Co-storage of Adrenaline and Noradrenaline with Met-Enkephalin-Arg\(^6\)-Gly\(^7\)-Leu\(^8\) and Met-Enkephalin-Arg\(^6\)-Phe\(^7\) in Chromaffin Cells of Hamster Adrenal Medulla*

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Summary. The co-existence of catecholamines with opioid peptides in adult hamster adrenal chromaffin cells was studied by Sternberger's peroxidase-antiperoxidase (PAP) method. Paraffin embedded serial sections (3-4 µm thick) of adrenal glands perfusion-fixed with either Bouin's fluid or 4% paraformaldehyde solution were immunostained using anti-adrenaline (AP16-2), -noradrenaline (NAP1-18), -Met-enkephalin-Arg\(^6\)-Gly\(^7\)-Leu\(^8\) (Met-Enk-Arg-Gly-Leu: R-0171, NE2-206) and -Met-enkephalin-Arg\(^6\)-Phe\(^7\) (Met-Enk-Arg-Phe: AP3-311) sera.

1. Selective and specific immunostaining for adrenaline and noradrenaline was obtained in tissues fixed in 4% paraformaldehyde solution. Bouin's fluid gave negative or unsatisfactory results.

2. Immunostaining with two kinds of Met-Enk-Arg-Gly-Leu (R-0171, NE2-206) and one kind of Met-Enk-Arg-Phe sera (AP3-311) was dependent upon the method of fixation used. The immunostaining in the Bouin-fixed tissues was stronger than that in the 4% paraformaldehyde-fixed ones.

3. Secretory granules in chromaffin cells were responsible for both adrenaline- and noradrenaline-like and Met-Enk-Arg-Gly-Leu- and Met-Enk-Arg-Phe-like immunoreactivities.

4. All adrenaline-storing (A) and noradrenaline-storing (NA) cells showed intense Met-Enk-Arg-Gly-Leu- and Met-Enk-Arg-Phe-like immunoreactivities. In the Bouin-fixed adrenal medulla, immunostaining of A cells with the two kinds of anti-Met-Enk-Arg-Gly-Leu sera (R-0171, NE2-206) was deeper than that of the NA cells. Immunostaining of A cells with the anti-Met-Enk-Arg-Phe serum (AP3-311) was apparently of the same intensity as that of NA cells. In 4% paraformaldehyde-fixed tissues, no correlation was seen between the distribution of A and NA cells and that of intensely and slightly Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe immunopositive cells.

The "masking" of immunoreactive residues in chromaffin granules by an aldehyde-noradrenaline complex may be one of the major causes of the decreased opioid peptide-like immunoreactivity in the 4% paraformaldehyde-fixed adrenal medulla.

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During the past six years, many kinds of opioid peptides have been isolated from the adrenal medulla (Stern et al., 1979; Kimura et al., 1980; Kilpatrick et al., 1981). It has been established that all the amino acid sequences of these opioid peptides are contained in a multi-hormone precursor (Noda et al., 1982; Gubler et al., 1982; Comb et al., 1982). This protein, termed preproenkephalin A (Kakidani et al., 1982), contains four copies of Met-enkephalin (Met-Enk) and one copy each of Leu-enkephalin (Leu-Enk), Met-enkephalin-Arg6-Gly7-Leu8 (Met-Enk-Arg-Gly-Leu) and Met-enkephalin-Arg6-Phe7 (Met-Enk-Arg-Phe) (Noda et al., 1982). Although results of biochemical and pharmacological studies have shown that adrenal medullary opioid peptides are co-stored and co-secreted with catecholamines (Viveros et al., 1979, 1980; Hanbauer et al., 1982; Wilson, Chang and Viveros, 1982; see also Carmichael, 1983), it remains unsettled whether they are produced and stored by both A and NA cells or whether there are special kinds of chromaffin cells for their production.

The distribution of noradrenaline-storing (NA) and adrenaline-storing (A) cells has been studied in a wide variety of vertebrate species (Bänder, 1954; Coupland, 1965). Eränkö (1955) first showed that NA cells are localized in the periphery of the adrenal medulla in the hamster. Schultzberg and co-workers (1978a, b), who first established the existence of an opioid peptide-like immunoreactivity in adrenal chromaffin cells, stated that most of them in the cat, a large proportion in the guinea pig, and a few in the rat contained Met-Enk-like immunoreactivity. Later, Linnoila and co-workers (1980) observed that about one-third of the adrenal chromaffin cells in man exhibited Met-Enk- and Leu-Enk-like immunoreactivities. However, all the above authors who investigated the immunocytochemical localization of opioid peptides in the adrenal medulla did not distinguish between A and NA cells, and therefore the cytological background of the co-existence of catecholamines with opioid peptides is still mostly unknown.

Identifying A and NA cells by the "Faglu" method (Furness et al., 1977), Pelto-Huikko, Salminen and Hervonen (1982) reported that, in the hamster, immunofluorescence-cytochemically demonstrable Leu-Enk-like immunoreactivity is restricted to A cells. On the other hand, in the rat, dog and cat, we (Kobayashi et al., 1983a) showed that immunoreactive Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe were not restricted to a particular type of adrenal chromaffin cells. We immunocytochemically identified A and NA cells by a peroxidase-antiperoxidase (PAP) method using anti-adrenaline and noradrenaline sera (Verhofstad et al., 1980, 1983). As compared with the combined "Faglu" and immunofluorescence methods, our methodologies seems more sensitive to and specific for the simultaneous demonstration of catecholamines and opioid peptides. Thus, for elucidation of the co-storage problem of catecholamines with opioid peptides in adrenal A and NA cells, it seems necessary to re-examine the hamster adrenal medulla by the same techniques as that which we used for the previous investigation of this problem in other laboratory animals.

**MATERIALS AND METHODS**

*Antisera*: Antisera for adrenaline and noradrenaline as well as for the Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe used in the present study are listed in Table 1. Data on the characterization of the antisera were published elsewhere (anti-adrenaline serum, AP16-2 and anti-noradrenaline serum, NAP1-18, Verhofstad et al., 1980, 1983; anti-Met-Enk-Arg-Gly-Leu sera, R-0171, Kobayashi et al., 1983b; NE2-206, Ikeda et al., 1982; anti-Met-Enk-Arg-Phe serum, AP3-311, Ikeda et al., 1982).
Animals: The adrenal glands of nine Syrian hamsters of either sex, weighing 100-150 g were used throughout the study (Table 2).

Fixation: a. Paraformaldehyde fixation. The animals were anesthetized with Nembutal (50 mg/kg i.p.) and perfused from the descending thoracic aorta with freshly prepared 4% paraformaldehyde solution buffered at pH 7.4 with 0.1 M phosphate buffer. Perfusion fixation was continued for several minutes. The adrenal glands were then cut in half with a razor blade, removed, carefully dissected free from the surrounding adipose tissue, and immersed in the fixative for 6-20 hrs at room temperature.

b. Bouin fixation. The anesthetized animals were first perfused with Ringer's solution for 1-2 min, and then with Bouin's fluid for several min. The adrenal glands were then isolated and further fixed in Bouin's fluid for 2-4 hrs at room temperature.

Immunocytochemistry: All fixed pieces of the adrenal glands were dehydrated through an ethanol series, treated with xylene, and embedded in paraffin (Merck, m.p. 57-60°C). Three to 4 μm thick serial sections were cut on a microtome and mounted on gelatin-coated glass slides.

Tissue sections were immunostained with the peroxidase-antiperoxidase (PAP) method (STERNBERGER, 1979). Details of the procedure are as follows: 1) After removal of paraffin in xylene and rehydration through a descending series of ethanol, the sections were washed in a phosphate buffered saline (PBS: 0.01 M, pH 7.2, with 0.14 M NaCl); 2) endogeneous peroxidase activity was inhibited by 3% H2O2 for 7 min; and 3) the sections were exposed to non-immune goat (for AP16-2, R-0171, NE2-206 and AP3-311) or rabbit (for NAPI-18) serum (not diluted) for 30 min at room temperature.

4) Then after removing excess non-immune goat or rabbit serum, the sections were

Table 1. Anti-catecholamine and anti-opioid sera used in the present study

| Antiserum to | Code | Working dilution* | Immunized animal | Carrier and coupler |
|-------------|------|------------------|------------------|--------------------|
| Adrenaline  | API6-2| 1 : 1,000        | Rabbit           | Bovine serum albumin by formaldehyde |
| Noradrenaline| NAPI-18| 1 : 4,000       | Sheep            | Bovine serum albumin by formaldehyde |
| Met-Enk-Arg-Gly-Leu | R-0171| 1 : 1,000       | Rabbit           | Ascaris protein by glutaraldehyde |
| Met-Enk-Arg-Phe | AP3-311| 1 : 500        | Rabbit           | Bovine thyroglobulin by carbodiimide |

*Tissue sections were incubated with the antiserum of this dilution

Table 2. Animals used in the present study

| Age (in days) | Sex | Body weight (g) | Fixative |
|--------------|-----|-----------------|----------|
| 68           | M   | 100             | 4% paraformaldehyde solution |
| 68           | M   | 100             | 4% paraformaldehyde solution |
| 68           | F   | 100             | 4% paraformaldehyde solution |
| 68           | F   | 110             | 4% paraformaldehyde solution |
| 123          | F   | 150             | 4% paraformaldehyde solution |
| 68           | F   | 100             | Bouin's fluid |
| 123          | M   | 125             | Bouin's fluid |
| unknown      | M   | 134             | Bouin's fluid |
| unknown      | M   | 125             | Bouin's fluid |
incubated with optimally diluted anti-adrenaline and -noradrenaline or anti-Met-Enk-Arg-Gly-Leu and -Met-Enk-Arg-Phe serum (Table 1) overnight at 4°C. The sections were washed in three changes of PBS; 5) treated with unlabelled goat anti-rabbit (1:200) (for API6-2, R-0171, NE2-206 and AP3-311) or rabbit anti-sheep IgG serum (1:200) (for NAP1-18) for 30 min at room temperature, and again washed in three changes of PBS; 6) applying rabbit (1:200) (for API6-2, R-0171, NE2-206 and AP3-311) or sheep PAP complex (1:200) (for NAP1-18) for 30 min at room temperature. Washing of the sections in three changes of PBS was followed by two changes of 0.05 M Tris•HCl buffer of pH 7.6; 7) finally, immunoreaction was revealed with a freshly prepared solution of 0.02% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H2O2 in 0.05 M Tris•HCl buffer of pH 7.6, and; 8) the sections were dehydrated through an ascending series of ethanol, cleared in xylene, mounted in Entellan (Merck), and examined and photographed with a Vanox AHB-LB microscope (Olympus).

Specificity control: Immunohistochemical reaction specificity was tested using the antisera preincubated overnight at 4°C with 10 µg-1 mg of corresponding catecholamine or 10–100 µg of corresponding opioid peptide per ml diluted antiserum (1:500–1:4,000) before application to the tissue section. Further control experiments were performed by PBS or non-immune rabbit and sheep sera. Also performed as a control experiment was the cytochemical staining by the antisera, preincubated overnight at 4°C not with the antigenic substance itself but with substances related to it (anti-noradrenaline serum preincubated with adrenaline; anti-adrenaline serum preincubated with noradrenaline; anti-Met-Enk-Arg-Gly-Leu sera preincubated with Leu-Enk, Met-Enk or Met-Enk-Arg-Phe; and anti-Met-Enk-Arg-Phe serum preincubated with Leu-Enk, Met-Enk or Met-Enk-Arg-Gly-Leu).

RESULTS

1. Histology
The boundary between the adrenal cortex and medulla in the hamster was distinct, as reported by previous authors (Wood, 1963; Coupland, 1965). Two types of chromaffin cells were distinguishable. The A cells were of a short, simple columnar epithelial type (about 20 µm in height and about 7 µm in width). They were localized in the central portion of adrenal medulla. The NA cells were cuboidal or polygonal in shape (about 20 µm in height and about 14 µm in the base), and were localized in the periphery of the adrenal medulla. In hematoxylin-eosin stained sections, A cells were more basophilic than NA cells.

The follicular arrangement of A and NA cells is shown in Figures 1A, B, 2A, B. Most of these so-called medullary follicles in the central portion of the adrenal medulla consisted of only A cells, while a few with only NA cells were seen in the periphery of this organ (Fig. 1A, B, 2A, B). “Mixed follicles” with both A and NA cells were also demonstrated (Fig. 2C, D). Both the A and NA cells were polarized with a nucleus on the side of follicular lumen and with a majority of secretory granules on the connective tissue front.

Cords, masses or follicles of both A and NA cells were surrounded by a thin layer of connective tissue elements. There were sinusoid capillaries and venules, both of which were lined by thin endothelial cells. In the space between the surface of chromaffin cell groups and vascular endothelial cells, a layer of connective tissue was intercalated. Schwann cells with dark and elongated nuclei were localized between
chromaffin cells (not illustrated). The hamster adrenal medulla also contained a few somata of sympathetic ganglion cells with a large vesicular nucleus (diameter, 10–12 μm) (not illustrated).

The contents of the lumen of the medullary follicles varied greatly. Erythrocytes were frequently seen, as reported by previous authors (GRAUMANN, 1956; Ito, 1958; GRYNSZPAN-WINOGRAD, 1975). In all probability, they were not an artefact due to perfusion fixation. Occasionally the lumen of the medullary follicles was filled with these extravasated erythrocytes. Profiles and/or debris of granulated leukocyte-like and lymphocyte-like cells were also seen. Colloid-like homogeneous masses often filled the follicular lumen.

2. Adrenaline and noradrenaline immunoreactive cells
Selective and specific immunostaining of A and NA cells with anti-adrenaline serum (AP16-2) and anti-noradrenaline serum (NAP1-18) respectively was obtained in the adrenal tissues of all the five hamsters perfusion-fixed with 4% paraformaldehyde solution. Bouin’s fluid gave negative or unsatisfactory results. In all four hamsters fixed in Bouin’s fluid, the background staining was intense, whereas neither A nor NA cells could be discriminated (not illustrated). Therefore, the following descriptions were restricted to the 4% paraformaldehyde fixed adrenal sections.

A cells were immunostained with the anti-adrenaline serum (AP16-2) as shown in Figures 1A, 2A, C, 3A and 6A. They were mainly localized in the central portion of the adrenal medulla. However, some were situated in the periphery of the adrenal medulla (Fig. 1A, 3A), especially in the narrow space between groups of NA cells which were adrenaline non-immunoreactive (Fig. 1A).

NA cells were intensely immunostained with the anti-noradrenaline serum (NAP1-18) (Fig. 1B, 2B, D, 4A, 6B). Groups of NA cells were localized in the peripheral parts of the adrenal medulla. They surrounded, though incompletely, centrally located A cells, which were weakly immunostained with the anti-noradrenaline serum (Fig. 1B). Intense noradrenaline immunoreactive cells and adrenaline non-immunoreactive cells agreed precisely as illustrated in Figures 1A, B and in Figures 6A, B.

Concentrated serum protein-like flocculent materials in the follicular lumen were often stained by anti-adrenaline and -noradrenaline sera; however, this was regarded as a non-specific background staining (vide infra).

Concerning the specificity controls, the preincubation of the anti-adrenaline serum (AP16-2, 1:1,000) with adrenaline (10 μg/ml) overnight at 4°C almost completely removed the specific immunostaining of the tissue section (compare Fig. 3A and Fig. 3B); hence, differentiation of A and NA cells was not possible in the specimen (Fig. 3B). Although both A and NA cells still showed a trace of coloration, this was properly regarded as a kind of background staining. Staining of the colloid-like masses in the follicular lumen remained unchanged after treatment with the anti-adrenaline serum (AP16-2, 1:1,000) with adrenaline (not illustrated). The preincubation of the same anti-adrenaline serum with noradrenaline (100 μg/ml) overnight at 4°C had no demonstrable effects on the specific immunostaining (not illustrated).

The preincubation of the anti-noradrenaline serum (NAP1-18, 1:4,000) with noradrenaline (10 μg/ml) overnight at 4°C greatly decreased the intensity of the specific immunostaining (Fig. 4A, B). Coloration of NA cells was not stronger than that of A cells, so that discrimination of these two kinds of chromaffin cells was impossible (Fig. 4B). The immunostaining of A cells was also significantly reduced (Fig. 4B). However, a trace of coloration on both A and NA cells was unable to be removed (Fig. 4B).
Fig. 1. Legend on the opposite page.
Fig. 2. A-D. Consecutive sections (3 μm thick) of adult Syrian hamster (No. 6) adrenal medulla fixed in 4% paraformaldehyde showing so-called medullary follicles. ×480. A and B. Medullary follicles consisting of A (2) and NA cells (1) respectively. The follicular lumen contains flocculent materials. Their coloration was immunologically non-absorbable, hence non-specific. C and D. A “mixed medullary follicle” consisting of A and NA cells. Asterisks indicate follicular lumen. A and C. Immunostaining using the AP16-2 antiserum (1:1,000). For coloration, the section was treated in DAB solution for 30 min. B and D. Immunostaining using the NAP1-18 antiserum (1:4,000). For coloration, the section was treated in DAB solution for 10 min.

Fig. 1. A and B. Consecutive sections (sectioned at 3 μm) of the adrenal gland of an adult Syrian hamster (No. 6) fixed in 4% paraformaldehyde immunostained for adrenaline (A) and noradrenaline (B). Structures marked by numerals (1-4) are variously-sized medullary follicles consisting of A (2, 3, 4) and NA cells (1) respectively. L lumen of the central vein. ×140. A. The dark adrenaline immunopositive cells represent A cells (A). Non-immunoreactive cells (NA) seen in the periphery of the adrenal medulla are NA cells. Cortical cells (Co) are unstained. Immunostaining was performed using the AP16-2 antiserum (1:1,000). For coloration, the section was treated in a DAB solution for 30 min. B. NA cells intensely immunopositive for noradrenaline, while A cells are weakly immunopositive. Immunostaining was performed using the NAP1-18 antiserum (1:4,000). For coloration, the section was treated in DAB solution for 10 min.
This may be due to non-immunologic background staining, or alternatively, to 1) physicochemical changes in immunogens of chromaffin cells (i.e. pre-existing noradrenaline) during the specimen preparation, and/or 2) gradual decomposition of noradrenaline (added to the diluted anti-noradrenaline serum as a test substance) upon exposure to light and air. Further experiments are required to test the absolute specificity of the immunostaining to noradrenaline. However, it was certain that the intensely-immunostained cells with anti-noradrenaline serum coincided in distribution and structure with the NA cells described by previous authors (Eränkö, 1955). Staining of the flocculent materials in the lumen of the medullary follicles with anti-noradrenaline serum was apparently unaffected by preincubation of the antiserum with noradrenaline (Fig. 4B). The preincubation of the anti-noradrenaline serum (NAPI-18, 1:4,000) with adrenaline (100 µg/ml), Met-Enk-Arg-Gly-Leu (100 µg/ml) and Met-Enk-Arg-Phe (100 µg/ml) overnight at 4°C had no demonstrable effects on the specific immunostaining (not illustrated).

3. Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe immunoreactive cells

*Bouin-fixed tissue:* In the Bouin-fixed adrenal gland, both A and NA cells were identified by cell type distribution patterns previously determined using anti-adrenaline and -noradrenaline serum in the 4% paraformaldehyde-fixed tissues (vide supra). The NA cell cytoplasm contained coarser granules, while A cells possessed finer cytoplasmic...
Immunostaining with all the three antisera against Met-Enk-Arg-Gly-Leu (NE2-206, R-0171) and Met-Enk-Arg-Phe (AP3-311) in the Bouin-fixed tissues was stronger than that in the 4% paraformaldehyde-fixed ones (Fig. 5).

All adrenal chromaffin cells from all four Bouin-fixed hamsters examined in the present study apparently contained both Met-Enk-Arg-Gly-Leu-like and Met-Enk-Arg-Phe-like immunoreactivities. The immunostaining intensity of individual A and NA cell was fairly uniform. The immunostaining of A cells with the two kinds of anti-Met-Enk-Arg-Gly-Leu sera (NE2-206, R-0171) was considerably deeper than that of NA cells (Fig. 5B, D). Immunostaining of both A and NA cells with the anti-Met-Enk-Arg-Phe serum (AP3-311) was distinct and intense (Fig. 5F). No difference in the immunostaining intensity was seen between A and NA cell groups (Fig. 5F).

Paraformaldehyde-fixed tissue: Neither Met-Enk-Arg-Gly-Leu-like nor Met-Enk-Arg-Phe-like immunoreactivities in adrenal glands from all five 4% paraformaldehyde-fixed hamsters was strong. When either the NE2-206 or R-0171 antiserum was used as the primary antiserum, long time treatment in a DAB solution (1–2 hrs) was required.
Fig. 5. Legend on the opposite page.
for the desired depth of immunostaining. Variations in the immunostaining intensity among A and NA cells were demonstrated (Fig. 5A, C, E). However, no correlation was seen between the distribution pattern of A and NA cells and that of cells intensely and/or slightly immunostained with any one of the two anti-Met-Enk-Arg-Gly-Leu (NE2-206, R-0171) and one anti-Met-Enk-Arg-Phe sera (AP3-311).

Specificity controls: The specificity of the immunostaining with the two anti-Met-Enk-Arg-Gly-Leu and one Met-Enk-Arg-Phe sera was examined by an absorption test. In both Bouin-fixed and 4% paraformaldehyde-fixed adrenal glands, no immunostaining of A and NA cells was obtained when the anti-Met-Enk-Arg-Gly-Leu (NE2-206, 1:500; R-0171, 1:1,000) and anti-Met-Enk-Arg-Phe (AP3-311, 1:500) sera were preincubated with 10–100 µg/ml of Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe respectively (not illustrated). Specific immunoreaction was demonstrated using the anti-Met-Enk-Arg-Gly-Leu sera (NE2-206, 1:500; R-0171, 1:1,000) preincubated with Leu-Enk, Met-Enk or Met-Enk-Arg-Phe (100 µg/ml) (not illustrated). The preincubation of the anti-Met-Enk-Arg-Phe serum (AP3-311, 1:500) resulted in no demonstrable effects on the specific immunostaining (not illustrated).

Staining of the flocculent materials in the lumen of the medullary follicles showed no changes after treating the anti-Met-Enk-Arg-Gly-Leu and -Met-Enk-Arg-Phe sera with Met-Ehk-Arg-Gly-Leu and Met-Enk-Arg-Phe (100 µg/ml) respectively (not illustrated).

DISCUSSION

1. Possible functional significance of medullary follicles in hamster adrenal medulla

The adrenal medulla of the hamster differs from that of other experimental animals on several morphological points (Knigge, 1954); hence it has been extensively studied. Such investigations have covered its histology (Graumann, 1956; Wood, 1963; Wood and Barrnett, 1964), fluorescence-histochemistry (Eränkö, 1955; Williams and Morris, 1970), postnatal histogenesis (Ito, 1958), and ultrastructure (Yates, Wood and Duncan, 1962; Yates, 1963; Arnold and Hager, 1967; Grynspan-Winograd, 1975; Benedeczky and Smith, 1972). One of the most characteristic features of the hamster adrenal medulla may be the formation of medullary follicles resembling thyroid follicles (Graumann, 1956; Ito, 1958; Al-Lami, 1970). Based on the results of his light and electron microscopic study, Al-Lami (1970) classified medullary follicles into three types: 1) "adrenaline follicles" consisting of A cells, 2) "noradrenaline follicles" consisting of NA cells, and 3) "mixed follicles." The existence of these three types of medullary follicles was confirmed in the present immunocytochemical study.

Fig. 5. A-F. Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe-like immunoreactivity in both A (A) and NA cells (NA) of adult Syrian hamsters (No. 4, 8). × 360. Met-Enk-Arg-Gly-Leu- and Met-Enk-Arg-Phe-like immunoreactivity in the Bouin-fixed adrenal medulla (B, D and F represent three consecutive sections of 3 µm in thickness) is apparently stronger than that in the 4% paraformaldehyde-fixed tissue (A, C and E represent three consecutive sections of 3 µm in thickness). Follicular lumen indicated by numerals (1, 2) contains erythrocytes. A and B were immunostained using the R-0171 antiserum (1:1,000); C and D, using the NE2-206 antiserum (1:500), and; E and F, using the AP3-311 antiserum (1:500). In the Bouin-fixed adrenal medulla, NA cells possess coarse granular cytoplasm while A cells show a more homogeneous cytoplasm. For coloration, 4% paraformaldehyde-fixed (A, C, E) and Bouin-fixed sections (B, D, F) were treated in the DAB solution for 1 hr and 10 min respectively.
Fig. 6. Legend on the opposite page.
The functional significance of the follicular arrangement of chromaffin cells in the hamster adrenal medulla is not known. GRAUMANN (1956) and Ito (1958) suggested that adrenal chromaffin cells first secrete catecholamines into the follicular lumen rather than into the blood stream. If this is the case, the follicular lumen should contain catecholamines. In the present study, however, materials filling the lumen of the medullary follicles showed no specific immunoreaction for catecholamines nor opioid peptides. The luminal contents were sometimes stained with the anti-catecholamine and -opioid peptide sera, but this apparent immunoreaction proved non-absorbable for catecholamines and/or opioid peptides. Therefore they were different from the granule substances of A and NA cells. The previous discussion by GRAUMANN (1956) and Ito (1958) that the medullary follicles in the hamster are the temporary reservoir of the secretion of adrenal chromaffin cells is unacceptable. AL-LAMI (1970) suggested, as one possible functional meaning, that the follicular lumen stored precursors of medullary secretion and that adrenal chromaffin cells rapidly reabsorbed them when quick synthesis of the secretion was necessary. However, the existence of erythrocytes, granulocytes and lymphocytes in the follicular lumen cannot be explained by this hypothesis. Furthermore, synthetic pathways for catecholamines and opioid peptides deduced from the study in other laboratory animals indicates that macromolecules or colloidal masses in the extracellular space cannot be the immediate precursors of secretory materials (KOBAYASHI, 1977; TRIFARO and POISNER, 1982). Further studies are needed to clarify the significance of the follicular arrangement of chromaffin cells in the hamster adrenal function.

2. Existence of Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe in both A and NA cells
The main purpose of the present study was to immunocytochemically examine the co-storage of catecholamines with opioid peptides within the two distinct types of chromaffin cells, i.e., adrenaline-storing (A) and noradrenaline-storing (NA) cells. It was shown that both A and NA cells in the hamster adrenal medulla contained immunoreactive Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe. This is in conflict with observations of PELTO-HUIKKO, SALMINEN and HERVONEN (1982) that opioid peptide like immunoreactivity is restricted to A cells. To explain this contradiction, the following methodological differences should be considered 1) PELTO-HUIKKO, SALMINEN and HERVONEN (1982) fixed bisected hamster adrenal glands by immersion for 3–4 hrs in 4% paraformaldehyde plus 0.2% glutaraldehyde buffered at pH 7.3 with phosphate buffer. On the other hand, the fixatives used in the present study were devoid of glutaraldehyde; 2) PELTO-HUIKKO, SALMINEN and HERVONEN (1982) used Vibratome sections, whereas the present study was performed using paraffin-embedded sections. The
reason for using paraffin sections in place of Vibratome sections is simply that the former are remarkably thinner than the latter and that the use of the thinner serial sections provides a more reliable demonstration of A and NA cells; 3) Pelto-Huikko, Salminen and Hervonen (1982) used an anti-opioid peptide serum which reacted with both Met-Enk and Leu-Enk. On the other hand, the anti-opioid peptide sera used in this study detects C-terminal portions of either Met-Enk-Arg-Gly-Leu or Met-Enk-Arg-Phe (Yanaihara et al., see Kobayashi et al., 1983b; Ikeda et al., 1982).

In a recent letter (personal communication to S.K.) Hervonen informed us that he was again unable to demonstrate opioid peptide-like immunoreactivity in NA cells of the hamster adrenal medulla. Hervonen and the authors hold the same opinion that differences in antibody specificity may be one of the major causes for the discrepancy in the occurrence of immunoreactive opioid peptides in NA cells. The results of the present study that the immunostaining in the Bouin-fixed adrenal medulla with the two kinds of anti-Met-Enk-Arg-Gly-Leu sera (R-0171, NE2-206) was deeper in A cells than in NA cells provides additional support for the idea that the antibody specificity is critical in the differential immunostaining of A and NA cells.

In the immunocytochemistry of the fixed tissue sections, information is obtained about the substance in their intracellular storage forms which are probably denaturat-ed by fixation, dehydration, embedding and sectioning procedures. Doubtless the immunoreactivity for a special substance is not identical to this substance in an isolated and pure form. Thus the results of the present study, as compared with those of Pelto-Huikko, Salminen and Hervonen (1982), indicate that the only possible way to interpret the nature of opioid peptide-like immunoreactivity in the hamster adrenal medulla is in terms of their immunostaining with the well-characterized anti-opioid peptide sera used under rigorously defined conditions.

At any rate, in the present study all the specificity controls performed showed that the immunostaining with anti-Met-Enk-Arg-Gly-Leu and -Met-Enk-Arg-Phe sera was specific to Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe. Thus the results of the present immunocytochemical study only suggest that both the A and NA cells of the hamster adrenal medulla probably possess Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe.

3. "Masking" of cytochemically demonstrable Met-Enk-Arg-Gly-Leu- and Met-Enk-Arg-Phe-like immunoreactivity

It has long been known that adrenal chromaffin cells contain cytoplasmic substances which are readily stained by basic dyes. These substances are empirically called basophilia. Chemical analysis of a fraction of chromaffin granules indicated that the main component of the basophilia in adrenal chromaffin cells is a kind of acidic protein called chromogranin A (possibly related to proenkephalin A; see Fischer-Colbrie et al., 1982) which exists in association with several different components such as catecholamines and adenine nucleotides. It was shown that, after fixation in aldehyde-containing fixa-tives, the A cells cytoplasm is more basophilic than that of NA cells.

Hopwood (1967, 1971) extensively studied the effect of fixation on the basophilia in adrenal chromaffin cells of various species, and pointed out that noradrenaline formed precipitates with the aldehyde fixative, whereas adrenaline did not give precipitates with it. He hypothesized that the aldehyde-noradrenaline complex "masks" basophilic residues of the proteinic components of chromaffin granules and thus selectively inhibits the staining of basophilia in NA cells.

The results of the present study showed that both Met-Enk-Arg-Gly-Leu-like and
Met-Enk-Arg-Phe-like immunoreactivities were apparently stronger in the Bouin-fixed adrenal medulla than in the 4% paraformaldehyde-fixed tissue and that both adrenaline-like and noradrenaline-like immunoreactivities were demonstrable in 4% paraformaldehyde-fixed, but not in Bouin-fixed tissues. Although the mechanism of basophilic staining with basic dyes is surely different from that of immunostaining for Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe with specific antisera, it seems likely that aldehyde-nor adrenaline complex inhibits the development of opioid peptide-like immunoreactivities in both A and NA cells. The occurrence of a considerable amount of noradrenaline-like immunoreactivity in A cells may account for the decreased Met-Enk-Arg-Gly-Leu- and Met-Enk-Arg-Phe-like immunoreactivities of A cells in 4% paraformaldehyde fixed tissue sections. On the other hand, a strong immunoreaction for Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe in the Bouin-fixed adrenal medulla is considered to be due to the liberation of Met-Enk-Arg-Gly-Leu and Met-Enk-Arg-Phe immunoreactive sites by Bouin's fluid-induced removal of chatecholamines from the coagulated chromaffin granule contents. However, experimental verification is needed to decide whether formaldehyde in Bouin's fluid does make an aldehyde-catecholamine complex.

According to calculations by WINKLER and CARMICHAEL (1982), 64 molecules of "enkephalin-like immunoreactivity (Met-Enk and Leu-Enk)" are co-stored in adrenal chromaffin granules with 1 dopamine-β-hydroxylase, 36 chromogranin A, 22,000 catecholamine, 4,900 ATP, 700 ADP, 210 AMP, 650 GTP, 370 UTP, 660 calcium, 190 magnesium, 880 ascorbic acid, 430 sialic acid, 250 sulfated hexosamine and 250 glucuronic acid molecules. These substances are condensed and form a storage complex within the chromaffin granules. Basic dyes and anti-Met-Enk-Arg-Gly-Leu and -Met-Enk-Arg-Phe sera histochemically and immunohistochemically conjugate with basophilic substances and immunoreactive sites respectively in this storage complex. Inhibition of the development of Met-Enk-Arg-Gly-Leu- and Met-Enk-Arg-Phe-like immunoreactivity may be due not only to co-stored proteinic substances and catecholamines but also to adenine nucleotides, metallic ions and carbohydrates. These substances, after fixation and other procedures of specimen preparation, may block the interaction between antigens and antibodies, and decrease immunostaining ability. Permeability to antibodies of the fixed chromaffin granules is believed to be an important factor which determines the immunoreaction intensity. If Bouin-fixed chromaffin granules are more permeable than 4% paraformaldehyde-fixed granules, a stronger immunoreaction is expected in Bouin-fixed tissues. Our preliminary electron microscopic study showed that both Bouin- and 4% paraformaldehyde-fixed chromaffin cells contained, in addition to swollen chromaffin granules, a considerable number of solid-looking ones (unpublished observation). It is likely that the free penetration of antibodies into these solid-looking granules is stopped at their periphery. Electron microscopic immunocytochemistry using a pre-embedding method (STERNBERGER, 1979) may be useful to test this hypothesis.

The present results that immunostaining of A cells for Met-Enk-Arg-Gly-Leu was stronger than that of NA cells in Bouin-fixed adrenal medulla may also be explained by possible differences in the permeability between fixed A cell granules and fixed NA cell granules. In this case, however, the present results fail to sufficiently explain how both A and NA cells in Bouin-fixed adrenal gland were of the same immunoreaction intensity against the anti-Met-Enk-Arg-Phe serum (AP3-311).
4. Species variation concerning cytochemically demonstrable opioid peptides

A remarkable species variation concerning the immunocytochemically demonstrable opioid peptide-like immunoreactivity in the adrenal medulla has been reported (SCHULTZBERG et al., 1978a, b; KOBAYASHI et al., 1983a, b). Results of radioimmunoassay studies indicated that relative amounts of opioid peptides and catecholamines differ from species to species (VIVEROS et al., 1979, 1980). It may be assumed that similar species variation takes place concerning relative amounts of opioid peptides and other granule components such as adenine nucleotides, metallic ions and carbohydrates. Thus the species variation concerning the cytochemically demonstrable opioid peptide-like immunoreactivity in the adrenal medulla may be ascribed to a possible interspecies variation in the molecular structure of opioid peptides on the one hand, and a species difference in the molecular organization of co-existing granule contents on the other.

Adrenal chromaffin cells are typical paraneurons (KOBAYASHI, 1977). Since adrenal medulla contains such a large amount of catecholamines and opioid peptides, the detailed study of each individual species using various fixatives and well-characterized anti-opioid peptide sera will prove useful in the elucidation of the biological significance of the co-existence of neurotransmitters with other substances in the whole neuroendocrine system.

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