*J. Anat.* (1989), **166**, pp. 213–225.
*With 2 figures*
*Printed in Great Britain*



# Localisation of chromogranin A and B, met-enkephalin-arg$^6$-gly$^7$-leu$^8$ and PGP9.5-like immunoreactivity in the developing and adult rat adrenal medulla and extra-adrenal chromaffin tissue

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(*Accepted* 28 *February* 1989)

## INTRODUCTION

Chromaffin tissue is widely distributed in vertebrates and is found in close association with parts of the peripheral sympathetic nervous system as well as in the mammalian adrenal medulla (for reviews, see Coupland 1965, 1980). Prior to birth the extra-adrenal chromaffin tissue is often precocious in development. Though only sparsely innervated or even non-innervated (as in the rabbit, Coupland & Weakley, 1968, 1970), extra-adrenal chromaffin tissue responds to severe hypoxia by the release of catecholamines (Brundin, 1966; Hervonen & Korkala, 1972). Adrenal medullary chromaffin tissue is, however, relatively slow to develop and mature in mammals.

In the newborn calf electric splanchnic nerve stimulation results in adrenomedullary release of catecholamines; however, the response is significantly less than that of 1 or 2 days old animals (Edwards, 1984); nevertheless, the proportions of adrenaline (A) and noradrenaline (NA) released are the same as in 2–5 weeks old animals. In rats, using insulin hypoglycaemia to induce neurogenic release of catecholamines from the adrenal medulla, it has been shown that functional innervation of the gland is not achieved before the end of the first week after birth (Bareis & Slotkin, 1978; Lau & Slotkin, 1979; Slotkin, Smith, Lau & Bareis, 1980; Slotkin 1986; Kirby & McCarty, 1987) and only becomes fully developed towards the end of the second postnatal week.

The maturation process relating to the innervation of the adrenal medulla is also reflected in the development and differentiation of chromaffin cells within the adrenal medulla and extra-adrenal chromaffin tissue as evidenced by ultrastructural appearance (Elfvin, 1967; Coupland & Weakley, 1968; El-Maghraby & Lever, 1980; Tomlinson & Coupland, unpublished) and the presence of catecholamines and enzymes involved in catecholamine synthesis in chromaffin cells (Verhofstad, Coupland, Parker & Goldstein, 1985). Furthermore there appears to be a delay in the biochemical maturation of catecholamine-storing vesicles during the same period (Slotkin, 1973, 1975).

In the current report a study has been made of the development of immunoreactivity to several peptide molecules known to occur in high concentrations and with specific distributions in the adult adrenal medulla and which may form useful markers for the process of the functional maturation of adrenal chromaffin tissues. These include chromogranins A and B, met-enkephalin and protein gene product 9.5 (PGP 9.5).

Over 70% of proteins in chromaffin granules are soluble and these were collectively named the chromogranins by Blaschko *et al.* (1967). Subsequently these soluble proteins have been shown to contain substantial proportions of the granule content of

dopamine-B-hydroxylase, enkephalin precursors and glycoprotein III as well as major acidic proteins rich in glutamic acid and usually referred to as chromogranins A, B and C (Winkler, 1976; Winkler, Apps & Fischer-Colbrie, 1986). Chromogranin A is the major component present in bovine chromaffin granules while chromogranin B is the predominant form in rat and human chromaffin granules. All are relatively widely distributed in endocrine and nervous tissue.

It is now apparent that at certain times and under particular conditions the synthesis and storage of opioid peptides is a characteristic feature of the adrenal medulla. Opioid peptides are co-stored with catecholamines in most species of adrenal examined (Schultzberg *et al.* 1978; Linnoila, Diaugustine, Hervonen & Miller, 1980; Kobayashi *et al.* 1983*a*) and are co-secreted with catecholamines (Viveros, Diliberto, Hazum & Chang, 1979). In addition nerve fibres immunoreactive to enkephalins have been described as being associated with chromaffin cells (Schultzberg *et al.* 1978; Kobayashi, Ohashi, Uchida & Yanaihara, 1983*c*; Pelto-Huikko, Salminen & Hervonen, 1985). The opioid peptide used in the present work was the octapeptide met-enkephalin-arg$^6$-gly$^7$-leu$^8$ (met-enk 8), an endogenous opioid peptide in bovine adrenal medulla (Kilpatrick, Jones, Kojima & Udenfriend, 1981) that comprises an amino acid sequence specifically found in the middle of the preproenkephalin A molecule (Noda *et al.* 1982). Hence the occurrence of met-enk 8 immunoreactivity suggests the presence of either preproenkephalin A or one of its derivatives.

Protein gene product 9.5 (PGP 9.5) is a soluble protein isolated from human brain. Studies on a wide range of species indicate that it represents a major protein component of neuronal cytoplasm (Jackson, Thompson & Thompson, 1985) and although its function is unknown, it has been proposed as a general marker for both neuronal and neuroendocrine tissue (Thompson *et al.* 1983; Gulbenkian, Wharton & Polak, 1987).

In this present work we have compared the distribution of chromogranin-like, met-enk 8-like and PGP 9.5 immunoreactivity in the developing adrenal medulla and extra-adrenal chromaffin tissue of rats from prenatal Day 16 to postnatal Day 15 and in adult animals.

## MATERIALS AND METHODS

The Wistar rats used were bred in the Nottingham University Medical School Animal Unit and maintained under standard conditions of temperature and light, 12 hours light: 12 hours dark. Food (Pilsbury diet 41B modified) and water were freely available. The day of the appearance of a cervical mucous plug was designated Day 0 of pregnancy. Tissues were obtained from fetuses on the 16th (E16) and 18th (E18) days of gestation, the day of delivery (Day 0), the 2nd, 7th, 11th and 15th postnatal days and 2–3 months after birth (adult).

### *Tissue preparation*

The fixative used was paraformaldehyde. Pregnant females were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (60 mg/kg). E16 fetuses were removed from the uterus, decapitated and an incision made in the anterior abdominal wall, after which they were immersed in 4% paraformaldehyde in 0·1 M phosphate buffer, pH 7·2 at 4 °C. It was possible to perfuse the vascular system of E18 fetuses briefly with fixative via the umbilical vein. In both cases a block of tissue containing the adrenals was dissected out and immersed in the above fixative for a further 4–6 hours. Postnatal animals were anaesthetised in the same way or by halothane inhalation (for neonates less than one week) and perfused through the left ventricle

with physiological saline containing a vasodilator (4 mM procaine hydrochloride) at 37 °C for 30 seconds followed by the paraformaldehyde fixative for 15 minutes. A tissue block containing the adrenal glands was then fixed by immersion for an additional 2 hours.

Previous experience had shown (Kent, Tomlinson & Coupland, 1987) that the antigenicity of met-enk 8 was maintained better with Bouin's fluid than with paraformaldehyde fixation. Tissues from half the animals in each group were therefore first perfused with 4% paraformaldehyde and then placed in Bouin's fixative for the period of immersion fixation (3–4 hours).

All tissues were kept overnight in phosphate buffer, pH 7·2, and in addition Bouin-fixed tissue was thoroughly rinsed in 30% alcohol before dehydration and embedding in paraffin wax. Serial sections cut at 5 $\mu$m were mounted on gelatinised slides.

### *Antisera*

All antisera were polyclonal and raised in rabbits. Chromogranin A and B antisera were prepared and characterised in the Department of Pharmacology, Innsbruck (Fischer-Colbrie & Frischenschlager, 1985) and were kindly made available by Professor Hans Winkler. Met-enkephalin-arg$^6$-gly$^7$-leu$^8$ antiserum was prepared in Shizuoko College of Pharmacy using met-enkephalin-arg$^6$-gly$^7$-leu$^8$ conjugated to ascaris protein (Ikeda *et al.* 1982) and characterised for immunocytochemistry by Kobayashi *et al.* (1983*b*). This was donated to the department by Professor N. Yanaihara. PGP 9.5 antibody was obtained from Ultraclone, Cambridge.

Immunogold GAR G5 was obtained from Janssen Pharmaceutical, Beerse, Belgium; bovine serum albumin from Sigma Chemical Company; Tween 80 from BDH Chemicals.

### *Immunocytochemical protocol*

Immunostaining was based on an immunogold–silver staining (IGSS) method described by Springall, Hacker, Grimelius & Polak (1984). Pretreatment with Lugol's iodine was necessary. Sections were washed in Tris-buffered saline (TBS) at pH 7·4 containing 0·5% Tween 80 up to the primary antibody stage and afterwards with TBS at pH 8·2. Primary antibodies, diluted with TBS, pH 7·4 and containing 0·1% bovine serum albumin (BSA), were applied for 18 hours at 4 °C. The secondary antibody was goat anti-rabbit immunoglobulin labelled with 5 nm colloidal gold (GAR G5) diluted 1:250 or 1:125 in TBS, pH 8·2, containing 0·8% BSA; sections were incubated in this mixture for 60 minutes at room temperature. Thorough washing in glass-distilled deionised water preceded enhancement of the gold labelling with the physical developer IntenSE 1 (Janssen Pharmaceutical, Belgium). Pale counterstaining with Mayer's haemalum was occasionally employed but adjacent stained sections were usually used for the identification of tissue components. The working dilutions of the antisera were determined by applying ascending dilutions from 1:100 to 1:1600 to sections of adult tissue.

In control experiments primary antibody was omitted or replaced with non-immune serum. In addition, whenever possible, tests with primary antiserum preadsorbed with the appropriate antigen were performed.

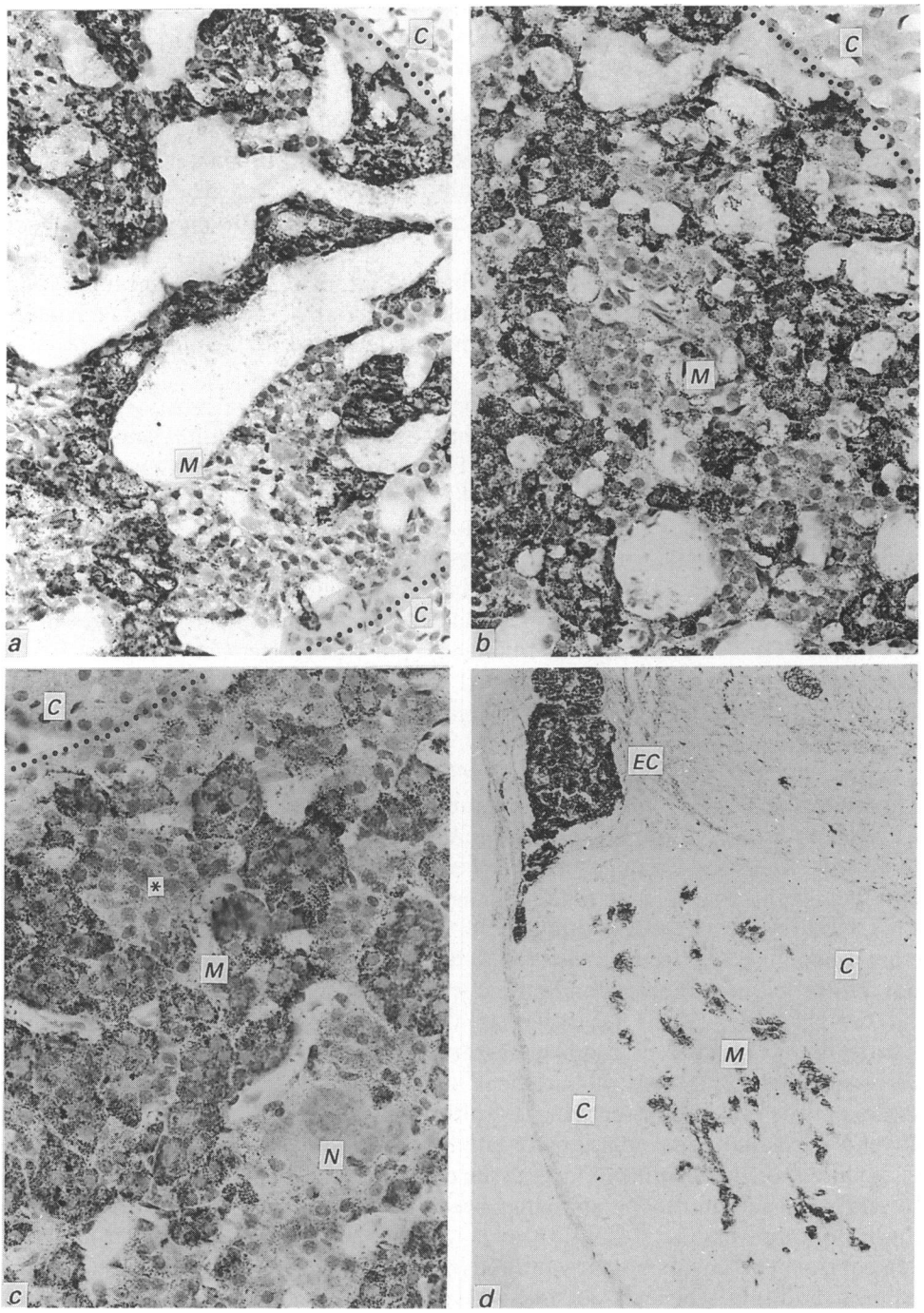


Fig. 1 (*a–d*). (*a*) Chromogranin A-like reactivity in the chromaffin cells of a 7 days old rat adrenal medulla. ×250. (*b*) Chromogranin A-like reactivity in the adrenal medullary cells of a 15 days old rat. ×250. (*c*) Adult adrenal medulla, the majority of the chromaffin cells are immunoreactive for chromogranin A. Some groups of cells (*) show a weak response. ×250. (*d*) Adrenal gland of an 18 days old (E18) fetus immunostained for PGP 9.5. A positive response can be seen in chromaffin cells arranged in clusters in the medulla and situated extra-adrenally at the medial pole of the gland. ×100. *C*, adrenal cortex; *EC*, extra-adrenal chromaffin tissue; *M*, adrenal medulla; *N*, neuronal soma.

## RESULTS

### *Chromogranins*

*Adult*

Chromaffin cells of the adrenal medulla were immunoreactive for both chromogranin A and B; in all cases there was no response in the cortical cells. Neuronal somata within the adrenal medulla were unreactive.

Comparing the responses at identical antibody dilutions, chromogranin A-like immunoreactivity was more intense than chromogranin B immunoreactivity. A series of increasing antibody dilutions revealed the response at 1:100 to be very intense and at 1:1600 very weak but still discernible with antibody to chromogranin A whilst the response for chromogranin B was weak at 1:400 and equivocal at a dilution of 1:800.

At the highest antibody titres all chromaffin cells were stained, although not uniformly, while at lower titres of both chromogranin antibodies groups of chromaffin cells with noticeably low levels of immunoreactivity were seen (Fig. 1*c*). Although these were not unequivocally identified as noradrenaline-storing (NA) cells they conformed to NA cells in their distribution pattern, appearance and proportionality with respect to the medullary cell population (20–30%). From the serial dilution tests it was apparent that dilutions of 1:200 for chromogranin A and 1:100 for chromogranin B antibodies resulted in equal intensities of reaction in adult medullary cells: these titres were chosen for the studies on developing adrenal glands.

*Developing adrenal medulla*

The earliest stage in which chromogranin A was detected was at 18 days gestation (E18) when a positive reaction was observed in only one of three fetuses: the reaction was faint but distinct and was associated with the cytoplasm of small nests of cells in the future medullary region of the adrenal gland. In the newborn animals weak reactivity to chromogranin A was consistently found in chromaffin cells, distributed unevenly throughout the medulla. The response at 7 days (Fig. 1*a*) was more intense and at 15 days (Fig. 1*b*) the pattern and intensity of immunostaining were similar to those of the adult. Groups of chromaffin cells under the capsule at the medial pole of the adrenal and nearby in the cortex were strongly stained in these animals. Chromogranin B was not detected in the adrenal glands of animals younger than 7 days although by 15 days the intensity of the staining was similar to adult levels.

*Extra-adrenal chromaffin tissue*

Chromaffin tissue, identified by its position and cellular characteristics, was seen to be weakly immunoreactive in the newborn. Extra-adrenal chromaffin tissue aggregated as a distinct para-aortic body (PAB) showed positive immunoreactivity in most chromaffin cells as did the scattered small groups of extra-adrenal chromaffin cells lying medial to the adrenal gland. Extra-adrenal chromaffin tissue never showed chromogranin B-like immunoreactivity. Table 1 summarises the findings.

### *Met-enkephalin-arg$^6$-gly$^7$-leu$^8$-like immunoreactivity*

*Adult adrenal*

A very few chromaffin cells gave a positive reaction for met-enk 8-like immunoreactivity, often fewer than 10 cells per section of adrenal medulla. Weak staining of cortical glomerulosa cells was a consistent finding. Positively stained nerve terminals associated with chromaffin cells were observed throughout the medulla

Table 1. *Relative intensity of immunostaining for chromogranin A and B in developing adrenal and extra-adrenal chromaffin tissue*

| Age | | Tissue | Chromogranin A | Chromogranin B |
|---|---|---|---|---|
| E16 | (3) | Adrenal medulla | − | − |
| E18 | (3) | | +/− | − |
| 0–2 days | (4) | | + | − |
| 7 days | (4) | | ++ | +/− |
| 15 days | (4) | | +++ | + |
| Adult | (4) | | ++++ | +++ |
| 0–2 days | (3) | PAB | +/− | − |
| | | Extra-adrenal chromaffin tissue | +/− | − |

The intensity of immunostaining was visually rated as absent (−); weak and variable (+/−); weak (+) to intense (++++). The figures in brackets indicate the number of animals in each group.

(Fig. 2*e*) but no reactive nerve fibres were found in the adrenal cortex or the capsule. It was not possible to distinguish between A and NA cells in these preparations.

*Developing chromaffin tissue* (Fig. 2)

Met-enk 8 reactivity was not detected in the adrenal medulla before birth but occasional clusters of cells at the medial pole of the adrenal gland, which were probably immature chromaffin cells, were positive and whenever the PAB was sectioned the majority of its chromaffin cell population was stained (Fig. 2*d*). In newborn animals the results were variable but the majority (4 out of 6) of neonatal medullae contained a higher proportion of stained chromaffin cells than did adult medullae (Fig. 2*a*). Extra-adrenal chromaffin cells were usually positive and the PAB stained more heavily than adrenal medullary chromaffin cells. Nerve terminals were unstained in the adrenal medullae of the newborn group (0–2 days) while, in the same sections, met-enk 8-like immunoreactivity of nerve terminals in adjacent sympathetic ganglia could be demonstrated.

In the adrenal medulla of a single animal from the 7 days old group, almost all the chromaffin cells showed some level of reactivity and in one of the 11 days old animals approximately half the medullary cells were stained. None of the remaining 7 or 11 days animals showed greater proportions of stained cells than did adult animals and staining in excess of adult levels was not seen after the 11th day.

Nerve terminals showed negative immunoreactivity at 7 days, a positive reaction being first detected in 11 days old rats (Fig. 2*b*). A fine network of silver-stained threads was observed surrounding some groups of chromaffin cells in all sections of 11 days old rats. In all 15 days old animals (Fig. 2*c*) the terminals were more densely stained and more uniformly distributed throughout the medulla. A similar pattern was observed in all adult animals.

In summary, the only met-enk 8 detected before birth was in the extra-adrenal chromaffin tissue. During the first postnatal week medullary chromaffin cells giving a positive reaction appeared in a greater proportion than in the adult adrenal. Immunostaining of the nerve terminals of the adrenal medulla was first detected during the second week of life and thereafter increased in intensity up to adult levels. Table 2 summarises the findings.

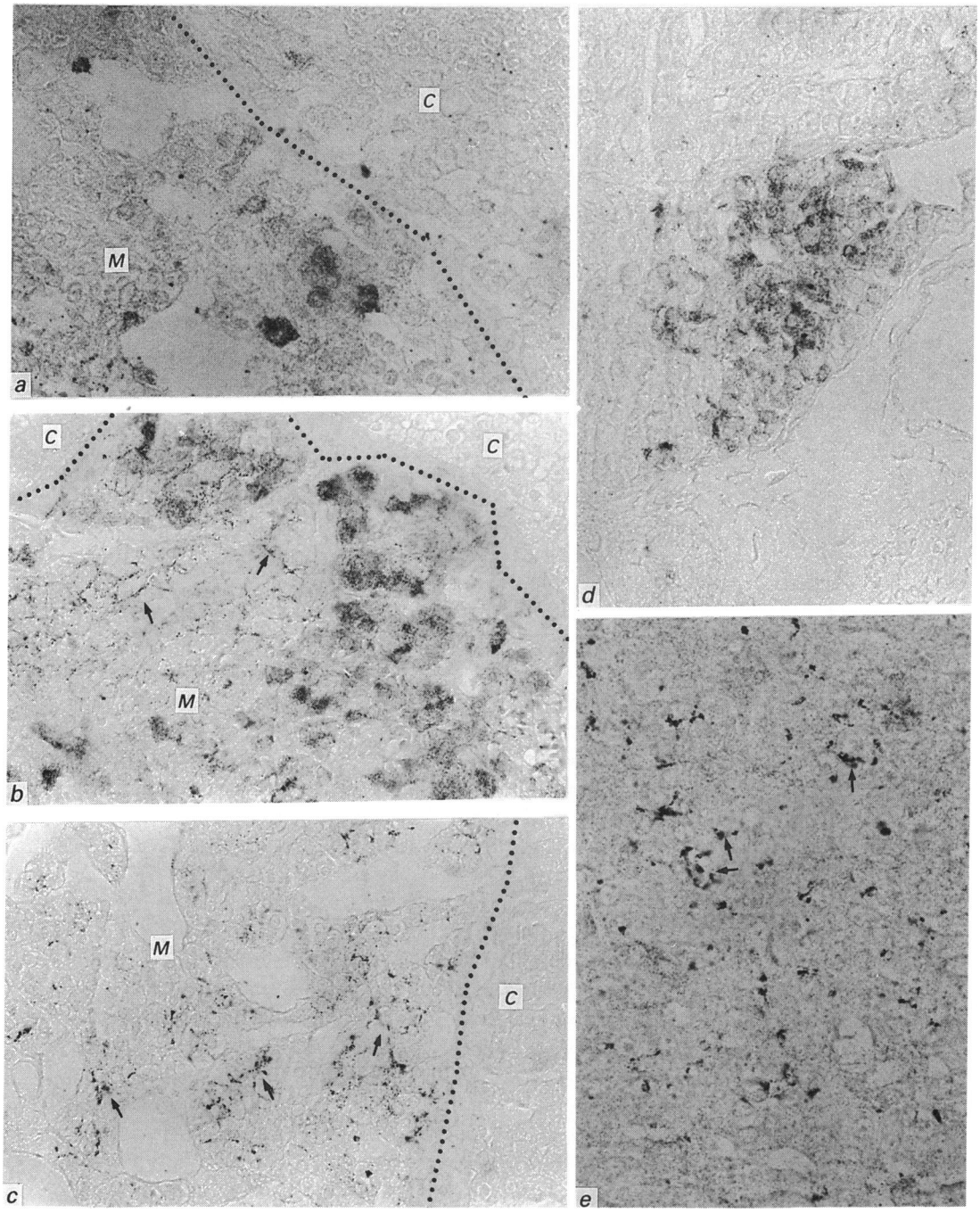


Fig. 2(*a–e*). Immunoreactivity for met-enk 8 demonstrated by immunogold–silver enhancement. (*a*) Met-enk 8-like reactivity in the adrenal medullary chromaffin cells of a one day old rat. ×350. (*b*) Adrenal medulla of an 11 days old rat showing immunoreactivity of chromaffin cells and the nerve terminals (arrowed) surrounding unstained cells. ×350. (*c*) Adrenal medulla from a 15 days old rat. Immunoreactivity can be seen in the nerve terminals (arrowed) and not in chromaffin cells. ×350. (*d*) Extra-adrenal chromaffin tissue (PAB) from a 2 days old rat. The majority of the cells contain met-enk 8-like immunostained material. ×250. (*e*) Adrenal medulla of adult rat. Immunoreactive nerve terminals (arrowed) are common; the chromaffin cells in this field are unreactive. ×400. *C*, adrenal cortex; *M*, adrenal medulla.

Table 2. *Relative intensity of immunostaining for met-enk 8 and PGP 9.5 of chromaffin tissue during development*

| Age | | Tissue | Met-Enk 8 | | PGP 9.5 |
|---|---|---|---|---|---|
| | | | Chromaffin cells | Nerve terminals | Chromaffin cells |
| E16 | (3) | Adrenal medulla | − | − | +++ |
| E18 | (3) | | − | − | +++ |
| 0–2 days | (6) | | + more than 1 in 10 cells stained | − | +++ |
| 7 days | (4) | | +* or − | − | n.a. |
| 11 days | (3) | | +† or − | + | n.a. |
| 15 days | (3) | | + or − | ++ | +++ |
| Adult | (4) | | + or − | +++ | +++ |
| E18 | (3) | PAB + extra-adrenal tissue | ++ | − | +++ |
| 0–2 days | (3) | | ++ | − | +++ |

The intensity of immunostaining was visually rated as absent (−); weak and variable (+/−); weak (+) to intense (+++). The figures in brackets indicate the number of animals in each group. n.a., not available.

* In one adrenal almost all chromaffin cells were immunoreactive.

† In one adrenal more than half the chromaffin cells reacted.

### *PGP 9.5-like immunoreactivity*

#### *Adult adrenal*

All adrenal medullary cells showed an intense reaction with this antibody with no evidence of discrimination between A and NA cells. It was not possible to identify any positively reacting nerve elements but this could have been due to the fact that staining was masked by the intense reaction of adjacent neuroendocrine cells. Cortical cells were uniformly negative.

#### *Developing chromaffin tissue*

Chromaffin cells of the extra-adrenal tissue, PAB and adrenal medulla also showed intense PGP 9.5-like immunoreactivity in E16 fetuses (Fig. 1*d*) and in the newborn. The intensity of the staining approached that of the adult adrenal medulla. Again due to limitations of morphological discrimination, identification of fibres and nerve terminals was not possible.

## DISCUSSION

Since this work began Schober, Fischer-Colbrie & Winkler (1989) have investigated the ontogenesis of chromogranin A and B in rat adrenal medulla by a dot immunobinding assay. Our results for chromogranin A are in agreement with theirs but the weaker reaction obtained with chromogranin B could not have been predicted from the immunoblotting. The most likely explanation for the discrepancy lies in the immunocytochemical (ICC) technique: the chromogranin B serum may have a lower sensitivity for the reactive epitopes remaining in tissue sections or this antigen may be more susceptible to the fixation procedures employed. Either possibility would fit the observations, since confirmed with Bouin-fixed tissue and a different batch of antibody (Kent, unpublished), that chromogranin B antisera always gave a weaker response

than chromogranin A antisera in the adult rat adrenal medulla where it is known that chromogranin B predominates (Fischer-Colbrie, Hagn & Schober, 1987).

The lack of staining in prenatal adrenal glands is not surprising since the total chromogranin content of the gland at prenatal day E17 is less than 0·1% of adult levels (Schober *et al.* 1989) and can only be detected by pooling glands from many animals. Postnatally our results are consistent with a gradually increasing concentration of chromogranins in the cytoplasm of the chromaffin cells, particularly marked during the first week and coinciding with a period when chromaffin granule numbers (Tomlinson & Coupland, unpublished) and levels of both chromogranin A and B measured by assay (Schober *et al.* 1989) rise steeply. The presence of cells at different stages of maturity could account for the variation in the intensity of the immunoreactivity which lessened with time. The suggestion that patches of less intensely immunopositive cells may be NA cells needs further investigation.

There is some disagreement between different laboratories about the details of the distribution of enkephalin-like immunoreactivity in the adult adrenal medulla, mainly in relation to the relative reactivity of A and NA cells. Variation between species accounts for some of the discrepancies but results on the rat have not always been consistent and the differences reported in this species are most likely to arise from the variety of antisera workers have used. The antiserum (R-0171) we used has been fully characterised for immunocytochemisty by Kobayashi *et al.* (1983*b*); it was raised against the octapeptide met-enkephalin-arg$^6$-gly$^7$-leu$^8$ (met-enk 8) which occurs only in the proenkephalin A molecule and therefore can be regarded as an indirect marker for the preproenkephalin gene, indicating the presence of proenkephalin A and/or its derivatives. In theory at least, sera against leu-enkephalin and met-enkephalin can also react with prodynorphin and proopiomelanocortin (POMC) respectively (Petrusz, Merchenthaler & Maderdrut, 1985). Our findings in the adult rat medulla are in agreement with those of Kobayashi *et al.* (1983*a*) who found, using met-enk 8 antiserum, less than 1% of medullary cells staining positively, the most striking feature being immunopositive nerve terminals surrounding the chromaffin cells (Kobayashi *et al.* 1983*c*). Pelto-Huikko *et al.* (1985), using three different antisera to either leu-enkephalin or met-enkephalin, also demonstrated a network of nerve fibres around A cells but not NA cells and only 3–5% of the A cells and no NA cells were immunoreactive. In view of the fact that, in contrast to other species, enkephalins are only present in trace amounts in the rat adrenal gland (Hexum, Yang & Costa, 1980) these are not unexpected findings, but they strongly suggest that a significant proportion of the total enkephalins measured by assay of the homogenised rat adrenal are derived from nerve endings. The weak immunoreactivity of adrenal medullary cells in the rat means that enkephalin-positive terminals can be easily identified.

Immunoreactivity to both met- and leu-enkephalin (Pelto-Huikko *et al.* 1985) and to met-enk 8 (Kobayashi *et al.* 1983*c*; Kondo, Kuramoto & Iwanaga, 1984) has been shown to be co-localised with catecholamines in chromaffin granules and confined to the large-cored synaptic vesicles of presynaptic, probably cholinergic (Kondo, Kuramoto, Wainer & Yanaihara, 1985), nerve terminals ending on chromaffin cells.

The earliest stage at which met-enk 8-like material was detected in nerve endings, between 7 and 10 days, coincides with the timing of the establishment of functional innervation of the adrenal medulla. Although the presence of nerve fibres and synapses on chromaffin cells has been described at prenatal stages (Daikoku, Kinutani & Sako, 1977) and at birth (Tomlinson & Coupland, unpublished), evidence based on the reflex response to insulin hypoglycaemia suggests that splanchnic nerve transmission does not become functional until the end of the first week of life (Lau,

Bartolome, Bartolome & Slotkin, 1987). During this first week, before met-enk 8-positive nerve terminals could be detected, the proportion of positive chromaffin cells was considerably higher than at later times and only approached the low levels of the adult after the appearance of positive nerve endings. Also, in some medullae, the nerve networks associated with unstained chromaffin cells seemed to be more prominent than those surrounding more heavily stained cells (Fig. 2*c*) and the chromaffin cells of the PAB, which contained no met-enk-8-positive nerve endings, were consistently immunopositive. These findings are in keeping with sparsely or non-innervated extra-adrenal cells. Taken together the observations suggest a reciprocal relationship between met-enk 8-like material present in nerve endings and the levels in chromaffin cell cytoplasm. The enkephalin content of the adult medulla is known to be regulated transsynaptically; denervation results in an increase in the number of leu-enkephalin-like positive cells (Schultzberg *et al.* 1978) and elevated enkephalin or proenkephalin stores (Lewis *et al.* 1981; Fleminger, Lahm & Udenfriend, 1984), with most of the 10–20 fold increase being due to the precursor molecule proenkephalin A (Yoburn, Franklin, Calvano & Inturrisi, 1987). Conversely, impulse activity and membrane depolarisation inhibit enkephalin biosynthesis (La Gamma, Adler & Black, 1984; La Gamma *et al.* 1985) in adult rats and a parallel effect may explain our findings in the neonate, i.e. the maturation of transsynaptic impulse-activity suppresses synthesis of enkephalins by medullary chromaffin cells. This would be consistent with recent denervation experiments (La Gamma & Adler, 1988) demonstrating that the normal development of adrenal medullary opiate peptides is dependent upon functional presynaptic terminals.

The presence of the PGP 9.5 molecule was detected at earlier stages than either chromogranins or enkephalins. There are several possible explanations inherent in the ICC technique such as the sensitivity of the antiserum and the resilience of the antigen but the distribution of PGP 9.5 staining throughout the neuron from the perikaryon to the axon terminals in several neuronal types in a wide range of mammals including man (Gulbenkian *et al.* 1987) and its occurrence in extracts from mammalian and non-mammalian nervous systems (Jackson *et al.* 1985) suggest that the protein is a fundamental component of the cytoplasm of cells of the neuronal lineage and, as such, would be expressed early in the ontogeny of the nervous system.

In the earliest stages we examined (E16), cell bodies and axons of both the central and sympathetic nervous system were strongly positive as were the extra-adrenal chromaffin cells, particularly the masses of cells at the medial pole of the adrenal. Thus chromaffin cells share an antigenic protein with other cells of neuronal origin which is expressed at very early stages in differentiation. Work to determine whether the PGP 9.5 neuronal marker can be used to study earlier stages in neurogenesis and neural crest migration is continuing.

## SUMMARY

The localisation of chromogranins A and B, met-enkephalin-arg$^6$-gly$^7$-leu$^8$ (met-enk 8) and protein gene product 9.5 (PGP 9.5) in the adrenal medulla and extra-adrenal chromaffin tissue has been studied in the developing rat by immunogold–silver staining.

In the adult rat adrenal the cytoplasm of all medullary chromaffin cells showed a positive response with chromogranin A and B; in each case occasional groups of cells with a low reactivity that may have been NA cells were seen. Chromogranin A was first detected in adrenal medullary and extra-adrenal chromaffin cells at 18 days of gestation whilst chromogranin B was not detected in animals younger than 7 days. In

15 days old animals the adrenal medullary response to A and B was of the same intensity as that seen in the adult.

Less than 1% of adult medullary chromaffin cells were responsive to met-enk 8 staining and medullary cells were unreactive in the fetus, with only extra-adrenal chromaffin tissue responding prenatally. During the first postnatal week immunoreactive cells appeared in the adrenal medulla in considerably greater proportions than in the adult gland. In contrast, positively stained nerve terminals associated with chromaffin cells and abundant in the adult adrenal were not detected during the first week of life. Immunoreactive nerve terminals were first seen early in the second week of life at a time when positive chromaffin cells were becoming less common.

PGP 9.5 was located in all chromaffin cells of the adult adrenal and was readily detected in chromaffin cells in the adrenal and in extra-adrenal locations of the earliest stage examined (E16).

Our findings suggest that the ontogenesis of the chromogranin-like immunostaining reflects the maturation of chromaffin granules and the PGP 9.5 immunostaining detected a protein common to cells of neuronal origin and expressed at an early stage of differentiation. The reciprocal relationship between the presence of enkephalins in chromaffin cells and in their presynaptic terminals merits further investigation.

The authors are grateful for generous gifts of antisera, to Professor N. Yanaihara for anti met-enk 8 serum and to Professor H. Winkler for antisera to chromogranins A and B. The technical assistance of Diane Johnson and secretarial skills of Ann Davis and Marie Walker-Greenwood are gratefully acknowledged.

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