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The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands: Identification by an antiserum against a synthetic PDH

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Summary. A high-titer antiserum has been obtained from two rabbits immunized with a glutaraldehyde conjugate of synthetic pigment-dispersing hormone (PDH) from *Uca pugilator* and bovine thyroglobulin. The antiserum blocked melanophore-dispersing activity of the peptide in vivo. In sinus glands (SG) of *Carcinus maenas*, *Cancer pagurus*, *Uca pugilator* and *Orconectes limosus*, electron-microscopic immunocytochemistry revealed sparsely distributed axon endings containing a distinct PDH-immunoreactive type of neurosecretory granules (diameter 90–130 nm). Exocytotic figures indicating release of the content of these granules into hemolymph lacunae were occasionally observed. Preservation of fine structure and antigenicity of the PDH granules were markedly dependent on the fixation procedure used. A preliminary experiment with *C. maenas* showed that preterminal axon dilatations near the basal lamina seemed to accumulate PDH-granules when animals were kept in complete darkness for three days. Immunodot blotting of fractions after high pressure liquid chromatography (HPLC) of extracts from SGs of *C. maenas* and *O. limosus* revealed a strongly immunoreactive substance at a retention time very similar to those of synthetic PDHs of *Uca pugilator* and *Pandalus borealis*. It is also coincident with a zone of biological activity. Thus, the antigen demonstrated by immunocytochemistry is identical or very similar to one of the known PDHs.

Key words: Pigment-dispersing hormone – Ultrastructural histochemistry – Immunocytochemistry – Neurosecretion – Crustaceans (*Carcinus maenas*, *Cancer pagurus*, *Uca pugilator*, *Orconectes limosus*)

The present study is part of a project to elucidate the neuronal associations and neurosecretory pathways of "native" (Greenberg and Price 1983) crustacean peptides. As "native" we consider neuropeptides that have thus far been demonstrated only in crustaceans. Evidence of their occurrence in other animal groups is, at present, only preliminary. This evidence is largely based on bioassays and preliminary biochemical characterization, and no final conclusions can be drawn due to lack of structural data (Rao 1985, unpublished results). Although a wealth of data on the occurrence, physiological significance and biochemistry of crustacean neuropeptides, especially from the eyestalk neurosecretory system, has accumulated (reviews: Kleinholz and Keller 1979; Keller 1983; Webster and Keller 1987), immunocytochemical studies have thus far been scarce because only a few peptides have been available in synthetic form or as pure native substances in sufficient quantities to permit the production of antisera. Thus far, these requirements are met only by the crustacean hyperglycemic hormone (CHH) and two chromatophorotropins, red pigment-concentrating hormone (RPCH) and pigment-dispersing hormone (PDH). The primary structures of RPCH (an octapeptide) and PDH (= DRPH, distal retinal pigment hormone, an octadecapeptide), both from *Pandalus borealis*, have originally been elucidated by Fernlund and Josefsson (1972) and Fernlund (1976). Rao et al. (1985) and Kleinholz et al. (1986) have recently elucidated the sequences of PDHs from *Uca pugilator* and *Cancer magister*, which are identical but differ from the PDH of *Pandalus* in six out of eighteen residues.

Antisera against CHH have been used to visualize its neurosecretory pathway in the eyestalk of different decapods (Jaros and Keller 1979; Van Herp and Van Buggenum 1979; Gorgels-Kallen et al. 1982; Keller et al. 1985). By use of antibodies directed against the N-terminal tetrapeptide of RPCH, the neuronal association of this neuropeptides was demonstrated in *Carcinus maenas* and *Orconectes limosus* (Mangerich et al. 1986). To extend such studies to another known crustacean neuropeptide we raised polyclonal antibodies against synthetic PDH of the *Uca/Cancer* type. An initial study in our laboratory, employing light-microscopic immunocytochemistry on eyestalks from *C. maenas* and *O. limosus*, revealed a surprisingly complex system of PDH-positive neurons (Mangerich et al. 1987). Axon projections to the principal neurohemal organ, the sinus gland (SG), and neurosecretory terminals in the SG were also demonstrated. Their perikarya could not be located with certainty, but it can be safely stated that they are not localized in the medulla-terminalis X-organ (XO). In this respect, the system is distinct from the CHH- and

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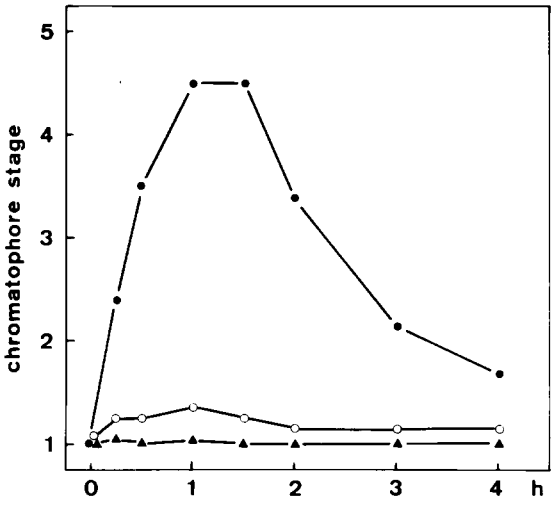


Fig. 1. Melanophore bioassay in *Uca pugilator* to demonstrate the presence of anti-PDH antibodies. Response to 50 fmol of synthetic PDH/animal preincubated with preimmune serum (●) or antiserum from the same animal (○). Controls (▲) injected with 1:25 diluted preimmune- or antiserum without PDH. Six animals were used for each point of the curves. For details, see Materials and methods

RPCH-neurosecretory pathways that consist of perikarya in or near the XO connected to the SG by the principal XO-SG tract.

It was the objective of the present study to supplement these light-microscopic results by a detailed electron-microscopic/immunocytochemical study of the neurosecretory terminals in the SG. We sought to identify them, to determine their frequency and arrangement among other terminals, and to characterize the type of granule that contains PDH. To assess whether a distinct granule type is involved, it seemed useful to include at least one other neuropeptide in the study; therefore, a new CHH antiserum was used to identify CHH terminals and granules for comparison. We studied four different species, i.e., *C. maenas* and *O. limosus*, which had been used in the light-microscopic study (Mangerich et al. 1987), *U. pugilator* because the antiserum was raised against the hormone of this species, and *Cancer pagurus*.

Biochemical evidence of a granular association of PDH was first provided by Pérez-Gonzales (1957) by means of differential centrifugation of homogenates of SGs from *U. pugilator*. The first authors to ascribe PDH to a morphologically characterized granule type were Aoto and Hisano (1985), who found, by electron microscopy, that certain axon profiles were depleted in their granule content by prolonged illumination and enriched in dark-adapted animals. This indicated specific response of PDH-terminals to light, which is consistent with the well-established fact that PDH-mediated melanophore dispersion and light adaptation of the compound eye are particularly dependent upon illumination (Rao 1985, for review).

Since it was not known whether the reactive antigen demonstrated in this study in *C. maenas*, *O. limosus* and *C. pagurus* was identical to PDH from *U. pugilator*, HPLC analyses combined with immunodot blotting and comparison of retention times of the reactive substances with those of PDHs from *U. pugilator* and *O. limosus* were carried out for two of the species, *C. maenas* and *O. limosus*.

Materials and methods

Animals

Carcinus maenas were obtained from the Netherlands Institute voor het Onderzoek van de Zee, Texel, and kept under 16L:8D cycle in recirculated artificial seawater. *Cancer pagurus*, collected by local fishermen were kept and prepared at the Laboratoire de Biologie Marine, Collège de France, at Concarneau. *Uca pugilator* from the seashore near Pensacola, Florida, were kept in artificial seawater under ambient light at room temperature. *Orconectes limosus* from the river Havel in Berlin were kept in running tap water under the same conditions as *C. maenas*. Except for *C. pagurus* all animals were fed pelleted cat food at libitum. All dissections were made during the light phase.

Fixation and embedding

Sinus glands (SG) of the crabs were dissected under chilled seawater or saline (Nordmann and Morris 1980), those of the crayfish in Harreveld saline (Van Harreveld 1936). The following fixation procedures were used:

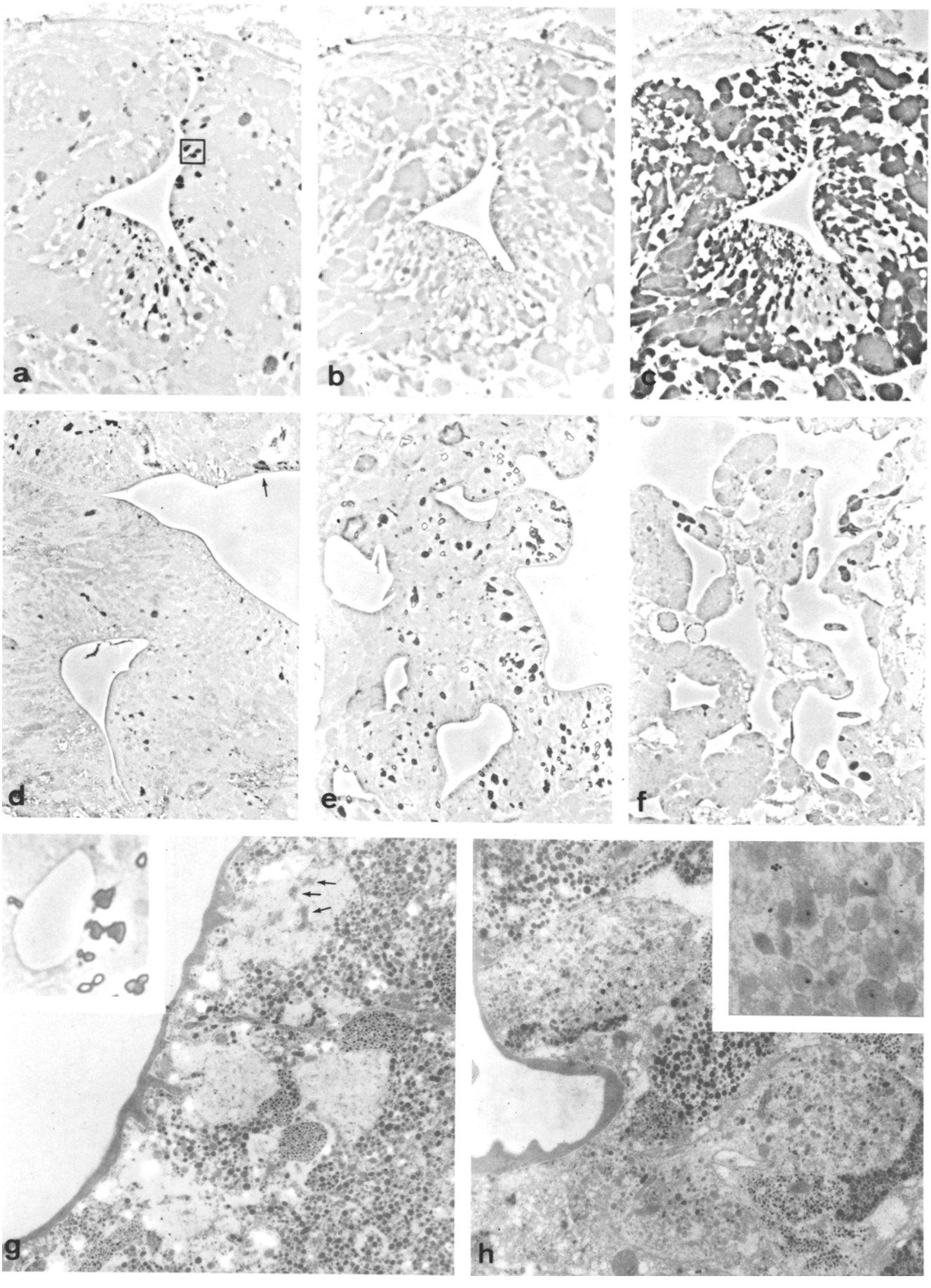
1) 3% Glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.4, containing 0.7 M sucrose, for 3 h at room temperature (RT); washing in the same buffer.

2) 4% Glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.4 containing 0.45 M sucrose and 2 mM $CaCl_2$, for 3 h at RT, washing overnight at 4° C in buffer and postfixation with buffered 1% OsO_4 according to Nordmann and Morris (1980).

3) 2% Paraformaldehyde, 2% glutaraldehyde and 0.1% picric acid in 0.1 M sodium cacodylate, pH 7.4, containing 0.5 M sucrose and 5 mM $CaCl_2$ for 2–3 h on ice, followed by washing overnight at 4° C in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.7 M sucrose and 5 mM $CaCl_2$. Postfixation in the dark at RT for 1 h with 1% OsO_4 buffered with 0.05 M sodium cacodylate, pH 7.4, containing 1.5% $K_3Fe(CN)_6$, 0.7 M sucrose, and 5 mM $CaCl_2$, followed by extensive washing in buffer (modified after Langford and Coggeshall 1980).

For SGs from *O. limosus* sucrose was omitted from the fixative and the washing buffer contained 0.35 M instead

Fig. 2. a–c Consecutive semithin sections through SG of *Carcinus maenas*. **a** PDH-immunoreactive axon profiles (AP) predominantly located around a hemolymph lacuna; **b** control to **a**, section treated with PDH-preabsorbed antiserum; **c** CHH-immunoreactive AP (fixation 2, PAP), ×240. **d–f** PDH-immunoreactive AP in semithin sections through the SG of *Cancer pagurus* (**d**), *Uca pugilator* (**e**), and *Orconectes limosus* (**f**). All three fixation 3, PAP, ×400. **g** Ultrathin section of SG from *Carcinus maenas* (×6500) shows loss of granule structure in PDH axon profiles after fixation 1. Mitochondria (*arrows*) are still visible. The same axon profiles are shown on an adjacent semithin section (inset, PAP, ×900). Despite loss of granules, the overall stainability of the AP is preserved. **h** SG of *C. maenas*. Osmium treatment (fixation 2); improved ultrastructure of membranes and granules but immunostaining (inset, GAR-Au 30) is still inadequate. Ultrathin section adjacent to the portion of the semithin section marked by a square in Fig. 2a. ×6500; inset ×37200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of 0.7 M sucrose. After dehydration in a graded ethanol series, the SGs were embedded in a low viscosity resin (Spurr 1969).

Antisera and gold conjugates

Synthetic PDH of the *Uca/Cancer* type, (1.8 mg, Rao et al. 1985) and bovine thyroglobulin (6.5 mg, Sigma) were dissolved in 1 ml sterile 0.1 M sodium phosphate buffer, pH 7.4. Under stirring at 0° C, 18 μmoles freshly prepared glutaraldehyde were added and the reaction allowed to proceed for 24 h at 0° C. The reaction mixture was then brought to RT and, after addition of another 18 μmoles glutaraldehyde, incubated for another 15 min. The conjugate was immediately dialyzed against 0.01 M PBS, changed several times, for 2 days at 4° C. Assuming a coupling yield of 50%, approximately 125 nmoles peptide conjugated to thyroglobulin were dissolved in 0.75 ml sterile PBS, emulsified with 0.75 ml Freund's complete adjuvant (Difco) and administered to two rabbits by multiple sub- and intracutaneous injections. Booster injections of 50 nmoles of peptide equivalents/animal were given 35 days later. After two ear bleedings to check the rise in antibody titer the rabbits were terminally bled at day 39 after the booster injections. The development of antibodies was monitored by immunocytochemistry using the sera from the different bleedings and by bioassays (see below).

The production of a new antiserum against the crustacean hyperglycemic hormone (CHH) purified from SG of *C. maenas* by means of HPLC (Keller and Kegel 1984) will be described elsewhere (Dircksen et al., submitted).

For the preparation of goat anti-rabbit (GAR)-colloidal gold conjugates, we followed the method of DeMey et al. (1981). GAR-IgG was prepared from crude goat anti rabbit serum (GAR, Nordic, Tilburg, Netherlands) by affinity chromatography on small protein A-Sepharose (Pharmacia, Freiburg, FRG) columns according to the manufacturer's description or obtained commercially as 7S ultracentrifuge fraction (GAR 7S, Nordic). Colloidal gold (30 or 40 nm) was prepared by reduction of tetrachloroauric acid (Merck, Darmstadt, FRG) with sodium citrate (Frens 1973) and coupled to predialyzed antibodies at pH 9.0 (=GAR Au 30, GAR Au 40). GAR coupled to 10 nm colloidal gold was obtained from Janssen Pharmaceutica (Beerse, Belgium) (Auroprobe^R, EM GAR G10).

Immunocytochemistry

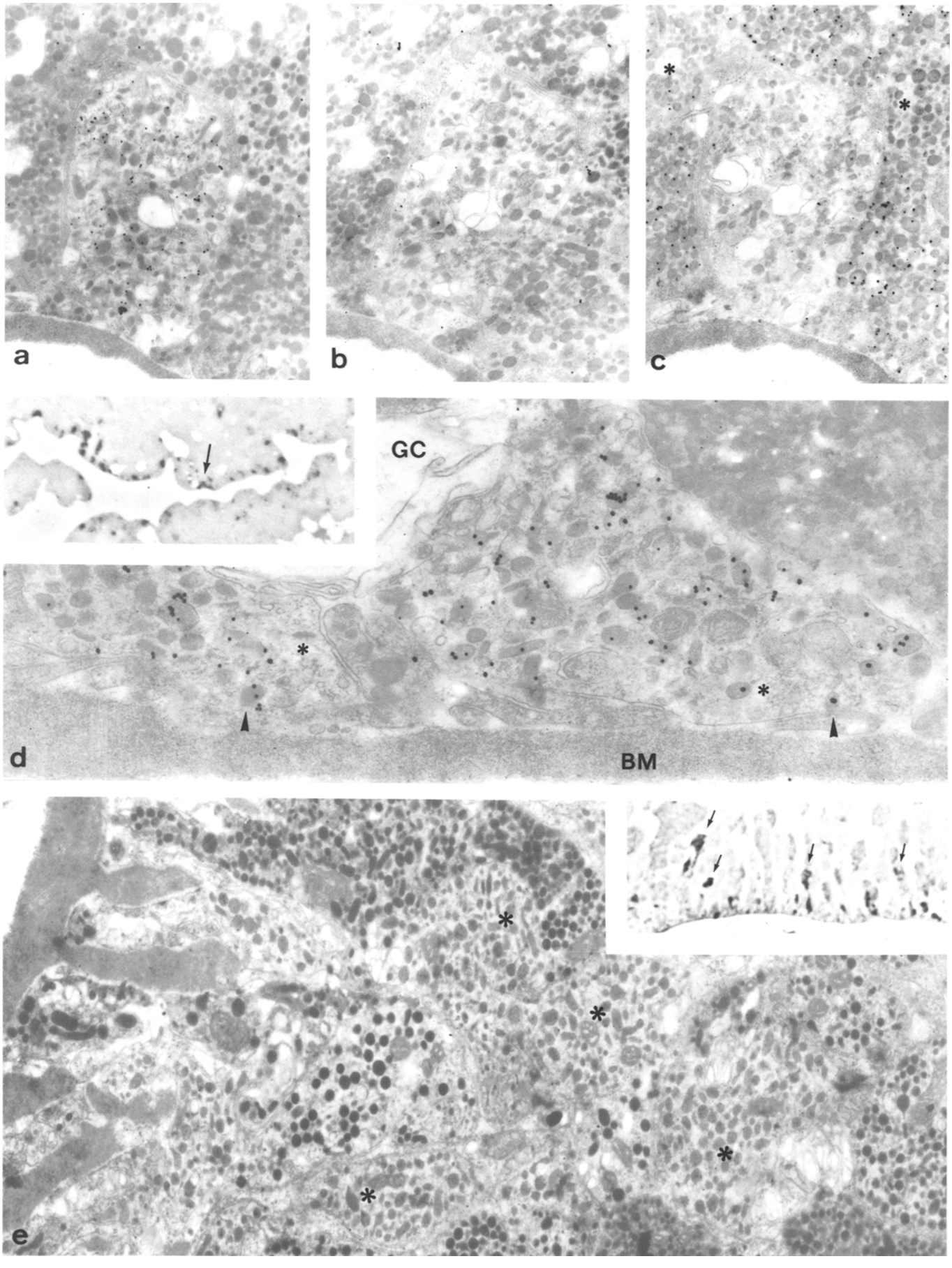
For light microscopy semithin sections (0.5–1 μm) were immunostained with the PAP-technique of Sternberger (1974). Resin was removed according to Maxwell (1977) and osmium removed by placing the slides in 1% aqueous Na-metaperiodate for 7 min before equilibration in 0.01 M PBS. Incubations with the anti-PDH serum diluted 1:12000 with PBS (containing 0.01% Na-azide) or anti-CHH serum diluted 1:6000 were carried out overnight or for 48 h at 4° C in a moist chamber. GAR serum (Nordic) diluted 1:40 with PBS and PAP (Nordic) diluted 1:150 with PBS without Na-azide were applied for 1 h at room temperature (RT). The peroxidase reaction was carried out for 2-5 min with 0.05% 3,3-diaminobenzidine and 0.015% H_2O_2 as substrates in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.9% NaCl.

For electron microscopy, postembedding on-grid labeling was carried out on serial ultrathin sections of silver interference color collected singly on 75–100 mesh Pioloform F-coated nickel grids. After floating on drops of freshly prepared 4% Na-metaperiodate for 1 h (Hearn et al. 1985; Bendayan and Zollinger 1983), thorough rinsing in distilled water and 0.01 M PBS, grids were floated on normal goat serum diluted 1:30 with PBS for 15 min at RT. The Na-metaperiodate treatment proved to be more adequate than Na-ethoxide- or H_2O_2-"etching". It appeared to remove osmic acid partially from the antigenic sites while preserving the typical osmium membrane contrast deeper in the immunostained section. Incubation schedules with primary antisera were the same as for semithin sections, and GAR-colloidal gold conjugates diluted with PBS (GAR Au 30 1:20, GAR G10 1:40) were applied for 1 h at RT. After brief counterstaining with uranyl acetate and lead citrate, sections were viewed on a Zeiss EM 109 electron microscope at 50 kV. Controls were performed by incubation with anti-PDH serum preabsorbed for 4 h at 37° C or overnight at 4° C with 10 nmoles synthetic PDH per μl anti-PDH. Addition of thyroglobulin did not block the anti-PDH serum.

HPLC and immunodot blotting

Sinus glands of *C. maenas* and *O. limosus* were dissected, lyophilized, and extracted by sonication in 2 N acetic acid and subjected to HPLC as described by Keller and Kegel (1984). For details of chromatographic conditions, see legends to Figs. 6 and 7. Peak fractions were collected manually, dried (Speec Vac, Savant Instruments Inc., Farmingdale, N.Y.) and redissolved in small volumes of 2 N acetic acid. Nitrocellulose (NC) strips (Schleicher and Schüll, BA 83) were soaked in a 2% aqueous BSA-solution for 10 min at room temperature, air dried and spotted with 1.5 or 2 μl of HPLC-fractions or peptide standards. After air drying, strips were fixed in paraformaldehyde vapor according to Larsson (1981) and washed in BTTBS (1% BSA, 1.0% Tween 20, 0.05 M Tris pH 7.4, 0.9% NaCl). They were then incubated sequentially with anti-PDH serum diluted 1:10000 in BTTBS overnight at 4° C and GAR-7S Au 40 (1:100) for 1 h at RT with extensive washing in BTTBS after each step, and visualized by silver enhancement according to Moeremans et al. (1984).

Fig. 3a–e. SG of *C. maenas*. **a–c** Consecutive sections showing: a PDH-immunoreactive axon profile (AP). **b** preabsorbed control; **c** three surrounding AP with CHH-immunoreactivity and two immunonegative AP (*asterisks*). **a–c** ×17400. **d** Enlarged view of a PDH-immunoreactive axon terminal marked by *arrow* in an adjacent semithin section (*inset*). Two exocytotic figures (*arrowheads*) and electron-translucent vesicles (*asterisks*) are visible. *GC* glia cell, *BM* basal lamina. ×37200, *inset*: PAP, ×320. **e** SG from an animal kept in the dark for three days. *Inset*: Strongly PDH-immunopositive (PAP) axon dilatations at a distance of 20–50 μm from the basement membrane (*arrows*, ×320). The adjacent ultrathin section shows accumulation of PDH granules in these dilatations (*asterisks*). GAR-G10. ×17200. Fixation 3 in all cases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Bioassay

To test whether the antiserum after the final bleeding was able to block the melanophore dispersing action of PDH in vivo, samples of 1 pmole of synthetic PDH were preincubated in 100 µl of a 1:25 dilution of antiserum or control serum in PBS, pH 7.4, for 2 h at RT, and 5 µl/animal (= 0.05 pmoles PDH) were injected into *U. pugilator* that had been destalked 24 h previously. The melanophore response was monitored by use of the Hogben and Slome (1931) chromatophore index. Appropriate aliquots of HPLC-fractions of SG-extracts were tested in the same manner after vacuum drying in the vacuum centrifuge (Speed Vac) and uptake in filtered sea water.

Results

Although both immunized rabbits developed a strong anti-PDH immunoresponse, one serum (code 3B3) proved to be of a higher titer than the other and was used throughout this study. It gave good immunostaining without background on sections at a dilution higher than 1:15000. In addition, this antiserum effectively blocked the melanophore dispersing activity of synthetic *PDH* in vivo (Fig. 1).

The PAP-staining patterns on semithin sections through the SGs of all four species showed that PDH-immunoreactive axon profiles and terminals are small and scarce (Fig. 2a, d, f). Only in *U. pugilator* were they somewhat more numerous (Fig. 2e). This is particularly clear by comparison with CHH-containing profiles and terminals (Fig. 2c), which are much more numerous and display a larger mean diameter. Fig. 2b shows that preabsorption of the antiserum with PDH completely abolishes the immunostaining. In *C. maenas*, PDH-terminals tend to cluster around the central hemolymph lacuna (Fig. 2a). Ultrathin serial sections of the SG of *C. maenas* demonstrated that CHH and PDH axon profiles are clearly distinct and that there is no co-localization of the two neuropeptides (Fig. 3a–c).

Three different fixation procedures were tested to achieve both preservation of ultrastructure and antigenicity. Glutaraldehyde (fixation 1) alone did not preserve the ultrastructure of the PDH granules, although the axon profiles exhibited considerable immunostaining on semithin sections (Fig. 2g). In an adjacent ultrathin section, mitochondria were still visible but granule structure was lost, whilst other granule types in surrounding axon profiles were preserved, although without membrane contours. Postfixation with OsO_4 (fixation 2) greatly improved ultrastructural detail but caused loss of antigenicity. By use of fixation 3, a combination of paraformaldehyde and glutaraldehyde followed by postfixation with OsO_4 in the presence of K-hexacyanoferrate (III), good preservation of both ultrastructure (especially membranes), and antigenicity were obtained (Fig. 3a–e). This procedure was chosen for the comparative work.

In the SG of *C. maenas*, PDH reactivity was associated with membrane-bounded granules of elongated or ellipsoidal, relatively irregular shape (diameter ~90–130 nm). They are clearly distinct from the larger (~120–200 nm) CHH-positive granules in adjacent axon profiles (Fig. 3a–c). Terminals abutting on the basal lamina around a hemolymph lacuna were particularly rich in mitochondria and showed occasional omega-shaped exocytotic figures (Fig. 3d). In SGs of *C. pagurus* and *U. pugilator*, a PDH-granule type very similar in size and shape to PDH-granules of *C. maenas* was found (Figs. 4a–e, 5a, b), and the CHH-granules, as demonstrated in *C. pagurus* (Fig. 4b, c), also resembled the respective type from *C. maenas*. Fig. 5 (a, b) shows that the label is confined to the granule population in an axon profile, whereas the axoplasm is virtually devoid of immunoreactivity.

In a preliminary experiment with *C. maenas*, it was observed that the content of PDH-granules in preterminal axon dilatations increased when animals were kept for three days in complete darkness (Fig. 3e).

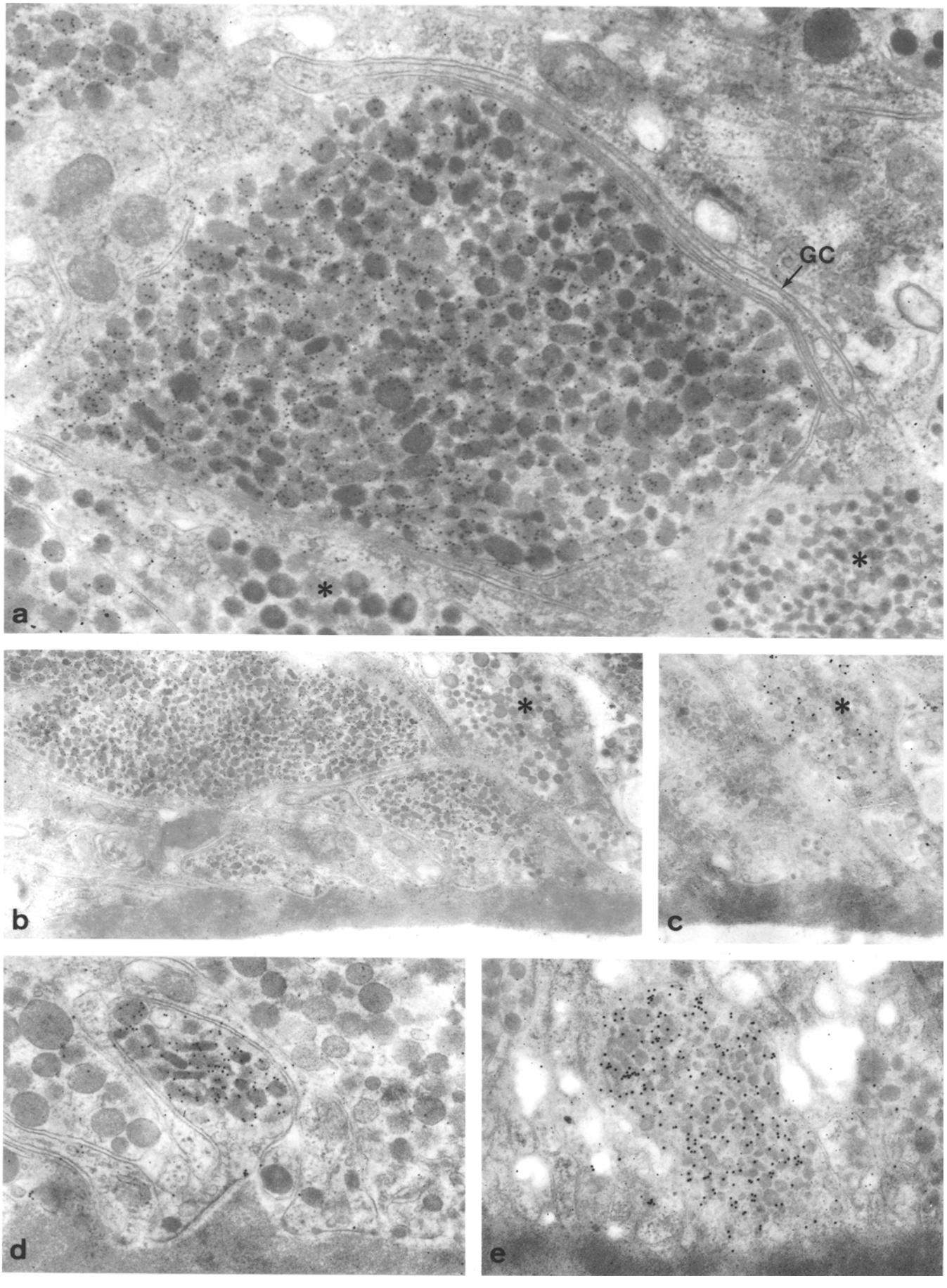
In *O. limosus* two types of PDH-granules were observed (Fig. 5c–f), one of which was similar in size and shape to that of the brachyuran species, and another, slightly larger one (180 nm). Both types may coexist in one axon profile. Frequently, the largest granules displayed broken membranes. They were not observed as exocytotic figures. The immunolabeling in both granule types is, under identical conditions, generally lower than in the brachyuran species.

Immunodot blotting analysis of HPLC-separated extracts from SGs of *C. maenas* and *O. limosus* shows only one of several biologically active peak fractions to react with the PDH-antiserum (Figs. 6, 7). The retention times of the immunoreactive fractions resembled those of synthetic PDH of *Pandalus* and *Uca*.

Discussion

In the PDH of the *Uca/Cancer* type, a Lys residue is found in position 13 instead of Arg in the PDH of *Pandalus*. This ε-amino group together with the free N-terminal obviously accounted for facilitated conjugation to thyroglobulin by glutaraldehyde, leading to a highly immunogenic complex. Only one booster injection was necessary to achieve high titers of specific antibodies as demonstrated by almost complete inhibition of melanophore dispersion in *U. pugilator* in vivo after preincubation of synthetic PDH (Fig. 1). The antiserum permitted clear, background-free localization of PDH-neurons in the eyestalks of *C. maenas* and *O. limosus* by light-microscopic immunocytochemistry (Mangerich et al. 1987). In the present study these observations were extended to semithin and ultrathin sections of SGs in order to identify PDH-axon profiles and neurosecretory granules. In the ultrastructural work, fixation problems had to be overcome that seemed to specifically affect PDH granules.

Fig. 4a–e. PDH-immunoreactive axon profiles (*AP*) and terminals in SG of *C. pagurus* and *U. pugilator*. **a, b** *C. pagurus*: PDH-positive AP and adjacent immunonegative AP with different granule types marked by *asterisks*. In **b**, the three AP to the left of the asterisk-marked immunonegative AP are PDH-positive, although the gold labeling is difficult to see at this magnification. GAR-G10. **a** ×37200, **b** ×15000. **c** Portion of a section adjacent to **b** showing CHH-immunoreactivity in AP marked by *asterisks* in **b** and **c**. GAR Au 30, ×15000. **d** *C. pagurus*: Terminal close to the basement membrane showing round and elongated PDH granules. ×34500. **e** PDH terminal in *U. pugilator*. ×34500. Fixation 3 used throughout. *GC* glia cell process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Fixation procedure 3 (for details, see Materials and methods) permitted the best on-grid immunolabeling and preserved the ultrastructure well.

The observed PDH-granule type can now be considered to be one identified type of approximately five types that have been described in *C. maenas* (Smith 1974) according to purely morphological criteria. Two other types have thus far been characterized in this species with regard to their neuropeptide content; i.e., CHH granules (diameter 120–200 nm, this study) and Leu-enkephalin-containing granules (82 ± 23 nm, Jaros et al. 1985). The PDH-immunoreactive type appears to correspond to Smith's (1974) type 4 and to type 2 in *U. pugilator* as described by Unglaub-Silverthorn (1975). The granules containing CHH correspond most likely to the type 5 described by Smith (1974) in *C. maenas*. Putative CHH granules (diameter 115–240 nm) have previously been identified by Gorgels-Kallen and Van Herp (1981) in the SG of *Astacus leptodactylus*. In size and shape they appear to correspond to the CHH granules in the SG of *C. maenas*. These comparisons are made under the assumption that different fixations do not markedly alter the pattern of granule types.

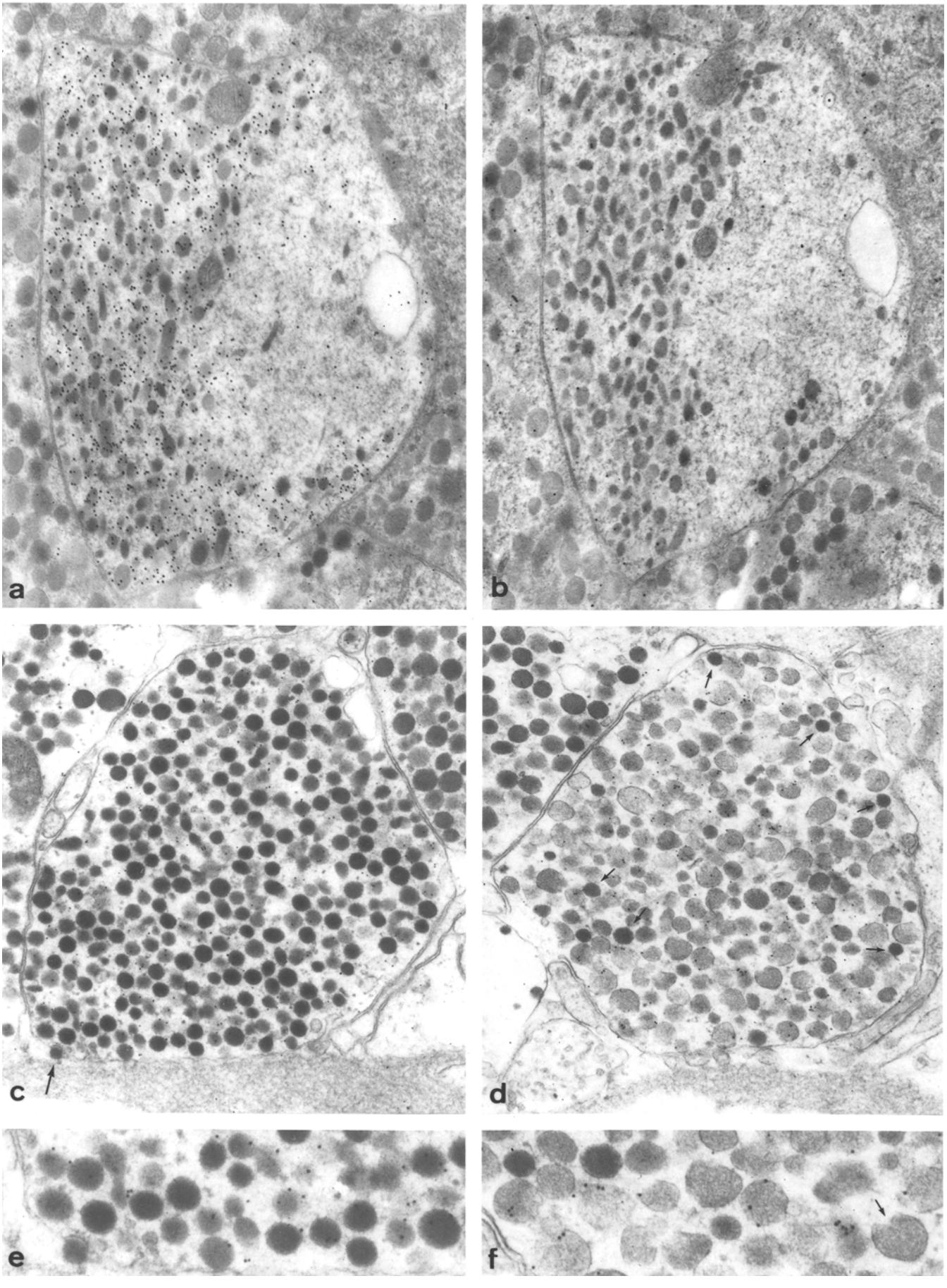
The immunocytochemical results demonstrate that the morphological granule classification may eventually be replaced by direct identification of granule contents. It may be possible to determine whether the number of granule types is actually representative of the number of neuropeptides in the SG and which neuropeptides are colocalized in one type of granule or in axon profiles.

In the SG of *O. limosus* a type of PDH granules apparently similar to the brachyuran type coexists together with a larger type in some axon profiles. The large granules often displayed broken membranes. We do not think that truly different types exist, rather, we propose that the larger granules might be indicative of an aging process that results in osmotically swollen cores. Nordmann (1977) has interpreted the occurrence of pale and swollen granules in the SG of *C. maenas* by this phenomenon. From studies on axon terminals from rat neurohypophysis Nordmann and Labouesse (1981) concluded that similar aging and osmotically sensitive granules may be destined for backtransport into axonal swellings behind the sites of exocytosis. In this context, it seems interesting that we never observed the larger granule type to undergo exocytosis, in contrast to the smaller ones. Close to the sites of release, aggregations of electron-translucent, 50 nm-vesicles and/or larger vacuoles were observed (Fig. 3d). These may reflect endocytotic processes of membrane retrieval after exocytosis, as proposed by Bunt (1969), Nordmann and Morris (1976, 1980) and Nordmann (1979).

The disappearance, in *C. maenas*, of granules from PDH-axon profiles that retained overall immunoreactivity in semithin sections, is obviously a fixation artifact. The same phenomenon was observed in proctolin-containing axon profiles in the pericardial organs (Dircksen, unpublished observations). It is therefore unlikely that this represents a degranulation event, as has been claimed for the SG of *Palaemon paucidens* as a result of strong continuous illumination (Aoto and Hisano 1985). However, that light has an effect on the PDH granule population was shown by our observation that three days of continuous darkness caused an increase of granule content in axon dilatations proximal to the sites of release in the SG of *C. maenas*. In this respect, both results agree and the same granule type may have been identified in both cases, in the study by Aoto and Hisano (1985) indirectly by virtue of the specific response to changes in illumination and in our study by direct identification.

From three of the four species used in this study, the structure of the PDH is not known, although it can be assumed – considering the identity of PDH from *U. pugilator* and *C. magister* (Rao et al. 1985; Kleinholz et al. 1986) – that the two related species *C. maenas* and *C. pagurus* have an identical or very similar PDH. To obtain information on the relationship of the reactive antigen to the known PDHs, we used HPLC fractionation of SG extracts from *C. maenas* and *O. limosus* and immunodot blotting, i.e., a method resembling that used for immunocytochemical detection on sections. As shown in Figs. 6 and 7, the retention times of the immunoreactive peak fractions are identical or very similar to those of synthetic PDH of *Uca* and *Pandalus*. The fact that only one very distinct immunoreactive fraction was revealed is surprising, considering that the zone of biological activity comprises several more HPLC fractions. It is well documented that PDH activity is separable into various fractions by ion exchange chromatography, electrophoresis and HPLC, and up to seven variants have been reported (Kleinholz 1970, 1972; Fernlund 1971; Keller 1977; Keller and Kegel 1984). Apparently, this heterogeneity cannot be explained by the existence of different forms in different structures, which may be concluded from the results that have been obtained by separation of complex extracts, e.g., the whole eyestalks. At least some heterogeneity is also found in isolated SGs (Keller 1977; Keller and Kegel 1984). Its nature is unknown at present. The known sequences are those of the predominant PDH-forms in *U. pugilator*, *P. borealis* and *C. magister*. Our finding that only one distinct fraction reacts with the antiserum indicates that the other bioactive substances in the HPLC profile are either structurally unrelated or related but different in their amino acid sequences. PDH of *Pandalus*, which differs from the hormone of *Uca* by six amino acid residues, does react, although to a slightly lesser degree (Figs. 6, 7). Therefore, the other bioactive but immunonegative substances probably differ even to a greater degree. This agrees with former findings that the SG contains substances with PDH activity that are quite different from the known PDHs (Keller and Kegel 1984).

Fig. 5a–f. PDH-immunoreactive axon profiles (AP) in *U. pugilator* and *O. limosus*. **a** *U. pugilator*: Cross-sectioned AP in the SG to show that immunostaining is restricted to the granule population while granule-free axoplasm is not stained. Note elongated granules. **b** Preabsorption control on section adjacent to **a**. GAR-G10. ×31000. **c–f** *O. limosus*: **c** Terminal showing the PDH-granules typical for this species. Exocytotic figure marked by *arrow*; **d** In another axon profile the granule type (*arrows*) as shown in **c** coexists with larger granules of lower electron density displaying broken membranes (*arrow* in **f**). **e** Enlarged portion of **c**; **f** enlarged portion of **d**. GAR G10. **c, d** ×28500; **e, f** ×58300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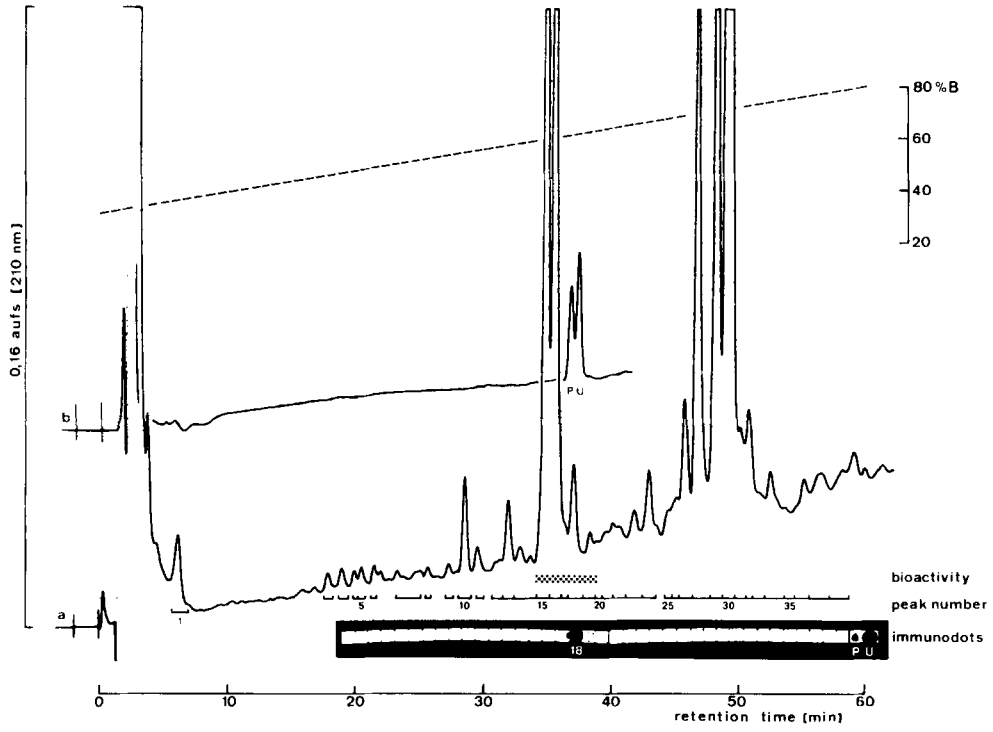

Fig. 6. HPLC of a 2 N-HAc extract of 20 SG from *C. maenas* (**a**) and a mixture of synthetic PDH of *Pandalus* (*P*) and *Uca* (*U*) under identical conditions (**b**). Immunodot blotting of 38 manually collected peak fractions on a nitrocellulose strip shows PDH-immunoreactivity in only one fraction (18) close to the retention times of P and U. At the right end of the strip, authentic samples of P and U (20 ng each) were spotted. Note lower immunoreactivity of P. Note that fractions 15–17 show biological activity but no immunological reactivity. A Waters μ-Bondapak phenyl column (0.39 × 30 cm) connected to a Vydac 5C-201 RP guard column was used. Gradient elution with solvent A: 0.11% TFA and solvent B: 0.10% TFA; 60% CH_3CN from 30% to 80% B in 1 h. Flow rate: 0.9 ml/min

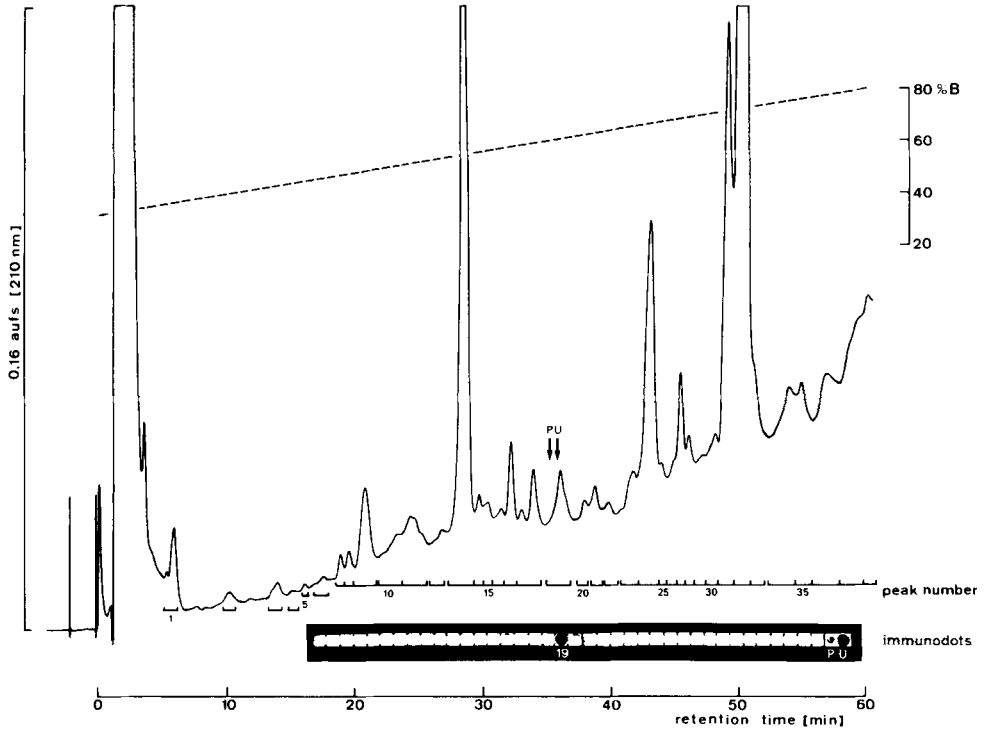

Fig. 7. HPLC of a 2 N-HAc extract of 30 SGs from *O. limosus* and immunodot blotting. Same methodology as in Fig. 6. The biological activity profile was not determined. Only fraction 19 showed PDH-immunoreactivity

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Note added in proof

Rao et al. (J Biol Chem 26:2672, 1987) have reported the structure of a PDH from an insect, *Romalea microptera*. It corresponds to the crustacean PDHs in consisting of 18 residues. The sequence shows 78% homology with the *Uca*/*Cancer*-type PDH.

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