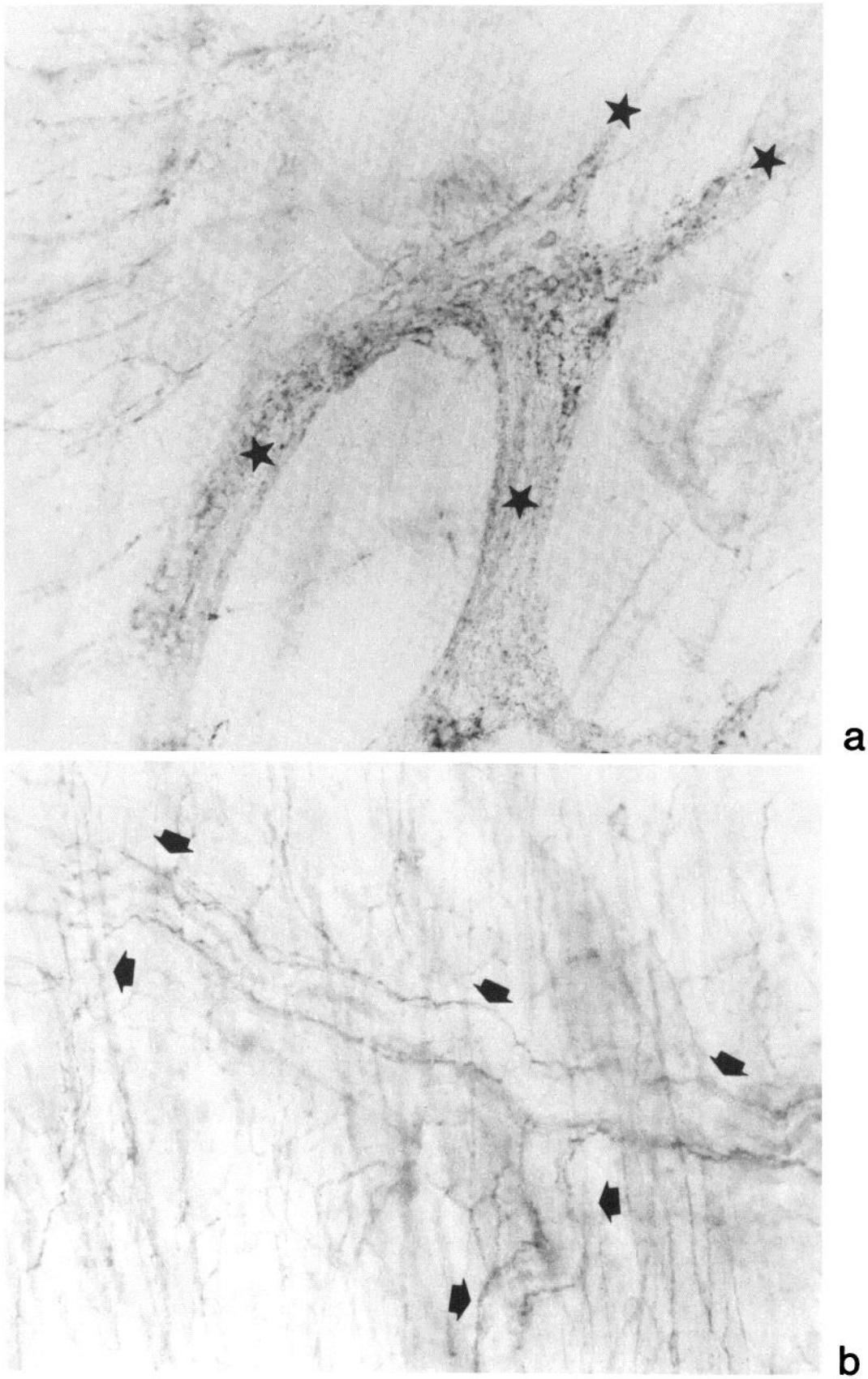


Figure 3. Whole mount preparations of cat stomach myenteric plexus—longitudinal muscle layer (*a*) and inner, circular muscle layer (*b*) stained with Met-enkephalin antibody KA_3 (PAP method). Note the presence of numerous beaded enkephalin nerve terminals in both ganglionic parts and interconnecting strands (*stars*) of the myenteric plexus. The ganglia also contain a fair number of enkephalin-immunoreactive nerve cell bodies. Only a few enkephalin fibers are detected in the external longitudinal muscle layer. In *b*, numerous enkephalin fibers surround a blood vessel (*arrows*) passing through the muscle layer. Note that the inner circular muscle is well supplied with enkephalin fibers running in parallel with the smooth muscle bundles. Magnification: *a*, × 150; *b*, × 120.

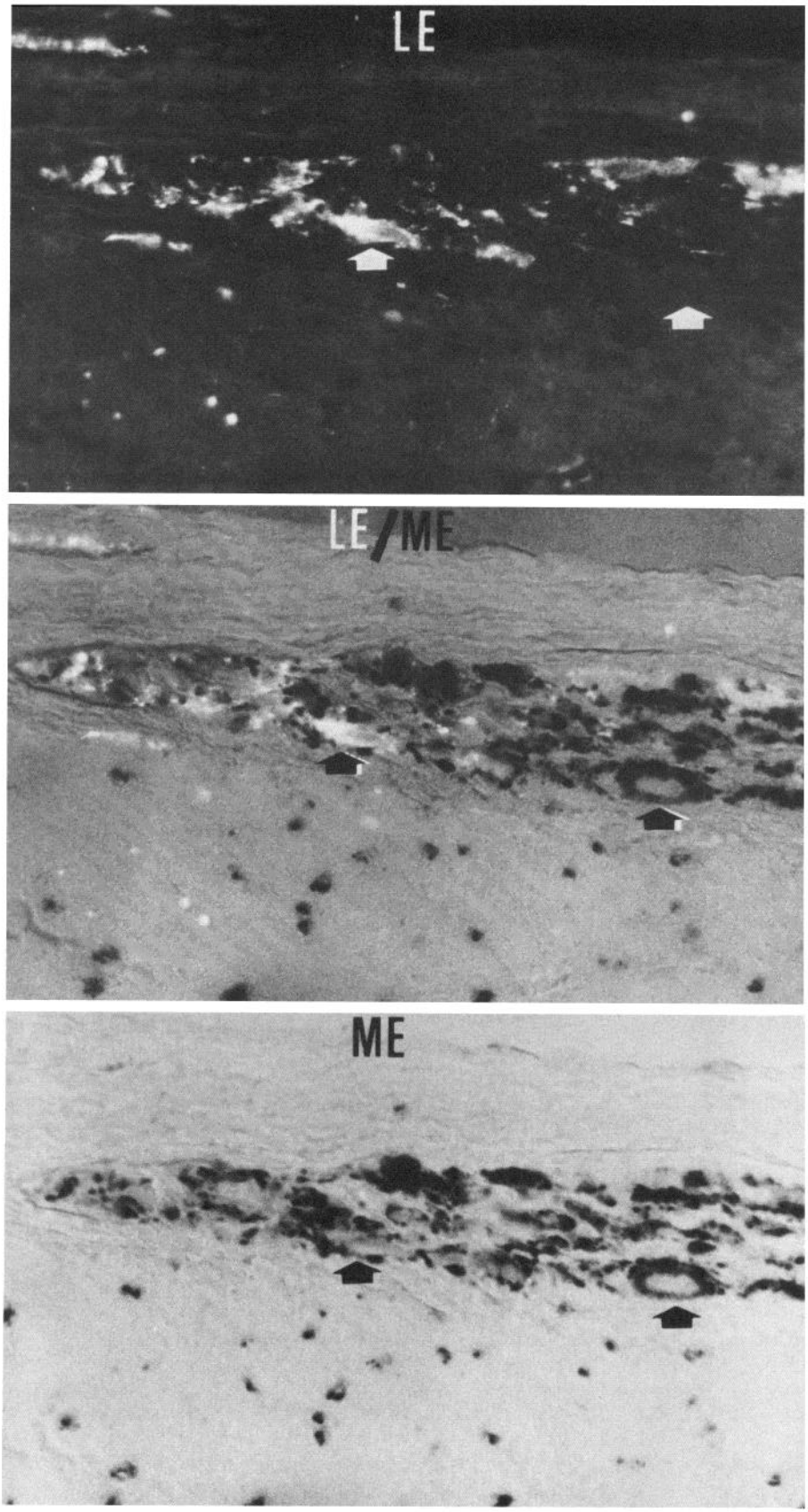


Figure 4. Simultaneous demonstration of Leu-enkephalin (*LE*)- and Met-enkephalin (*ME*)-immunoreactive nerve cell bodies and terminals in the cat corpus-fundus region of the gastric muscle wall. This section was first stained for Met-enkephalin immunoreactivity using Leu-enkephalin-preabsorbed antiserum M14 and the PAP method (*c*). Subsequently, antibodies, but not the peroxidase reaction product, were eluted and the section was stained for Leu-enkephalin using the oxidation-L_2 sequence and indirect immunofluorescence (*a*). In the section photographed in fluorescent light and phase contrast (*b*), it is easy to see that the immunofluorescence and the PAP reaction product reside in separate nerve cell bodies (*arrows*) and terminals. By comparing *a* and *b* with *c*, it is also evident that the Leu-enkephalin immunofluorescent cells and terminals are devoid of PAP reaction product and, hence, of immunocytochemically detectable Met-enkephalin. Thus, from experiments such as this, we conclude that the gut contains Leu-enkephalin-immunoreactive nerves that are not Met-enkephalin immunoreactive. Magnification × 580.

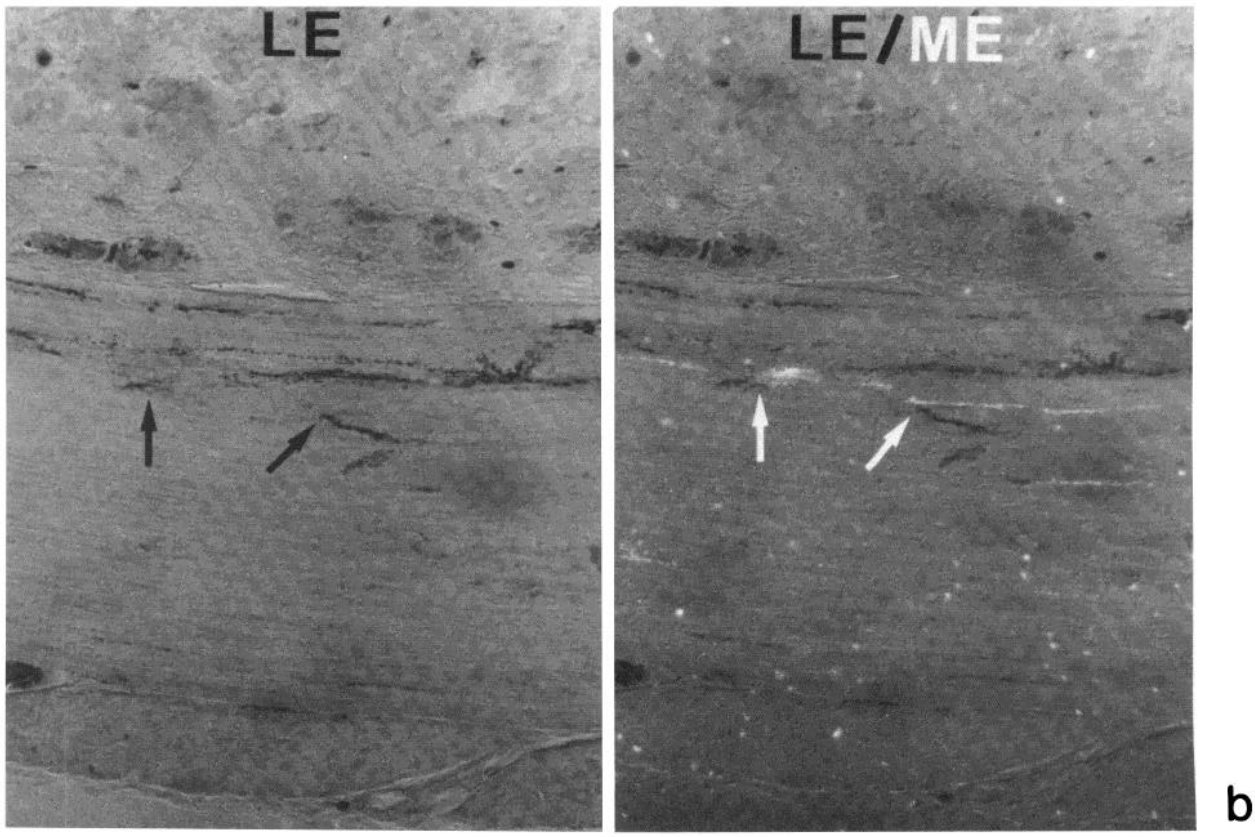


Figure 5. Cat duodenum stained for simultaneous visualization of Leu-enkephalin (*LE*) and Met-enkephalin (*ME*) immunoreactivity by procedures identical to those described in Figure 4. In this experiment, however, we stained first for Leu-enkephalin by the PAP procedure (Met-enkephalin-preabsorbed antiserum SM) and, after antibody elution, for Met-enkephalin using indirect immunofluorescence (Leu-enkephalin-preabsorbed antiserum M14). Note that, in this sequence, which is the reverse of that used in Figure 4, numerous Met-enkephalin immunofluorescent nerve fibers are devoid of PAP reaction product (*arrows*) and, hence, of detectable Leu-enkephalin immunoreactivity. Thus, from results such as this, we conclude that the gut contains Met-enkephalin-immunoreactive fibers that are not Leu-enkephalin immunoreactive. Magnification × 110.

brane is often indistinct and sometimes appears disrupted (Figs. 7 and 8).

Interestingly, the terminals displaying both Leu- and Met-enkephalin immunoreactivity contained a mixture of granular vesicles, resembling those seen in exclusively Met-enkephalin-immunoreactive terminals, and opaque vesicles, resembling those seen in exclusively Leu-enkephalin-immunoreactive terminals. In addition, the latter, "mixed" terminals also contained numerous small agranular vesicles. In all types of terminals, the latter vesicles often occupied a distinct pole of the terminal.

Immunocytochemical staining on grids. We were concerned with sectioning artifacts in the above identification procedures since, apparently, the distribution of the various vesicle subtypes was not random in the terminals. Therefore, our data were substantiated by direct immunocytochemical staining on grids.

Use of the PAP procedure of Sternberger (1979) confirmed the presence of both reactive and unreactive larger vesicles in nerve terminals stained for either Met- or Leu-enkephalin. Unfortunately, the contrast of the specimens was too low to allow ready distinction between the different subtypes of larger (granular/opaque) vesicles (Fig. 8).

Use of colloidal gold techniques (the GLAD procedure as well as the protein A-colloidal gold procedure) allowed, however, contrasting of the immunocytochemically stained specimens and thereby distinction between the vesicle subtypes (Figs. 8 and 9). The results confirmed that the large granular vesicles were associated with Met-enkephalin immunoreactivity and that the large opaque vesicles were associated with Leu-enkephalin immunoreactivity. In terminals containing both large opaque vesicles and large granular vesicles, some labeling for Met-enkephalin also was seen occasionally over the former vesicles. The colloidal gold techniques also permitted further subdivision of the large granular vesicles. Thus, in some of these vesicles, the core was highly electron dense. Such electron-dense granular vesicles varied in their stainability with Met-enkephalin antisera from completely negative to strongly stained. A second subclass of granular vesicles had a core of medium electron density and these vesicles were invariably strongly stained with Met-enkephalin antisera. Occasionally, these vesicles also reacted weakly with Leu-enkephalin antisera. Since such vesicles sometimes were difficult to distinguish clearly from the large opaque vesicles in etched and immunocytochemically stained sections, we are at present hesitant in making a distinct subdivision of Leu- and Met-enkephalin-immunoreactive vesicles on

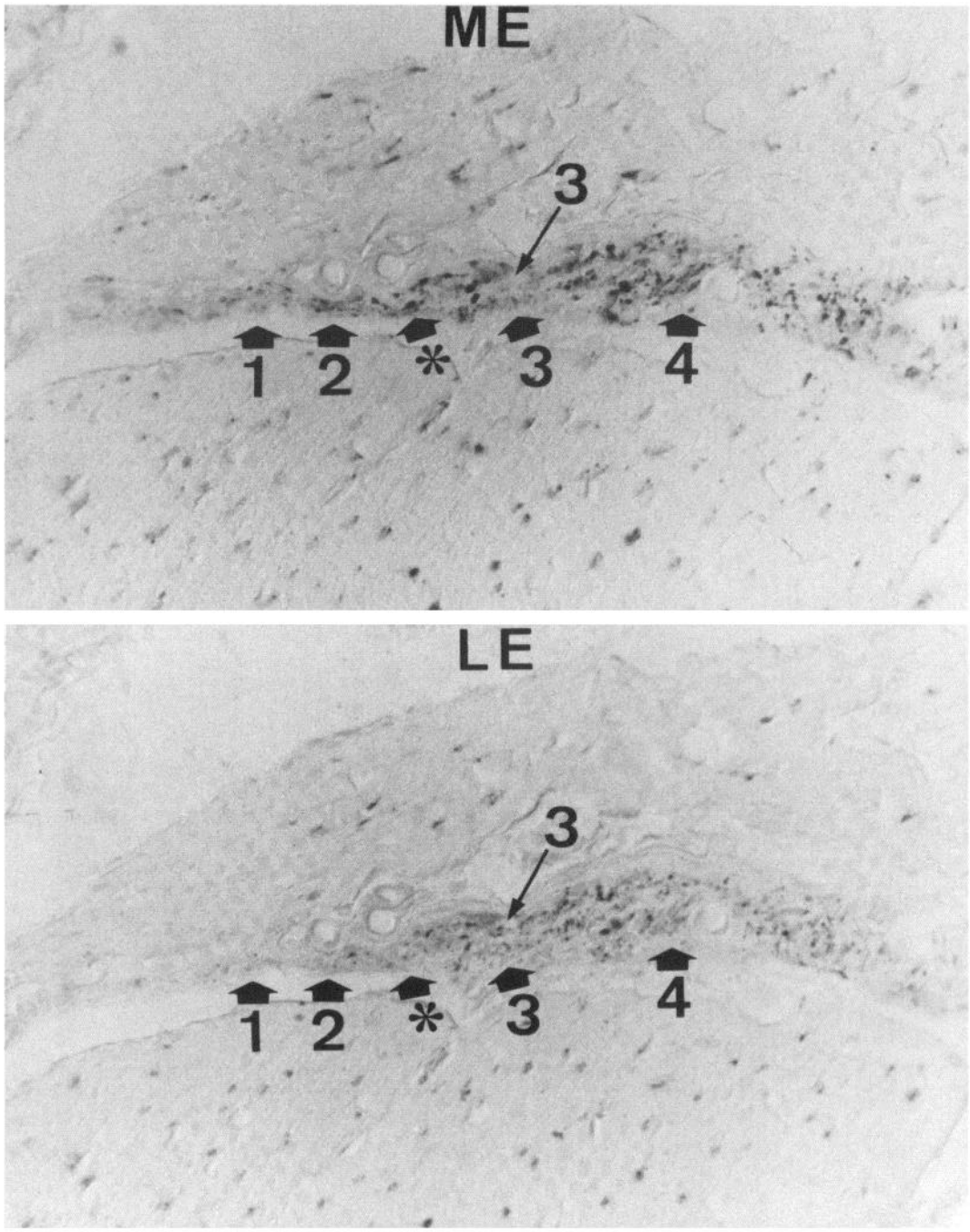


Figure 6. Cat antropyloric region of stomach stained for sequential visualization of Met-enkephalin (*ME*) and Leu-enkephalin (*LE*). The section was first stained for Met-enkephalin (*a*; Leu-enkephalin-preabsorbed antiserum M14, PAP method, 3-amino-9-ethylcarbazole development) and photographed. Subsequently, all antibodies and all peroxidase reaction product were removed, the completeness of the elution was checked as described in the text, and the section was restained for Leu-enkephalin immunoreactivity (*b*; Met-enkephalin-preabsorbed antiserum SM, PAP method). By carefully comparing the two pictures, it can be seen that many nerve profiles contain both Met- and Leu-enkephalin immunoreactivity but also that some regions of the myenteric plexus (such as those indicated with the numbers *1*, *2*, and *4* and the *asterisk*) contain Met-enkephalin-immunoreactive, but Leu-enkephalin-unreactive, terminals and that others (such as the region indicated by *3*) contain terminals that are Leu-enkephalin immunoreactive, but Met-enkephalin unreactive. Results like this allow us to conclude that the feline gut contains at least three types of enkephalin-immunoreactive nerves, including those exclusively Met-enkephalin immunoreactive (as in *areas 1*, *2*, *4*, and *asterisk*), those exclusively Leu-enkephalin immunoreactive (as in *area 3*), and those immunoreactive for both Met- and Leu-enkephalin. The reader may wish to make comparisons in the muscle coat outside of the myenteric plexus, where all three types of terminals also can be seen. Magnification × 350.

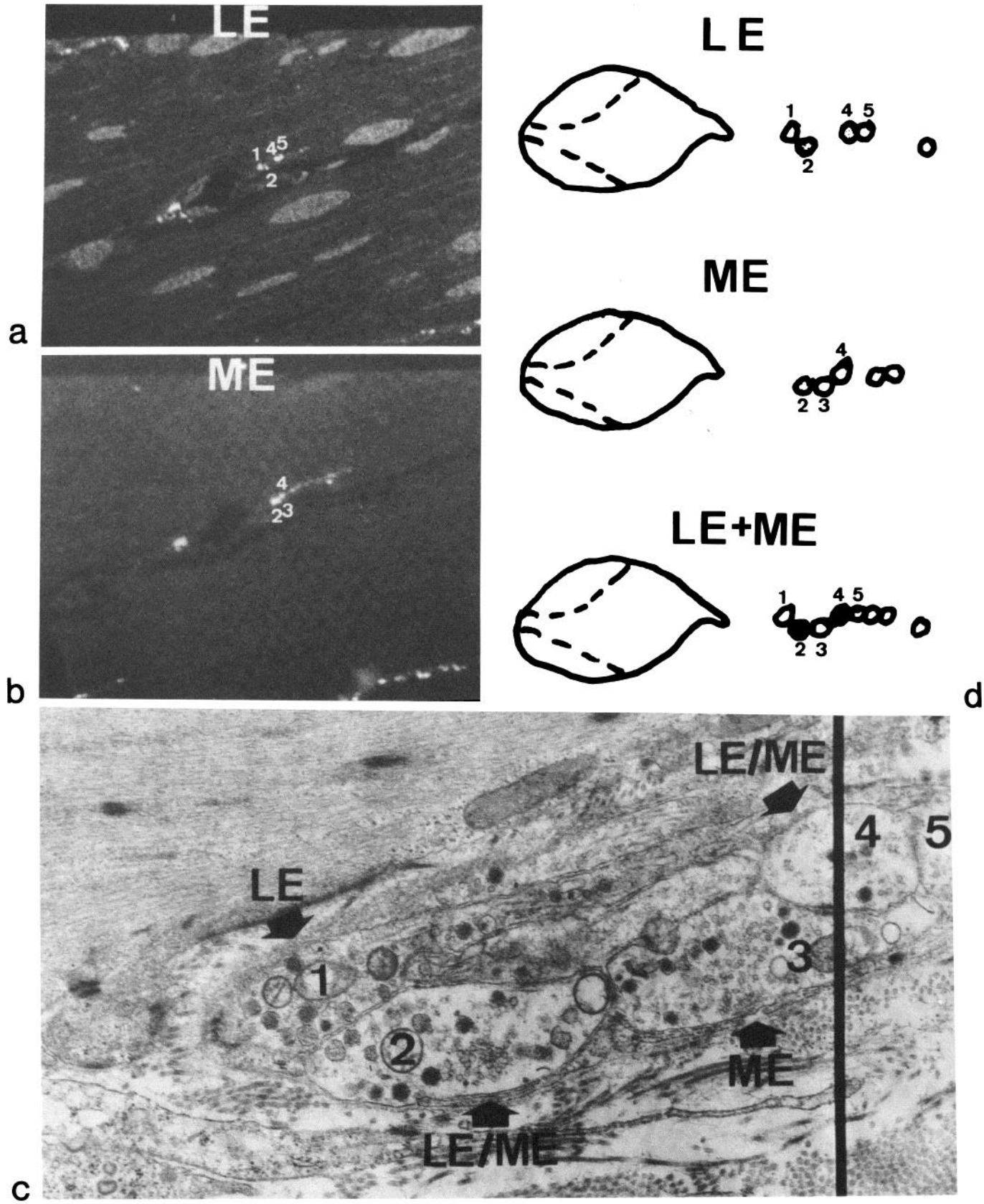


Figure 7. Three adjacent ultrathin sections of cat antropyloric gastric muscle wall. Section 1 (*a*) was stained for Leu-enkephalin (*LE*) immunoreactivity (Met-enkephalin-preabsorbed antiserum SM, indirect immunofluorescence) and section 3 (*b*) was stained for Met-enkephalin (*ME*) immunoreactivity (Leu-enkephalin-preabsorbed antiserum M14, indirect immunofluorescence). The intervening section 2 (*c*) was examined in the electron microscope and the immunofluorescent terminals in sections 1 and 3 were

ultrastructural grounds only (Fig. 9). Labeling for either Met- or Leu-enkephalin was never present over the small, clear vesicles or over other types of terminals. Absorptions of the antisera with the corresponding peptide completely abolished labeling. Occasionally, mixed Met/Leu-enkephalin-immunoreactive terminals were seen to make synaptic contacts with nonimmunoreactive nerve cell bodies of the myenteric plexus (Fig. 9).

To summarize, the above data indicate that exclusively Met-enkephalin-immunoreactive terminals contain small agranular vesicles, devoid of detectable enkephalin immunoreactivity, as well as larger granular vesicles. Exclusively Leu-enkephalin-immunoreactive terminals contain similar small agranular and immunologically unreactive vesicles as well as larger opaque vesicles. Finally, a third type of terminal contains nonimmunoreactive small clear vesicles and both large granular and large opaque vesicles that look similar to those seen in the above described terminals. The granular vesicles seen in these "mixed" terminals seem to be exclusively associated with Met-enkephalin immunoreactivity. With regard to the large opaque vesicles of the "mixed" terminals, our data definitely show the presence of Leu-enkephalin immunoreactivity but do not allow us to exclude the coexistence of Met-enkephalin immunoreactivity. At present, therefore, we caution against overinterpretation of the morphological appearances of these vesicle subtypes. It is presently impossible to exclude the possibility that the different vesicle morphologies may reflect different stages in the processing of granule-bound precursor(s).

Discussion

Our results concerning the distribution of immunoreactive enkephalin nerves in the feline gut agree with previous observations made in other species (Elde et al., 1976; Hughes et al., 1977; Larsson et al., 1979; Linnoila et al., 1978; Polak et al., 1977; Schultzberg et al., 1980). In general, both Leu- and Met-enkephalin-immunoreactive nerves showed similar distributional profiles. Blood vessels supplying the gut, however, were more commonly associated with Met-enkephalin-immunoreactive nerves than with Leu-enkephalin-immunoreactive nerves. The nerves supplying the vessels (mainly small arteries) were terminal-like and beaded and occurred at the zone of junction between the vascular media and adventitia, suggesting true innervation. The occurrence of a possible Met-enkephalinergic innervation of the quantitatively important gastrointestinal vascular bed is of interest in view of the documented effects of enkephalins on blood pressure and hypertension (cf., Di Giulio et al., 1979; Konturek et al., 1978). In the cat, such vascular innervation appeared most abundant in the proximal gut and, so far, has not been seen in extragastrointestinal blood vessels (L. -I. Larsson and K. Stengaard-Pedersen, unpublished observations).

The finding of separate populations of Met- and Leu-enkephalin-immunoreactive nerves confirms a previous report (Larsson et al., 1979) and raises important questions concerning the biosynthetic pathway(s) for the enkephalins. Before embarking upon a discussion of the implications of our finding of Met-, Leu-, and Met/Leu-enkephalin-immunoreactive nerves, it is essential to point out that, as demonstrated in model-staining experiments (Table I), many of the antisera used in this study do not react only with pentapeptide enkephalins but also may recognize COOH-terminally extended enkephalin congeners. Therefore, the immunoreactivity detected is specific for the presence of a Leu- or Met-enkephalin-like sequence but does not allow us to conclude whether the pentapeptides per se or larger (precursor?) forms are detected. Unfortunately, since it has yet to be isolated, the postulated common 30,000- to 50,000-dalton precursor to Met- and Leu-enkephalin (Kimura et al., 1980) cannot be tested for immunological reactivity. However, since enkephalin antibodies previously have been reported to be unable to react with this molecule and since it occurs in low concentrations (Kimura et al., 1980; Rossier, 1981), its contribution to the immunocytochemical staining is less likely.

Our finding of a separate population of Leu-enkephalin-immunoreactive nerves goes along with recent observations that at least two distinct molecules, α-neoendorphin (Kangawa et al., 1979) and dynorphin (Goldstein et al., 1979), contain the Leu-enkephalin sequence. Since the common precursor to Leu- and Met-enkephalin contains only one Leu-enkephalin copy (Kimura et al., 1980; Rossier, 1981), it is evident that at least two different Leu-enkephalin-containing peptides occur independently. Whether these peptides represent precursor forms or messengers in their own right is unknown. Both α-neoendorphin and dynorphin-(1–13) react with the Leu-enkephalin-directed antibodies used in this study, and these molecules therefore may contribute to the staining observed.

Our finding of a population of nerves that simultaneously react with both Met- and Leu-enkephalin antisera seemingly goes along with the finding of a precursor common to the two pentapeptides. Simultaneously, however, we do detect another population of nerves, immunoreactive only with Met-enkephalin antisera. Since the common precursor is believed to contain six or seven copies of Met-enkephalin (Kimura et al., 1980), it is presently difficult to know whether all Met-enkephalin-

identified. As can be seen from the drawing (*d*), made by tracing the contours of neighboring structures and immunoreactive terminals in sections 1 and 3 on transparencies, two of the terminals (*numbers 2* and *4*) are both Leu- and Met-enkephalin immunoreactive, whereas those remaining are either Leu- (*numbers 1* and *5*) or Met-enkephalin immunoreactive (*number 3*). When identified at the ultrastructural level, it can be seen that all immunoreactive terminals contain small agranular ("cholinergic-like") vesicles and that the exclusively Leu-enkephalin-immunoreactive terminal (*number 1*) contains large opaque vesicles of medium electron density, whereas the exclusively Met-enkephalin-immunoreactive terminal (*number 3*) contains more electron-dense vesicles with a clear halo, separating the granular core from the surrounding membrane. The terminals displaying both Met- and Leu-enkephalin immunoreactivity (*numbers 2* and *4*) contain two types of vesicles, morphologically resembling those seen in the exclusively Leu- and Met-enkephalin-immunoreactive terminals, respectively. Magnification × 21,100.

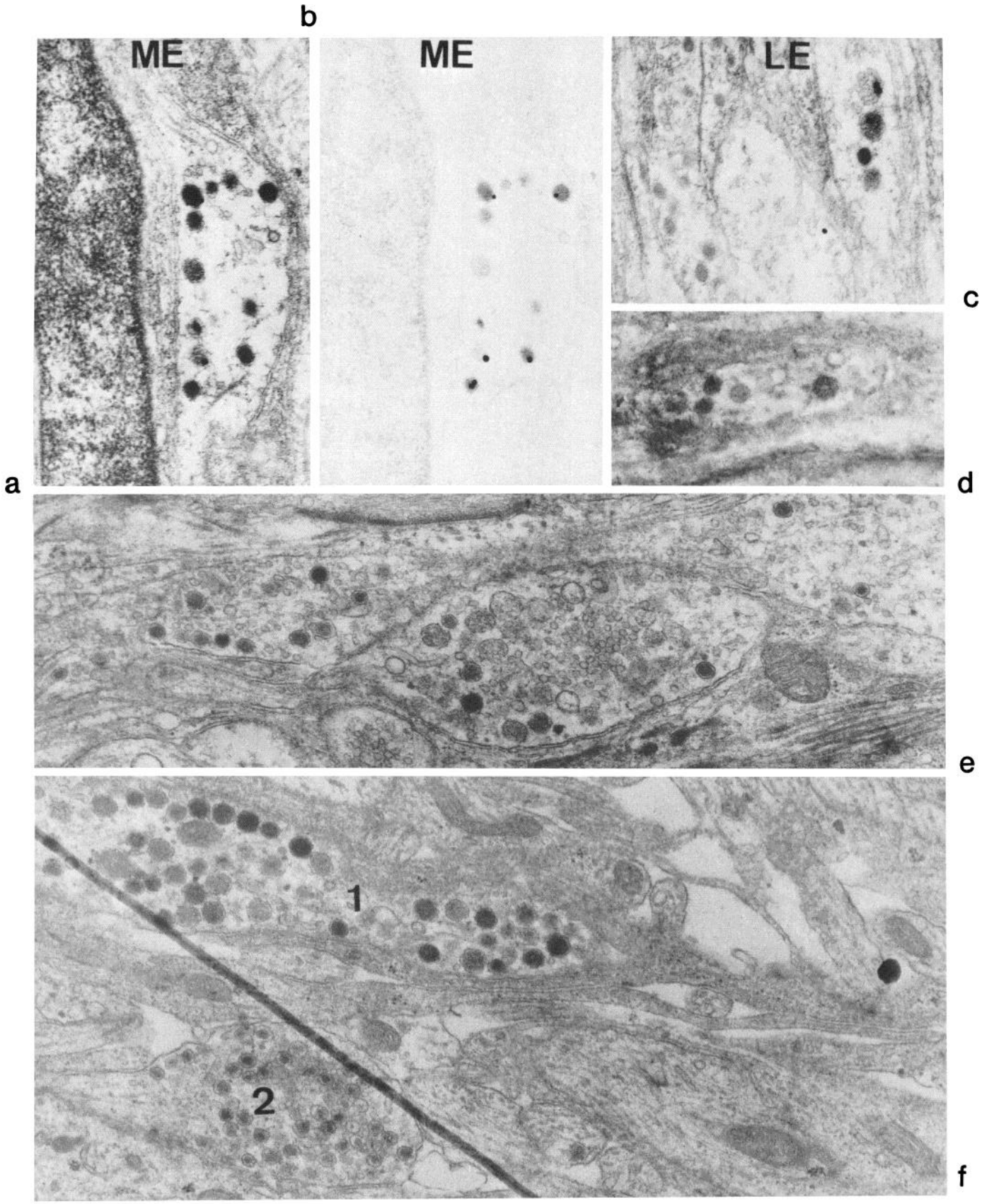


Figure 8. Ultrastructural immunocytochemistry of Met (*ME*)- and Leu-enkephalin (*LE*) in cat duodenum. Ultrathin sections were stained on grids with the gold-labeled antigen detection (GLAD) method for revealing Met-enkephalin immunoreactivity (*a* and *b*). The terminal shown is printed both in an optimally (*a*) and a suboptimally exposed (*b*) version since the granular vesicles in this optimally contrasted section are sufficiently electron dense to mask the gold particles, indicating the site of Met-

containing peptides so far isolated form a part of it. Only β-endorphin can, with some confidence, be believed to be absent from the precursor (Rossier, 1981). The contributions of β-endorphin and its fragments, α- and γ-endorphin, to our staining results with the KA_3 antibody cannot be excluded, but since the M14 antibody, which does not recognize α-, β-, or γ-endorphin, gave quantitatively and qualitatively identical results, the presence of the endorphins is an unlikely explanation of our results.

The occurrence of the three above-mentioned types of enkephalin-immunoreactive nerves may be explained in two ways. Do multiple and independent Met- and Leu-enkephalin congeners (precursors?) exist? This view is, as pointed out above, supported by the discoveries of α-neoendorphin and dynorphin. Alternatively or additionally, does the processing of the common, nonimmunoreactive Met/Leu-enkephalin precursor differ between neurons? This view also can be supported by several arguments. Thus, the amino acid sequence of peptide I contains both the Met- and Leu-enkephalin sequences. The sequence (15–39) of peptide I corresponds to the entire sequence of peptide E, which still contains one copy each of Met- and Leu-enkephalin (Rossier, 1981). Interestingly, however, the 22-amino-acid peptide BAM 22P (Mizuno et al., 1980) corresponds to the sequence (1–22) of peptide E (Rossier, 1981). BAM 22P contains one Met-enkephalin copy but contains only the 2 NH_2-terminal amino acids of the Leu-enkephalin copy present in peptide E (Table II). Therefore, if BAM 22P is formed *in vivo* in some tissues by the putative biosynthetic sequence: peptide I → peptide E → BAM 22P, the single Leu-enkephalin copy present in the common large precursor is automatically destroyed by cleavage (Table II). Only studies on the biosynthesis of these molecules can confirm or refute this view. Presently, therefore, the possibility that a differentiated post-translational processing of a single large common precursor may (partially or totally) explain the differentiated distributions of Met- and Leu-enkephalin cannot be dismissed. Much evidence is beginning to accumulate in other cell systems for a differentiated processing of other precursors to secretory peptides. Thus, in the anterior pituitary, cleavage of the common 31,000-dalton ACTH-β-LPH (pro-opiomelanocortin) precursor proceeds to ACTH-(1–39) and β-LPH, whereas in the intermediate lobe, the same precursor is processed further to α-MSH (*N*-acetylated and amidated ACTH-(1–13)), CLIP (ACTH-(18–39)), and *N*-acetylated β-endorphin (β-LPH-(61–91)) (for references, see Eipper and Mains, 1978). Similarly, the postulated pancreatic glucagon precursor, glicentin, seems to be processed to glucagon in A cells but to exist as a larger molecule in intestinal L cells (see Thim and Moody, 1981) and a differentiated processing of several other hormones/neuropeptides, including gastrin and cholecystokinin, has been postulated (for references, see Larsson, 1980, 1981d). In all instances studied, such selective processing seems to lead to the destruction of one type of biological activity and the creation of another (cf., ACTH-(1–39)/α-MSH, glicentin/glucagon, and CCK-8/CCK-4).

The observations of a differentiated distribution of Met- and Leu-enkephalin in the gut and in the brain (Larsson et al., 1979; K. Stengaard-Pedersen and L. -I. Larsson, manuscript in preparation) goes along with the observations of a differentiated release of the enkephalins (Corbett et al., 1980) and of a differentiated activity of Leu- and Met-enkephalin in at least one biological system (Gibson et al., 1980). The distribution of Met- and Leu-enkephalin-immunoreactive nerve terminals in brain also agrees with the distribution of two types of opiate receptors with different sensitivity to the enkephalins (Goodman et al., 1980).

Our electron microscopical identification of Met-, Leu-, and Met/Leu-enkephalin-immunoreactive terminals also agrees with the above data. Interestingly, Met- and Leu-enkephalin-immunoreactive vesicles, which should be considered to belong to the class of "p-type" vesicles (Baumgarten et al., 1970), have distinct ultrastructural properties, which also allow their identification in preparations not immunocytochemically stained. All three types of terminals also contain small clear vesicles of the type seen in, e.g., cholinergic terminals. The latter vesicles do not seem to be associated with immunoreactive enkephalins. Whether they contain any other transmitter-like molecule is unknown, although it is of interest to note that a co-storage of acetylcholine and enkephalins has been postulated in nerves innervating the adrenal medulla (cf., Kumakura et al., 1980; Schultzberg et al., 1978). These data agree with a report by Pickel (1979) on enkephalin-immunoreactive nerve endings in the brain, which were described as containing both large vesicles and small clear vesicles. Further ultrastructural characterization of the vesicles was not possible in that study due to the use of pre-embedding PAP staining, which obscured a significant part of the vesicle morphology.

Previously, many other neuropeptides, like the vasoactive intestinal polypeptide (Larsson, 1977) and substance P (Pickel et al., 1977), have been ultrastructurally localized to large p-type vesicles. This study, employing optimally fixed, contrasted, and osmicated specimens, to our knowledge, represents the first in which the ultra-

---

enkephalin immunoreactivity (Leu-enkephalin-preabsorbed antiserum M14 and Met-enkephalin-coated gold particles). Note that, in this mixed terminal (*a* and *b*), the gold particles are almost exclusively associated with the large granular ("ME-type") vesicles but that, in addition, weak labeling over one large opaque granule occurs. As discussed in the text, it is presently impossible to determine whether large opaque vesicles in such mixed terminals also contain Met-enkephalin immunoreactivity. In *c* and *d*, Leu-enkephalin immunoreactivity has been localized by the protein A-gold technique (*c*) or by the PAP method (*d*) in mixed terminals. Note in *c* that Leu-enkephalin immunoreactivity appears exclusively associated with the large opaque vesicles. In *d*, PAP reaction product, indicating Leu-enkephalin immunoreactivity, is localized over four out of five vesicles in a terminal. The reactive vesicles have diameters and contours compatible with those of the large opaque vesicles but cannot, in contrast to the case with the gold techniques, be accurately identified due to lack of ultrastructural detail. In *e*, a conventional electron micrograph of mixed terminals, containing both granular and opaque vesicles as well as small agranular vesicles is displayed. In *f*, a mixed terminal (*number 1*) is shown along with a presumptive adrenergic (*number 2*) terminal. Note the difference in size between the granular vesicles of the Met/Leu-enkephalin terminal and the adrenergic terminal. Magnification × 32,000.

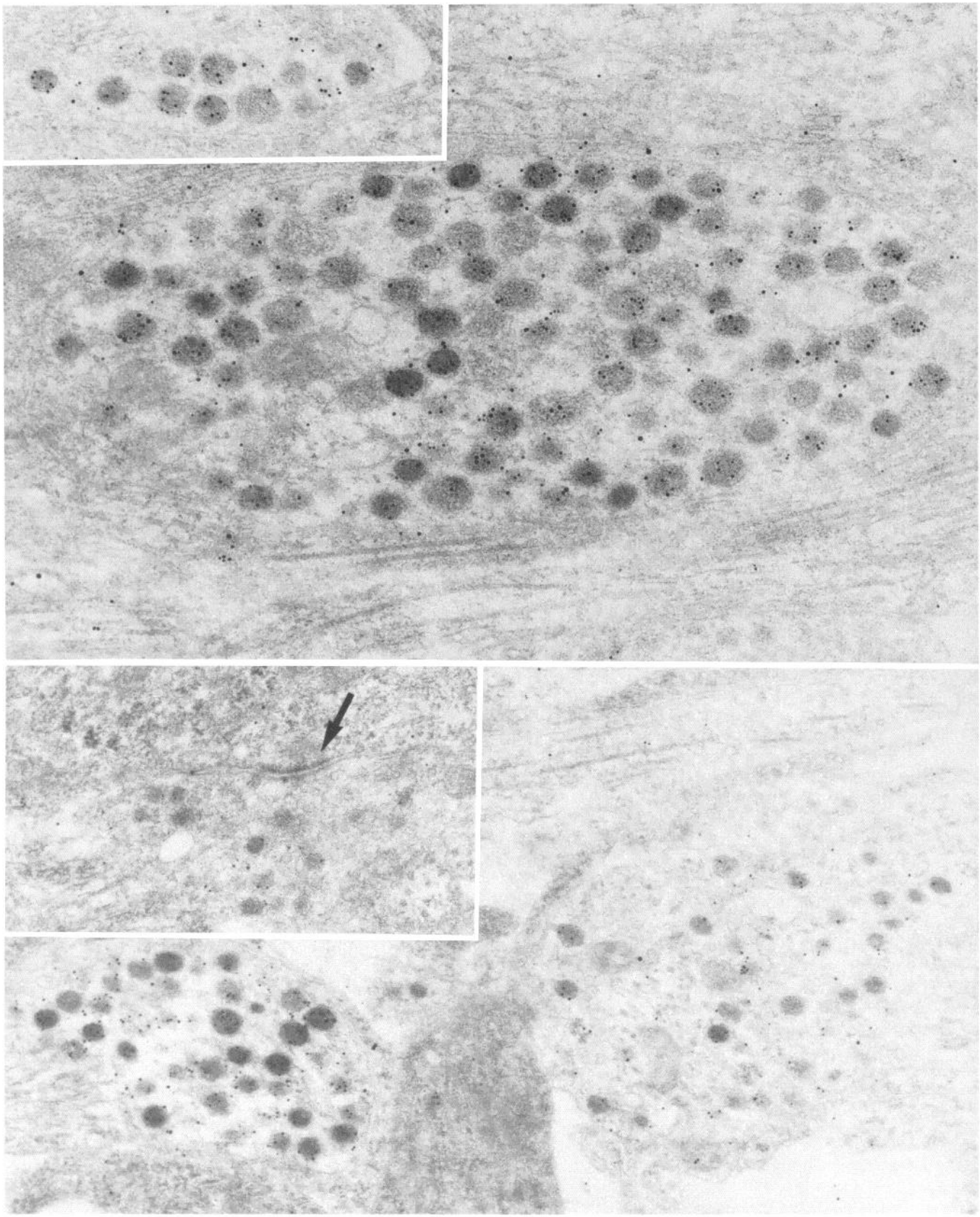


Figure 9. Ultrastructural immunocytochemistry of Met- and Leu-enkephalin in cat stomach. Ultrathin sections were stained on grids using the same procedures as in Figure 8. In *a* is shown a mixed enkephalin terminal stained with an antiserum reacting with both Met- and Leu-enkephalin (L_2). Note the presence of colloidal gold particles, indicating sites of Met/Leu-enkephalin immunoreactivity over all types of vesicles. In the *inset* to *a* is shown a similar mixed terminal stained with Leu-enkephalin/Sepharose-preabsorbed antiserum M14. Note the presence of gold particles, indicating Met-enkephalin immunoreactivity over large granular vesicles and weak or no labeling over large opaque vesicles. Note also that some vesicle subtypes look intermediary between large opaque and large granular vesicles. In the *inset* to *b* is shown a similarly stained mixed terminal, which is making a synaptic contact (*arrow*) with a neuronal cell body of the myenteric plexus. Magnification: *a*, × 44,000; *b*, × 33,000.

TABLE II

Amino acid sequences of some enkephalin congeners

These sequences were collected from Goldstein et al., 1979; Kangawa et al., 1979; Mizuno et al., 1980; and Rossier, 1981.

Peptide I:	Ser-Pro-Thr-Leu-Glu-Asp-Glu-His-Lys-Glu-Leu-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu
Peptide E:	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu
BAM 22P:	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly
Leu-enkephalin:	Tyr-Gly-Gly-Phe-Leu
Met-enkephalin:	Tyr-Gly-Gly-Phe-Met
α-Neoendorphin:	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg--
Dynorphin:	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile--
β-Endorphin:	Tyr-Gly-Gly-Phe-Met-Thr-Ser--

structural details of peptidergic nerve terminals have been analyzed.

Our studies also reveal that different p-type terminals contain different types of vesicles and that the peptide content of these may differ. Previous studies, employing conventional electron microscopy, have emphasized the ultrastructural heterogeneity of gut p-type terminals (Burnstock, 1975; Daikoku et al., 1975; Fehér et al., 1974; Gabella, 1972). We wish to emphasize that our designation of these vesicles as "large granular" and "large opaque" is purely descriptive and does not intend to correlate to the "LGV" and "LOV" vesicles of Burnstock (1975). As indicated by Burnstock (1975), these vesicles may be subdivided into several different subtypes. Finally, our results reveal a considerable heterogeneity between vesicles within peptidergic nerve terminals. This is particularly evident in the "mixed" Met/Leu-enkephalin terminals, which contain both small clear vesicles and large granular and large opaque vesicles. Interestingly, evidence for similar granule heterogeneity in peptide-secreting endocrine cells, not only with respect to morphology but also with respect to peptide content, is beginning to emerge (cf., Larsson, 1981c; Larsson and Stengaard-Pedersen, 1981). Whether these observations reflect a continuous processing of precursors to active, immunoreactive forms or a differentiated packaging of different secretory products in different vesicles or granules is currently unknown.

References

Baumgarten, H. G., A. -F. Holstein, and Ch. Owman (1970) Auerbach's plexus of mammals and man: Electron microscopic identification of three different types of neuronal processes in myenteric ganglia of the large intestine from rhesus monkeys, guinea pigs and man. Z. Zellforsch. Mikrosk. Anat. *106:* 376–397.

Burnstock, G. (1975) Comparative studies of purinergic nerves. J. Exp. Zool. *194:* 103–134.

Childers, S. R., R. Simantov, and S. H. Snyder (1977) Enkephalin: Radioimmunoassay and radioreceptor assay in morphine dependent rats. Eur. J. Pharmacol. *46:* 289–293.

Corbett, A. D., H. W. Kosterlitz, and A. T. McKnight (1980) Differential release of methionine- and leucine-enkephalin. J. Physiol. (Lond.) *308:* 66P.

Costa, M., R. Buffa, J. B. Furness, and E. Solcia (1979) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. Histochemistry *65:* 157–165.

Daikoku, S., C. Ikeuchi, and S. Miki (1975) Electron microscopic studies on developing intramural ganglia of the small intestines in human and rabbit fetuses. Acta Anat. (Basel) *91:* 429–454.

Di Giulio, A. M., H. -Y. T. Yang, W. Fratta, and E. Costa (1979) Decreased content of immunoreactive enkephalin-like peptide in peripheral tissues of spontaneously hypertensive rats. Nature *278:* 646–647.

Eipper, B. A., and R. E. Mains (1978) Existence of a common precursor to ACTH and endorphin in the anterior and intermediate lobes of the rat pituitary. J. Supramol. Struct. *8:* 247–267.

Elde, R., T. Hökfelt, O. Johansson, and L. Terenius (1976) Immunohistochemical studies using antibodies to leucine-enkephalin: Initial observations on the nervous system of the rat. Neuroscience *1:* 349–351.

Fehér, E., K. Csányi, and J. Vajda (1974) Comparative electron microscopic studies on the preterminal and terminal fibres of the nerve plexuses of the small intestines employing different fixation methods. Acta Morphol. Acad. Sci. Hung. *22:* 147–159.

Gabella, G. (1972) Fine structure of the myenteric plexus in the guinea pig ileum. J. Anat. *111:* 69–97.

Gibson, A., S. L. Hart, and A. Shabib (1980) Leucine-enkephalin and methionine-enkephalin produce opposing effects on plasma corticosterone levels in ether-stressed mice. Br. J. Pharmacol. *70:* 509–511.

Goldstein, A., S. Tachibana, L. I. Lowney, M. Hurikapiller, and L. Hood (1979) Dynorphin-(1–13), an extraordinarily potent opioid peptide. Proc. Natl. Acad. Sci. U. S. A. *76:* 6666–6670.

Goodman, R. R., S. H. Snyder, M. J. Kuhar, and W. S. Young (1980) Differentiation of delta and mu opiate receptor localizations by light microscopic autoradiography. Proc. Natl. Acad. Sci. U. S. A. *77:* 6239–6243.

Graham, R. C., Jr., U. Lundholm, and M. J. Karnovsky (1965) Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. J. Histochem. Cytochem. *13:* 150–152.

Hughes, J., H. W. Kosterlitz, and T. W. Smith (1977) The distribution of methionine-enkephalin and leucine-enkephalin in the brain and peripheral tissues. Br. J. Pharmacol. *61:* 639–647.

Jones, B. N., A. S. Stern, R. V. Lewis, S. Kimura, S. Stein, S. Udenfriend, and J. E. Shively (1980) Structure of two adrenal polypeptides containing multiple enkephalin sequences. Arch. Biochem. Biophys. *204:* 392–395.

Kangawa, K., M. Matsuo, and M. Igarashi (1979) α-Neo-endorphin: A "big" leu-enkephalin with potent opiate activity from porcine hypothalami. Biochem. Biophys. Res. Commun. *86:* 153–160.

Kimura, S., R. V. Lewis, A. S. Stern, J. Rossier, S. Stein, and S. Udenfriend (1980) Probable precursors of [Leu]enkephalin and [Met]enkephalin in adrenal medulla: Peptides of 3–5 kilodaltons. Proc. Natl. Acad. Sci. U. S. A. *77:* 1681–1685.

Konturek, S. J., W. Pawlik, K. M. Waluś, D. H. Coy, and A. V. Schally (1978) Methionine-enkephalin stimulates gastric secretion and gastric mucosal blood flow. Proc. Soc. Exp. Biol. Med. *158:* 156–160.

Kumakura, K., A. Guidotti, H. Y. -T. Yang, L. Saiani, and E. Costa (1980) A role for the opiate peptides that presumably coexist with acetylcholine in splanchnic nerves. In *Neural Peptides and Neuronal Communication,* E. Costa and M. Trabucchi, eds., pp. 571–580, Raven Press, New York.

Larsson, L. -I. (1977) Ultrastructural localization of a new neuronal peptide (VIP). Histochemistry *54:* 173–176.

Larsson, L. -I. (1979) Simultaneous ultrastructural demonstration of multiple peptides in endocrine cells by a novel immunocytochemical method. Nature *282:* 743–746.

Larsson, L. -I. (1980) Peptide secretory pathways in GI tract: Cytochemical contributions to regulatory physiology of the gut. Am. J. Physiol. *239:* G237–G246.

Larsson, L. -I. (1981a) A novel immunocytochemical model system for specificity and sensitivity screening of antisera against multiple antigens. J. Histochem. Cytochem. *29:* 408–410.

Larsson, L. -I. (1981b) Peptide immunocytochemistry. Prog. Histochem. Cytochem. *13:* 85.

Larsson, L. -I. (1981c) Adrenocorticotropin-like and α-melanotropin-like peptides in a subpopulation of human gastrin cell granules: Bioassay, immunoassay and immunocytochemical evidence. Proc. Natl. Acad. Sci. U. S. A. *78:* 2990–2996.

Larsson, L. -I. (1981d) Immunocytochemistry of secretory peptides: Immunochemical dissections of peptide diversity. J. Histochem. Cytochem. *29:* 1032–1042.

Larsson, L. -I., and K. Stengaard-Pedersen (1981) Enkephalin/endorphin-related peptides in antropyloric gastrin cells. J. Histochem. Cytochem. *29:* 1088–1098.

Larsson, L. -I., S. Childers, and S. H. Snyder (1979) Met- and leu-enkephalin immunoreactivity in separate neurones. Nature *282:* 407–410.

Linnoila, R. I., R. P. DiAugustine, R. J. Miller, K. J. Chang, and P. Cuatrecasas (1978) An immunohistochemical and radioimmunological study of the distribution of [Met^5]- and [Leu^5]-enkephalin in the gastrointestinal tract. Neuroscience *3:* 1187–1196.

Mizuno, K., N. Minamino, K. Kangawa, and H. Matsuo (1980) A new family of endogenous "big" met-enkephalins from bovine adrenal medulla: Purification and structure of docosa (BAM-22P) and eicosapeptide (BAM-20P) with very potent opiate activity. Biochem. Biophys. Res. Commun. *97:* 1283–1290.

Pickel, V. M. (1979) Immunocytochemical localization of neuronal antigens: Tyrosine hydroxylase, substance P and enkephalin. Fed. Proc. *38:* 2374–2380.

Pickel, V. M., D. M. Reis, and S. E. Leeman (1977) Ultrastructural localization of substance P in neurons of rat spinal cord. Brain Res. *122:* 534–540.

Polak, J. M., S. R. Bloom, S. N. Sullivan, P. Facer, and A. G. E. Pearse (1977) Enkephalin-like immunoreactivity in the human gastrointestinal tract. Lancet *1:* 972–974.

Rossier, J. (1981) Enkephalin biosynthesis: The search for a common precursor. Trends Neurosci. *4:* 94–97.

Roth, J., M. Bendayan, and L. Orci (1978) Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. J. Histochem. Cytochem. *26:* 1074–1081.

Schultzberg, M., J. M. Lundberg, T. Hökfelt, L. Terenius, J. Brandt, R. P. Elde, and M. Goldstein (1978) Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. Neuroscience *3:* 1169–1186.

Schultzberg, M., T. Hökfelt, G. Nilsson, L. Terenius, J. F. Rehfeld, M. Brown, R. Elde, M. Goldstein, and S. I. Said (1980) Distribution of peptide- and catecholamine-containing neurons in the gastrointestinal tract of rat and guinea pig: Immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin, and dopamine-β-hydroxylase. Neuroscience *5:* 689–744.

Sternberger, L. A. (1979) *Immunocytochemistry,* Ed. 2, John Wiley & Sons, New York.

Thim, L., and A. J. Moody (1981) The primary structure of porcine glicentin (proglucagon). Regul. Pept. *2:* 139–150.

Tramu, G., A. Pillez, and J. Leonardelli (1978) An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. J. Histochem. Cytochem. *26:* 322–324.