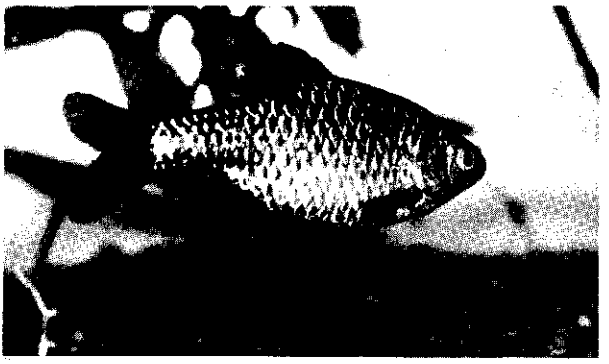
Enteroendocrine cells of the cyprinid fish, *Barbus conchonius*.



Barbus conchonius (Hamilton-Buchanan, 1822),
male at natural size

Promotor: dr. L.P.M. Timmermans, hoogleraar in de algemene dierkunde
Co-promotor: dr. J.W.M. Osse, hoogleraar in de algemene dierkunde

Jan H.W.M. Rombout

Enteroendocrine cells of the cyprinid fish, *Barbus conchonius*

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op woensdag 10 september 1980
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISN - 118084 - 03

Aan mijn ouders
Aan Joke en Jeroen

STELLINGEN

1. Hoewel er diverse aanwijzingen zijn voor een neurale herkomst van de enteroendocriene cellen is een herkomst uit de neurale lijst onwaarschijnlijk.

Dit proefschrift.

2. Het voorkomen van catecholamines in de granula van gastro-enteroendocriene cellen van hogere vertebraten wijst op een terugkoppelingssysteem met het centrale zenuwstelsel via sensorische neuronen onder de basaalmembraan.

Fujita T. en Kobayashi S., In: "International review of cytology" suppl.6 eds. Bourne G.H. en Danielli J.F., Academic Press, London, pp. 187-233 (1977).
Newson B., Ahlman H., Dahlström A., Das Gupta T.K. en Nyhus L.M., Acta Physiol. Scand. *105*, 521-523 (1979).

3. Celvermeerdering vindt bij vissen vanaf het larvale stadium veelal plaats door proliferatie van reeds functionerende cellen.

Dit proefschrift.
Proefschrift H.W.J. Stroband (LH, 10 september 1980).

4. De veronderstelling van Le Douarin e.a. dat de chorda, eventueel in combinatie met de neurale buis, het orgaan is dat direct of indirect de neurale lijst cellen induceert tot adrenerge differentiatie is aan bedenkingen onderhevig.

Le Douarin N.M., Teillet M.A. en Le Lièvre C., In: "Cell and tissue interactions", eds. Lash J.W. en Burger M.M., Raven Press, New York, pp. 11-27 (1977).
Patterson P.H., Ann. Rev. Neurosci. *1*, 1-17 (1978).

5. De hypothese van Fichtelius dat het darmepitheel van vertebraten beschouwd moet worden als een primair lymfoid orgaan is zeker voor beenvissen zeer aannemelijk.

Fichtelius K.E., Exp. Cell Res. *49*, 87-104 (1968).

6. De huidige opvatting dat *Rhabdospora thelohani* (Laguesse, 1895) een holocriene secretorische cel zou zijn in plaats van een visseparasiet dient betwijfeld te worden.

Leino R.L., Cell Tiss. Res. *155*, 367-381 (1974).
Desser S.S. en Lester R., Can. J. Zool. *53*, 1483-1495 (1975).
Grünberg W. en Hager G., Anat. Anz. *143*, 277-290 (1978)
Mattey D.L., Morgan W. en Wright D.E., J. Fish. Biol. *15*, 363-370 (1979).

7. De op kleurmarkeringsexperimenten gebaseerde gastrulatiebeschrijvingen en "fate maps" dienen gezien de beperkingen van deze methode met de nodige voorzichtigheid bekeken te worden.

Ballard W.W., Rev. Roum. Biol. -Zool. *18*, 119-135 (1973).

8. Aangezien de wet op de proefdieren (1977) geënt is op in de bevolking levende gevoelens zou het bezit van een sensorisch zenuwstelsel, in plaats van het bezit van een wervelkolom, als criterium voor registratie centraal dienen te staan.

9. De huidige economische problemen zijn gelet op het rapport van de club van Rome (1972) het gevolg van een "struisvogelpolitiek", hetgeen niet wil zeggen dat uitvoerig naar de aanwezige grondstoffen gekeken is.

Proefschrift van J.H.W.M. Rombout
Enteroendocrine cells of the cyprinid fish, *Barbus conchonius*.
Wageningen, 10 september 1980.

Contents

General introduction

History of the gastro-enteric endocrine cells

Heidenhain (1870) was the first to find in the fundus gland of dogs and rabbits a small cell type that was selectively stained brown after fixation with a solution containing dichromate. The first description of a cell type with a distinct bottle shape in the intestinal epithelium of a lizard, was presented by Nicolas (1891). Ciaccio (1906) called these cells enterochromaffin cells because of their chromaffinity. They are still known as enterochromaffin cells despite attempts to give them other names: Kultschitzky cells, Schmidt cells, Ciaccio cells, Masson cells (named after authors who described them in 1897, 1905, 1906 and 1914 respectively), yellow cells (Schmidt, 1905), basal-granulated cells (Kaufmann-Wolf, 1911; Clara, 1933), chromaffin cells (Kull, 1925) and "Helle Zellen" (Feyrter 1952).

Several authors (Ciaccio, 1907; Danish, 1924; Kull, 1925; Tehver, 1930) have suggested an endocrine function for the enterochromaffin cells because of the basal location of the secretory granules. On the basis of the presence of an apical cell process, first described by Clara (1933) and Patzelt (1936), some investigators ascribed an exocrine function to these cells. Feyrter (1953) even proposed that they may be engaged both in exocrine and endocrine activities. Recently electron microscopic studies of Kobayashi & Fujita (1974) and Osaka et al. (1974) have indicated the exocytotic release of the secretory granules at the base of these cells, and this is considered as convincing evidence of the endocrine nature of the enterochromaffin cells (= EC cell).

Most of the earlier workers believed that there is only one type of enterochromaffin cell. Erspamer (1966) gave the following description: "all these cells which can after formaldehyde fixation give a chromaffin, argentaffin, diazo-coupling and alkaline thioindoxyl reaction and give a yellow formalin induced fluorescence (FIF)". The reactive substance in their secretory granules respon-

sible for these reactions is 5-hydroxytryptamine, also called serotonin or (by earlier workers) enteramine (Erspamer & Asero, 1952). With different silver stains, two main types can be distinguished in the EC cells:

1. argentaffin cells, which are able to reduce silver salts (Masson, 1914);
2. argyrophil (Hamperl, 1932) or argentophil cells (Erspamer, 1937), which can only fixate silver salts and a reducing agent is required to demonstrate the presence of these cells.

Erspamer (1938) and Vialli & Erspamer (1939) suggested that these different silver staining properties were to be attributed to distinct phases in the secretory cycle of one enterochromaffin cell type. More recent electron microscopical results of Singh (1967), who found both argentaffin and argyrophil granules in one and the same cell, seem to support this suggestion. However, after 1967, many electron microscopical and immunohistochemical studies indicated a number of distinct cell types in the gastrointestinal epithelium of amphibia and birds, amounting to about 12 cell types in mammals. The argentaffin cell corresponds to the serotonin containing EC cell, which is found throughout the digestive tract. Most of the other cell types, with different locations in the gastrointestinal tract, are argyrophil. Therefore the name enterochromaffin cell (= EC cell) should be abolished as a general term for gastro-enteric endocrine cells.

In mammals most cell types of the pancreatic islets are present in the gastrointestinal epithelium, this may be explained by their common origin. Therefore, many authors use the term Gastro-Entero-Pancreatic (GEP) endocrine system for the endocrine elements of digestive tract and pancreas together (cf. Fujita & Kobayashi, 1974).

Function

As a result of comparative studies on the endocrine cells of gut and pancreas, the "one cell-one hormone" hypothesis, as demonstrated for pancreatic islet cells, may also be applied to the gastro-enteric endocrine cells. The diversity in endocrine cell types in the mammalian digestive tract is directly related to the number of gastrointestinal hormones that can be demonstrated to be present. At present the biological activity and the amino acid sequences of the following 8 gastrointestinal hormones are known: gastrin, secretin,

cholecystokinin-pancreozymin (CCK-PZ), gastric inhibitory peptide (GIP), vasoactive intestinal peptide (VIP), motilin, somatostatin and substance P (cf. Fujita & Kobayashi, 1977). Also a glucagon-like peptide (GLI or enteroglucagon, recently called glicentin by Pearse, 1979) has been demonstrated in the gastrointestinal epithelium. Based on recent studies neurotensin (Helmstaedter et al., 1977a,b; Sundler et al., 1977; Frigerio et al., 1977; Polak et al., 1977) and possibly some other substances (Grossman et al., 1974) must be considered as gut hormones. The current information on the GEP endocrine system of mammals is summarized in table I.

Table I: GEP-endocrine cells in mammals

Cell type	Location	Hormone
(A	p	glucagon
A (AL	s	glucagon
(L or EG	i	glicentin or enteroglucagon
B	p	insulin
D	p, s, i	somatostatin
D_1 (P)	p, i	bombesin ?
D_2 (F,PP)	p	pancreatic polypeptide (PP)
EC_1 (EC_{sp})	p, s, i	serotonin, substance P
EC_2 (EC_m)	i	motilin
ECL	s	histamin
G (GA	s	mainly gastrin 17
(GI	i	mainly gastrin 34
H	i	vasoactive intestinal peptide (VIP)
I	i	cholecystokinin-pancreozymin (CCK-PZ)
K	i	gastric inhibitory peptide (GIP)
N	i	neurotensin
S	i	secretin

Abbreviations: i: intestine; p: pancreas; s: stomach.
References : Forssmann (1976); Fujita & Kobayashi (1977); Solcia et al. (1978); Buchan et al. (1979); Pearse (1979), Buchan & Polak (1980).

Because of the similarity in chemistry of the gut hormones, two "families" can be recognized (Grossman, 1976; Dockray, 1976): one containing caerulein, gastrin and CCK-PZ, the other containing secretin, glucagon, GIP and VIP. These similarities may suggest a common evolutionary history for hormones of one "family". Therefore it is supposed that caerulein, only known to be present in fish and amphibia, is assumed to be the ancestral hormone for gastrin and CCK-PZ (Larsson & Rehfeld, 1977).

Nearly all GEP endocrine cells of birds and mammals contain biogenic amines: serotonin in EC-cells and catecholamines in other cell types.

Stimulation and granule release

In the cardia, pylorus, and intestine of mammals the basal-granulated endocrine cells are pyramidal or bottle-shaped; they are located on the basement membrane, and extend towards the lumen with a cytoplasmic process covered with a tuft of microvilli. This type of basal-granulated cell is called the "open" type (Fujita & Kobayashi, 1974). The apical part of this cell type is rich in pinocytotic invaginations and vesicles and sometimes a single cilium may extend into the gut lumen (Fujita & Kobayashi, 1971; Kataoka, 1974). Fujita & Kobayashi (1974, 1977) proposed a chemoreceptory function for the "open" endocrine cells, which receive chemical information with their apical end; this stimulus activates or inhibits the basal granule release via an unknown mechanism of intracellular transmission. Each cell type reacts upon specific stimuli with the exocytotic release of granules (Kobayashi & Fujita, 1974; Osaka et al., 1974; Fujita & Kobayashi, 1977). These authors consider the "open" endocrine cells as "taste cells of the gut", however, the synaptical connection with nerves, as present on gustatory cells, are lacking on these cells of the gut. So this "open" type of cell is believed to represent the most primitive endocrine cell, which is self-sufficing in its functioning.

In the gastric corpus of several mammalian species endocrine cells are generally without the cytoplasmic process to the lumen and located flat on the basement membrane. These "closed" types are supposed to react to physical stimuli caused by the food in the intestinal lumen, e.g. pressure on the wall, tension and temperature changes (Fujita & Kobayashi, 1974, 1977).

For the gastrin-producing G cell, which occurs in both "closed" and "open" types, it was suggested that the release of hormone is under direct vagal control (Grossman 1963; Elwin & Uvnäs, 1966), but recent electron microscopical

and pharmacological studies have failed to demonstrate synaptical connections of nerves with these G-cells (cf. Fujita & Kobayashi, 1977). Therefore, Uvnäs-Wallensten (1978) suggests gastrin release directly from gastrin-containing vagal branches.

Endocrine or Paracrine activity

The view of local endocrine activity, proposed by Feyrter (1953) under the name paracrinia, has recently been reinstated for endocrine cells of the digestive tract. It becomes more and more accepted that several substances in these cells do not act as hormones on remote targets by circulating in the blood, but as locally acting substances, that affect neighbouring cells (paracrine). Especially the secreted amine components must be considered as primarily local hormones or even neurotransmitters. Fujita (1976a) proposed that the secretion of transmitter-like substances may stimulate the nerves in the lamina propria or Meissner's plexus and may transmit information from the gut to the central nervous system. This hypothesis has recently been supported by the description of sensory-like neurons in the mammalian gut mucosa, just underneath the epithelium (Newson et al., 1979).

Also of somatostatin, the inhibitor of the release of growth hormone and of the secretion of insulin, glucagon and gastrin, it is believed, presumably for its very short half-life in blood, that its paracrine activities may be more important as its endocrine activities. Larsson et al. (1979) clearly proved the local regulatory function of somatostatin-producing D cells of the stomach by demonstrating long slender basally located processes of this cell type. The processes, localized between epithelium and basement membrane, terminate often in bulbous swellings on G cells, parietal cells and possibly also on other endocrine as well as exocrine cell types. Similar processes are seen in other endocrine cell types, e.g. EC cells, EG cells, COOH-terminal gastrin immunoreactive cells and other unidentified cell types (Larsson & Rehfeld, 1978; Larsson et al., 1979; Buchan & Polak, 1980). The assumption of paracrine activity of at least some of the cell types justifies an extra interest in the regulation of digestion.

Origin of GEP endocrine cells

It has long been believed that GEP endocrine cells originate in the endoderm, although Kull (1925) and Dias-Amado (1925) have already reported

that the basal-granulated cells of the gut first appear in the lamina propria and later penetrate into the mucosal epithelium. Danish (1924) even concluded that human enterochromaffin cells have the same origin as sympathetic nerve cells and migrate together with them in the gut; subsequently, the enterochromaffin cells enter the mucosal epithelium, while the nerve elements remain in the submucosa to form the plexus of Meissner. However, additional support for a neural origin of the enteroendocrine cells had to wait until 1968, when Pearse (1968, 1969, 1973) regarded the GEP endocrine cells as important members of the APUD series: a group of endocrine cells with the APUD characteristic (acronym stands for Amine Precursor Uptake and Decarboxylation). As the APUD cells share this amine-handling mechanism with a number of neurons and neural crest derived pigment cells and as some of the APUD cells originate in the neural crest (e.g. ultimo-branchial and thyroid C cells, carotid body type I cells, adrenomedullary cells and cells of the paraganglia), a neural crest origin was suggested for all members of the APUD series. At present over 40 endocrine cell types belong to the APUD series (Pearse, 1979; Pearse & Takor Takor, 1979). Several cell types belonging to this series were already described by Feyrter (1953) as "Helle Zellen" which constitute the "Diffuse Endokrine Organe".

After applying the formalin induced fluorescence technique (FIF) to mouse embryos, for histochemically demonstrating amine containing cells, Pearse & Polak (1971) described migration of neural crest cells into gut mucosa and pancreas. Unfortunately, however, experimental embryological studies failed to support a neural crest origin for GEP endocrine cells; Pictet et al. (1976) carried out neural crest extirpation; Le Douarin & Teillet (1973) and Andrew & Kramer (1979) applied neural crest transplantation with a stable nuclear marker and Andrew (1976a,b) with ^{3}H-thymidin as marker; Andrew (1963, 1974) and Rawdon et al. (1980) explanted presumptive gut before the arrival of neural crest cells. As a consequence Pearse & Takor Takor (1976) have revised their hypothesis on the origin of all members of the APUD series as being derivatives of neurectoderm or specialized (placodal) ectoderm. Recently Fontaine & Le Douarin (1977) even excluded the neurectoderm as a possible source of gut endocrine cells, by transplanting whole epiblasts from quail to chick (stable nuclear marker technique). Further evidence against a neural crest origin of GEP endocrine cells was provided by the existence of mixed endocrine-exocrine cells in the pancreas (Melmed, 1979). These results support the hypothesis of Cheng & Leblond (1974a,b) suggesting a "unitarian" origin of all intestinal epithelial cells, including enteroendocrine cells; mainly on

the basis of a similar turn-over time and the presence of the same marker organelle (phagosome) in both endocrine cells and enterocytes of the small intestine of the mouse. However, recently Fujita & Kobayashi (1977) and Tsubouchi & Leblond (1979) concluded from their experiments a very long life cycle for mammalian enteroendocrine cells compared to that of other epithelial cells.

As discussed above, direct and unequivocal evidence of a neurectodermal or endodermal origin of GEP endocrine cells is still not available. More and more circumstantial evidence for a neural origin is provided by different research groups. Next to neurotransmitter-like substances several neuropeptides have been demonstrated in the endocrine cells of the gastrointestinal tract (Pearse, 1977) and the enterohormones gastrin (Vanderhaegen et al., 1975), CCK (Pearse, 1977), VIP (Bryant et al., 1976; Larsson et al., 1976a,b; Said & Rosenberg, 1976) motilin (Yanaihara: cited by Fujita & Kobayashi 1977) and glucagon (Lorén et al., 1979) have been found in the brain with immunohistochemical techniques. Moreover Schmechel et al. (1978) have shown neurone-specific enolase (NSE; EC 4.2.1.11), one of the three iso-enzymes of the glycolytic enzyme enolase, and isolated from mammalian brain, to be present not only in neurons but also in cells of the APUD series, including pancreatic islet cells. Also the presence of the basal cytoplasmic processes of some gut endocrine cells, which are possibly comparable to neurosecretory neurons, may be in support of a neural origin (Larsson et al., 1979). Following the earlier conclusions of Kull (1925) and Dias-Amado (1925), Osaka & Kobayashi (1976) described the penetration of different endocrine cell types from the lamina propria into the mucosal epithelium of 4-5 months old human fetuses; this suggests an extra-endodermal, possibly a neural origin.

Fujita (1976) proposed that the GEP endocrine cells are paraneurons. The "family of paraneurons", which are "brothers of neurons" represents a larger system than the APUD series. More recently, Pearse (1977, 1979) and Pearse & Takor Takor (1979) arranged all members of the APUD series in the "Diffuse Neuro Endocrine System" (DNES), the third division of the nervous tissue, that is "complementary but essentially similar to the two senior divisions, called respectively somatic and autonomic". With regard to the GEP endocrine cells Pearse (1977) adjusted the APUD concept to: "the 40 cells of the APUD series, producing amines and/or peptides active as hormones or as neurotransmitters, are all derived from neuroendocrine-programmed ectoblast". No direct experimental evidence is available for or against this hypothesis.

Enteroendocrine cells in fishes

Endocrine cells are found in the alimentary tract of all classes of chordates and also in some groups of invertebrates (cf. Clara, 1933; Erspamer, 1958; Fujita & Kobayashi, 1977). Kataoka (1974) even recognized 6 different cell types in the digestive tract of a frog, on the basis of the ultrastructure of the secretory granules.

Studies on gut endocrine cells of fish are scarce and mainly based on light microscopy. Except Kull (1925) and Uggeri (1938), several of the early microscopists did not find endocrine cells in the gastrointestinal tract of teleosts (Cordier, 1926; Clara, 1933; Vialli & Erspamer, 1933). More recently Erspamer (1958) described enteroendocrine cells in some teleost species, but no serotonin and hence no argentaffinity was found (Kull, 1925; Uggeri, 1938; Erspamer, 1954, 1958; Fänge, 1962), except in eel and trout (Read & Burnstock, 1968) and *Mugil auratus* (Gabe & Martoja, 1971).

Recently at least three types of gastro-enteric endocrine cells have been described for some cartilaginous fish species (Gabe & Martoja, 1972; Tagliafierro & Faraldi, 1976; Tagliafierro et al., 1976) and for *Chelmon rostratus* (Teleostei; Ling & Tan, 1975), two types for *Protopterus annectens* (Dipnoi; Gabe, 1973), *Mugil auratus* (Teleostei; Gabe & Martoja, 1971) and *Perca fluviatilis* (Teleostei; Noaillac-Depeyre & Gas, 1979), and one type for *Chimaera monstrosa* (Holocephali; Tue, 1975), *Hoplosternum thorocatum* (Teleostei; Huebner & Chee, 1977) and for some cyclostomes (Van Noorden, 1972; Van Noorden & Pearse, 1974; Östberg et al., 1976). Only the results for *Chelmon rostratus*, *Hoplosternum thoracatum*, *Perca fluviatilis* and cyclostomes have been obtained with electron microscopy.

Enteroendocrine cells have not yet been described for the stomachless digestive tract of cyprinids. Compared to mammals, cyprinids may have a less complicated endocrine regulatory system in the gut because of the absence of peptic digestion and intestinal multicellular glands.

For the present study a tropical species of the cyprinids, *Barbus conchonius* is used, because fertilized eggs can easily be obtained. The results will be presented in 6 chapters, 5 of which are publications:

Chapter 1: morphology and histology of the digestive tract of the adult *B. conchonius*, and the presence and structure of several types of enteroendocrine cells.

Chapter 2: endocrine cell types of the pancreatic islets in relation to the enteroendocrine cells.

Chapter 3: distribution and possible functions of the enteroendocrine cells.

Chapter 4: embryonic development of the digestive tract and the possible origin of the enteroendocrine cells.

Chapter 5: migration and differentiation of neural crest cells and their possible contribution to the enteroendocrine cells.

Chapter 6: proliferation and differentiation of intestinal epithelial cells during development, and renewal and origin of the enteroendocrine cells.

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Cell and Tissue Research

Chapter I

Enteroendocrine Cells in the Digestive Tract of *Barbus conchonius* (Teleostei, Cyprinidae)

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Summary. Just as in other cyprinids, three zones can be distinguished in the digestive tract of *Barbus conchonius*. A fat absorptive zone (65–75%), including the intestinal bulb, is followed by a protein absorptive zone (25–35%) and a small ion and water absorptive zone (< 5%). The main characteristics of these zones are described.

Four types of enteroendocrine cells can be distinguished between the intestinal epithelial cells. The number decreases in the caudal direction, and there are very few in the protein absorptive zone. All the enteroendocrine cells are argyrophilic and differ mainly in the size and shape of their secretory granules. Serotonin producing and hence argentaffin cells have not been found. Amine precursor uptake and decarboxylation (APUD) by the enteroendocrine cells of adult fishes has not been observed. The possible functions of the enteroendocrine cells are discussed.

(Auto-)phagosomes, common in epithelial cells of the gut of *B. conchonius*, show similar staining characteristics as the enteroendocrine cells; their function is discussed.

Key words: Enteroendocrine cells (Cyprinidae) – APUD – (auto-)phagosomes – Ultrastructure.

Introduction

The endocrine function of the basal granulated cells or enterochromaffin cells in the gastrointestinal tract of vertebrates is generally accepted. These cells are

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characterized by argentaffinity (serotonin-production) or argyrophilia. Based on ultrastructural features, 6 to 11 types of enteroendocrine cells can be distinguished in mammals (Capella et al., 1969; Forssmann et al., 1969; Vassallo et al., 1969; Pearse et al., 1970; Ferreira, 1974; Zaviačič et al., 1976).

The search for enteroendocrine cells in fishes, however, has been less successful. Enterochromaffin cells were found only in some species (review Pentillä, 1966). More recently, argyrophil cells were observed in several species (Erspamer, 1954, 1958; Gabe and Martoja, 1971, 1972; Van Noorden et al., 1972; Gabe, 1973; Van Noorden and Pearse, 1974; Östberg et al., 1976). Serotonin-containing or argentaffin cells were never found in teleosts or cyclostomes, with the exception of *Anguilla occidentalis australis, Salmo trutta* and *S. irideus* (Read and Burnstock, 1968) and *Mugil auratus* (Gabe and Martoja, 1971). Electron microscopic studies have been carried out only on cyclostomes (Van Noorden, 1972; Van Noorden and Pearse, 1974; Östberg et al., 1976). With this method, only one enteroendocrine cell type was found.

With biochemical methods several enterohormones (secretin, cholecystokinin-pancreozymin) have been found in the intestine of teleosts (Nilsson, 1970; Barrington and Dockray, 1972; Dockray, 1974, 1975). This suggests that different types of enteroendocrine cells may be present in the digestive tract of these fishes. For the study of the enteroendocrine system in fishes, a species of cyprinids has been selected; the absence of stomach and multicellular exocrine glands in the intestinal wall makes the cyprinids particularly suitable for studying the enteroendocrine system.

This study is based on the intestine of *B. conchonius*, which has not been described before. For this reason, a detailed description will be presented.

Materials and Methods

Adult specimens of *B. conchonius*, reared in our laboratory at 23° C and fed with Trouvit pellets (Trouw & Co., Putten, Holland) were killed by decapitation.. The intestine was dissected and parts were fixed according to various methods.

Histological Staining Methods. As a control for the methods to be used, several mice were killed and parts of the duodenum were dissected. Parts of the gut of fish and mouse were fixed in the following solutions: 10% neutral formol, Helly, 2% glutaraldehyde buffered with 0.1 M cacodylate pH 7.2, or frozen in liquid nitrogen followed by freeze-drying and postfixation for 2 h in paraformaldehyde vapour at 80° C (Falck, 1962). The material was vacuum-embedded in Paraplast Plus (Shwerwood) and sectioned serially at 3 microns. The sections were stained with PAS, Masson-Hamperl's argentaffin reaction (Singh, 1964a), the argentaffin reaction according to Jacquier Burtner and Lillie (1949), the argyrophil reaction according to Singh (1964b), MacConnaill's lead haematoxylin (Solcia, 1969), Schmorl's ferricyanide test (Pearse, 1972) and the diazo-reaction with Fast Garnet GBC (Pearse, 1972).

Formalin – Induced – Fluorescence (FIF) for the Demonstration of Serotonin. Parts of the intestine were fixed according to Falck (1962). Sections of 3 microns were examined with a Zeiss fluorescence Standard microscope with an HBO-50 superpressure mercury lamp and with reflector FT 420, selection filters BP 405: 5, BP 405: 14 and barrier filter LP 418 (epi-illumination).

Amine – Precursor – Uptake and Decarboxylation (APUD). In vivo-incubation: Adult fishes were injected intraperitoneally with L-dihydroxyphenylalanine (100 mg L-DOPA/kg body weight) or with 10–40 µCi L-3,4-dihydroxy(ring 2,5,6-^{3}H)phenylalanine (spec. act.: 26 Ci/mmol, The Radiochemical

Centre, Amersham, England). After 4 h incubation, the animals were killed and the intestine was fixed according to Falck (1962) or in 10% neutral formol. The sections were examined with the fluorescence microscope or prepared for radioautography using Kodak NTB-2-emulsion.

In vitro-incubation: Small fragments of intestine were incubated for 1 h at 25° C in an aerated Tyrode-solution containing ^{3}H-L-DOPA (35 µCi/ml.). The material was treated as described above and prepared for radioautography.

Electron Microscopy. Small parts of intestine of 10 adult fishes were fixed for 15 min at 0° C in a mixture containing 1% OsO_4 and 2% glutaraldehyde buffered with 0.1 M cacodylate pH 7.2 and postfixed for 1 h at 0° C in a mixture containing 1% OsO_4, 2% glutaraldehyde and 1% potassium dichromate. The tissues were embedded in epoxy-resin and ultrathin sections were cut on an LKB-Huxley microtome. Sections were mounted on copper-grids and stained with saturated uranyl acetate followed by Reynold's lead citrate (1963). Photographs were taken with a Philips EM 300 electron microscope.

Results

Morphology and Histology of the Digestive Tract

The length of the stomachless digestive tract of *B. conchonius* is variable (2 to 4 times the standard length, the total length without tail), and an exact description of the curvatures of the intestine cannot be given. The first part of the gut, the intestinal bulb, is a straight tube, about one fifth the length of the intestine. The diameter of the intestinal bulb depends upon the feeding conditions, but is larger than the diameter of the remaining part of the gut. The intestinal wall is very thin. Its mucosa and submucosa are arranged in intestinal folds of variable height. After feeding, the distension of the gut results in lower folds and an increasing distance between folds. In the intestinal bulb, a tenfold increase of the diameter is possible.

The pancreatic duct and bile duct enter the intestinal bulb anteriorly after about 7% of the length of the gut. The gut is surrounded by the hepatopancreas containing the gallbladder and the spleen.

As in other cyprinids, three morphologically and functionally different zones can be distinguished in the digestive tract. The first zone is covered with an epithelium containing many osmiophilic vacuoles mainly localized in the apical part of the fold. This fat absorptive zone has a length of 65–75% of the whole length of the gut. The second part of the gut, a protein absorptive zone (25–35%), is characterized by the presence of many PAS-positive "supra-nuclear bodies" and by a distinct pinocytotic activity in the apical parts of all epithelial cells from the base to the top of the fold. The size of these bodies increases markedly in the caudal direction.

The third zone of the intestine, probably an ion- and water absorptive zone (rectum), is very short (< 5%). The transition between the different zones is rather abrupt.

The intestinal epithelium mainly consists of absorptive cells (diameter 3 µm, length 30 µm) with long slender microvilli. Many goblet cells (about 6%), regularly scattered over the whole intestinal epithelium, and enteroendocrine cells can be observed between the absorptive cells. Many PAS-positive round to oval structures (up to 12 µm) are present in the epithelium, and are remarkably similar to the serotonin producing EC cell in mammals, as regards argentaffinity, argyrophilia, lead haematoxylin-positivity and the yellow FIF-reaction (Fig. 1.). Electron

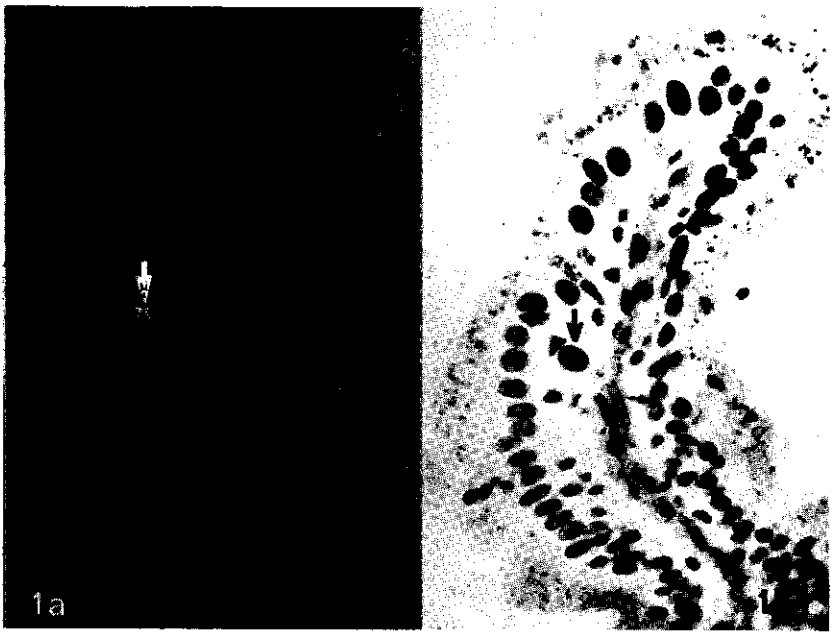

Fig. 1 a and b. Cross section through an intestinal fold of the fat absorptive zone. Fixation 10% formalin. **a** yellow formalin-induced fluorescence (FIF). **b** argyrophil staining in the same section. Note the oval structure in the epithelium

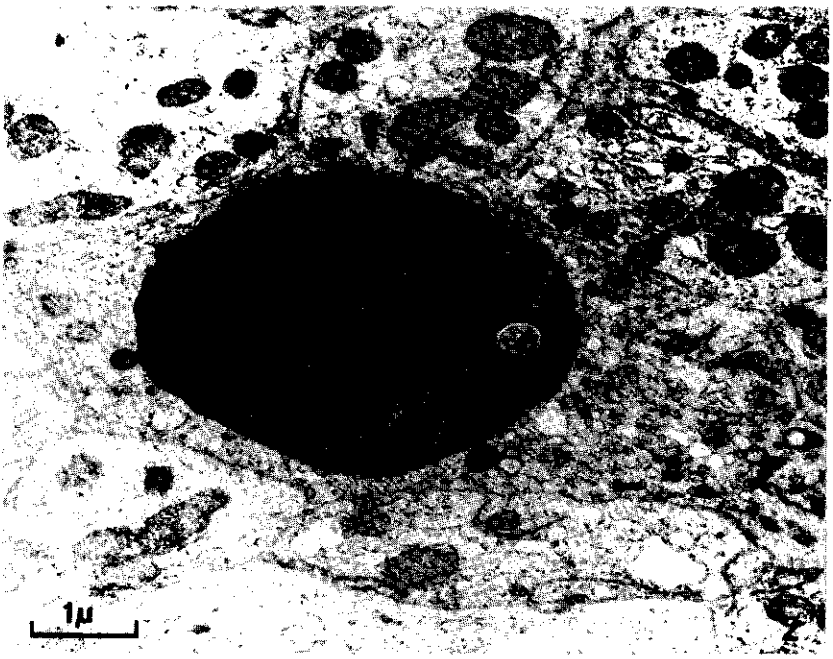

Fig. 2. Electron micrograph of the basal part of a fat absorptive cell in the intestinal bulb. Note the large (auto-)phagosome and the irregular shape of the cell. × 13,500

Table 1. Comparison of different staining methods after various fixations on enteroendocrine cells in the intestinal bulb of *B. conchonius* (B) and the duodenum of the mouse (M)

Staining		Fixation			
		Falck	Formol	Helly	Glutaraldehyde
Argentaffin I	M	++	++	++	++
	B	−	−	−	−
Argentaffin II	M	+++	±	++	++
	B	−	−	−	−
Argyrophil	M	+++	+	++	++
	B	++	−	−	−
Lead haematoxylin	M	+++	+++	++	+++
	B	±	±	−	−
Schmorl	M	+++	±	+	±
	B	+	−	−	−
Azo-coupling	M	+	+	++	++
	B	−	−	−	−
FIF (serotonin)	M	+++	−	−	−
	B	−[a]	−[a]	−	−

− = negative; ± = dubious; + = weak positive; ++ = positive; +++ = strong positive. Argentaffin I: Singh (1964a) Argentaffin II: Jacquier Burtner and Lillie (1949)

[a] Yellow fluorescence of (auto-)phagosomes

microscopical examination proved these structures to be (auto-)phagosomes (Fig. 2). Most of them are located in the basal parts of absorptive cells, some of which have an irregular shape.

The submucosa and epithelium are invaded by many lymphoid cells, macrophage-like cells and granulocytes (eosinophilic leukocytes and mast cells).

Enteroendocrine Cells

Light microscopical and ultrastructural observations have clearly shown the presence of enteroendocrine cells in the digestive tract of *B. conchonius*, but it proved difficult to demonstrate enteroendocrine cells with selective stains for light microscopy. For this reason the histological reactions were also carried out on the duodenum of the mouse (Table 1). Fixations according to Falck (1962) followed by argyrophilic staining according to Singh (1964 b) proved to be the most suitable method for examining the enteroendocrine cells in *B. conchonius* (Fig. 3). Lead haematoxylin and Schmorl's ferricyanide produced a weak positive reaction. By applying these methods, two cell types can be distinguished: an "open type" with a cytoplasmic process extending to the lumen and a "closed type", located close to the basement membrane and isolated from the lumen. Enteroendocrine cells of the "open type" outnumber the cells of the "closed type". A slightly coloured zone was visible above the nucleus of most cells of the "open type". The highest percentage of enteroendocrine cells (3% of the epithelial cells) is found in the first part of the

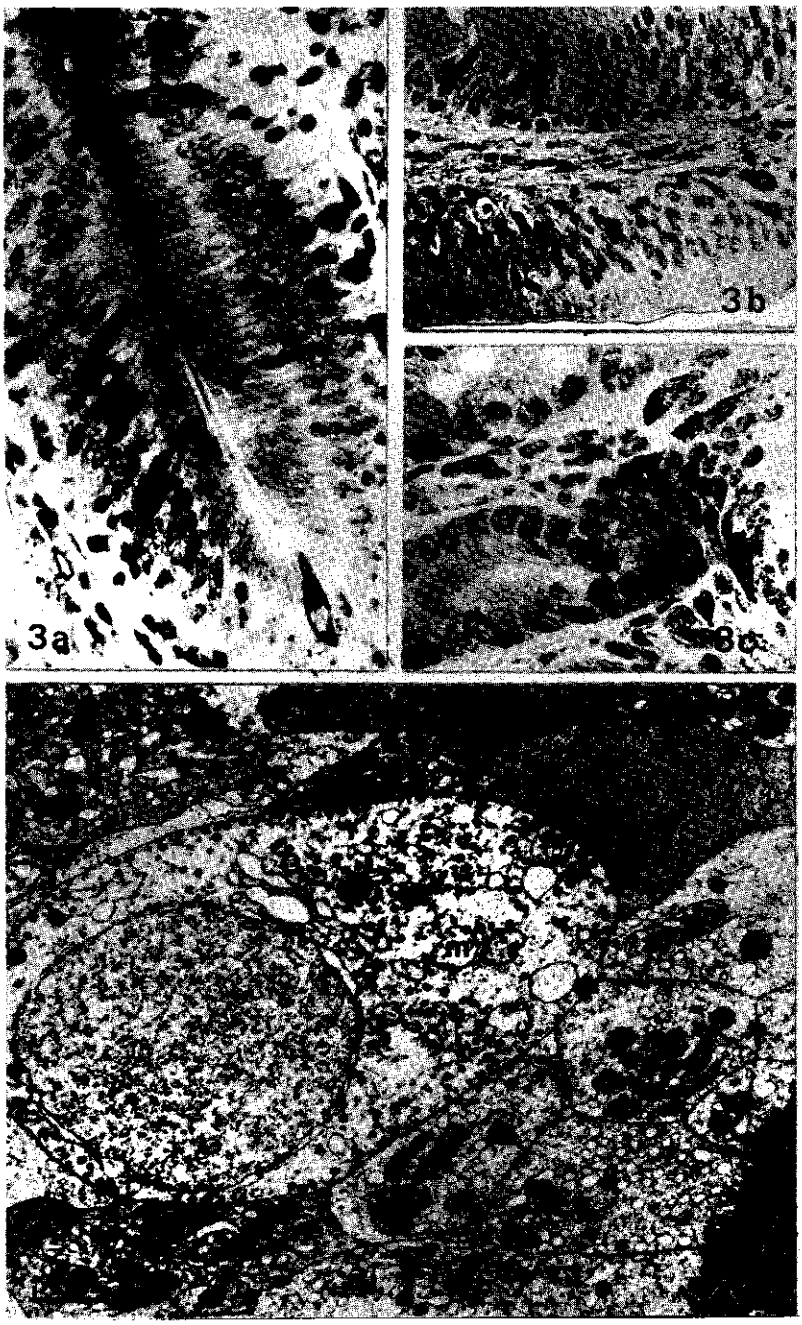

Fig. 3a–c. Intestinal bulb of *B. conchonius* **a** and **b** and duodenum of the mouse **c**. Argyrophil staining after fixation according to Falck. **a** basal part of two intestinal folds with enteroendocrine cells of the "open" type. **b** enteroendocrine cell of the "closed" type (arrow). **c** Note the darkly stained enteroendocrine cells

Fig. 4. Electron micrograph of a type I enteroendocrine cell. Note the small electron dense granules (123 nm), *bm*, basement membrane; *G*, Golgi apparatus; *L*, lysosome; *m*, swollen mitochondrion. ×9900

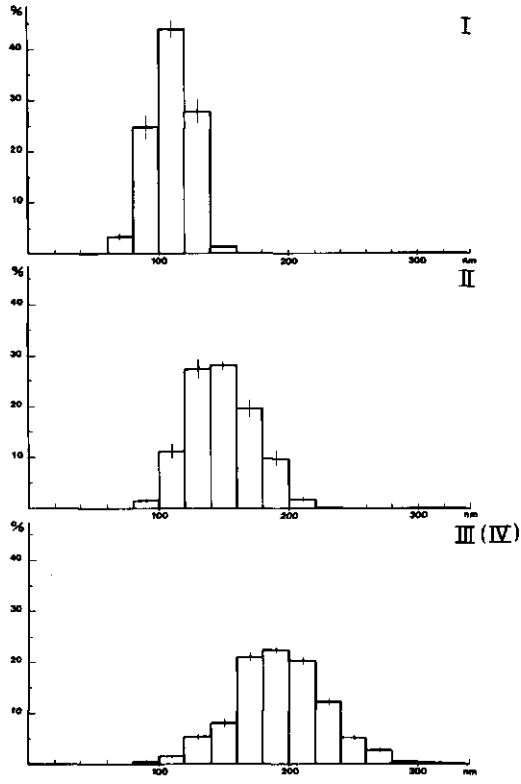

Fig. 5. Size distribution curves of the granules of the four types of enteroendocrine cells. All the granules of the cells examined for Table 2 were measured along their longest axis

Table 2. Enteroendocrine cells in *B. conchonius* (electron microscopy)

Type	Granules			Cell shape	Number[b]
	size (nm)[a]	SE	shape		
I	123	1.4	round-slightly oval	closed	5
II	170	4.5	round-slightly oval	open	8
III	231	3.3	round-slightly oval	open	12
IV	237	5.0	round-irregular	open?	7

[a] The average diameter of the largest granules (30% (III, IV), 40% (II) and 45% (I) of all the granules)
[b] The number of cells examined

intestinal bulb. This percentage decreases in the caudal direction, and in the protein absorptive zone the enteroendocrine cells are scarce.

A FIF-reaction of serotonin or chromaffinity (Helly) has not been observed. Also no amine precursor uptake and decarboxylation (APUD) after administration of (^{3}H)-L-DOPA in vivo or in vitro has been examined. With radioautography some labelled cells were seen in the submucosa, the muscularis and also in the spleen.

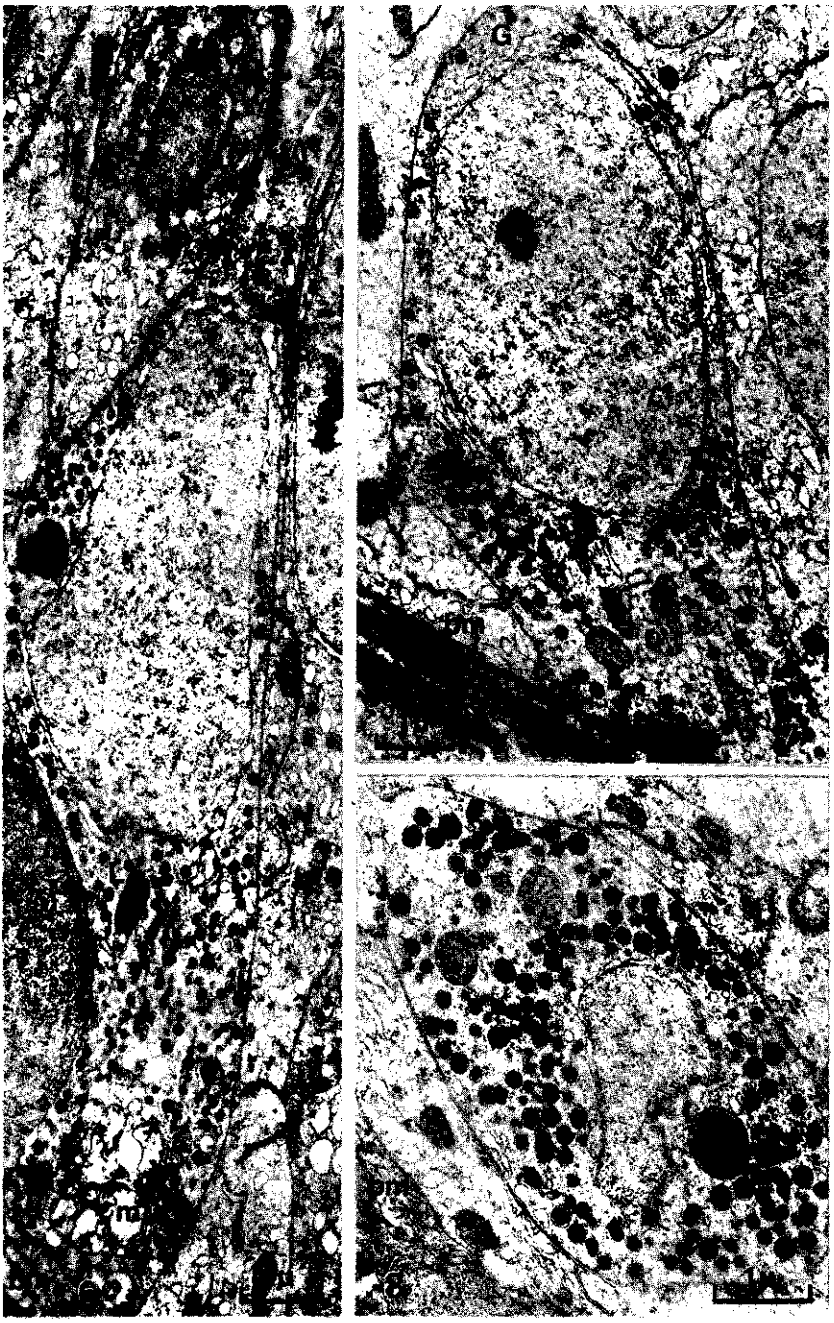

Fig. 6. Electron micrograph of a type II cell. Note the size of the granules (170 nm). ×9900

Fig. 7. Electron micrograph of a type III cell. Note the large granules (231 nm). ×9900

Fig. 8. Electron micrograph of a type IV cell. Note the round to irregular granules (237 nm). ×12,600

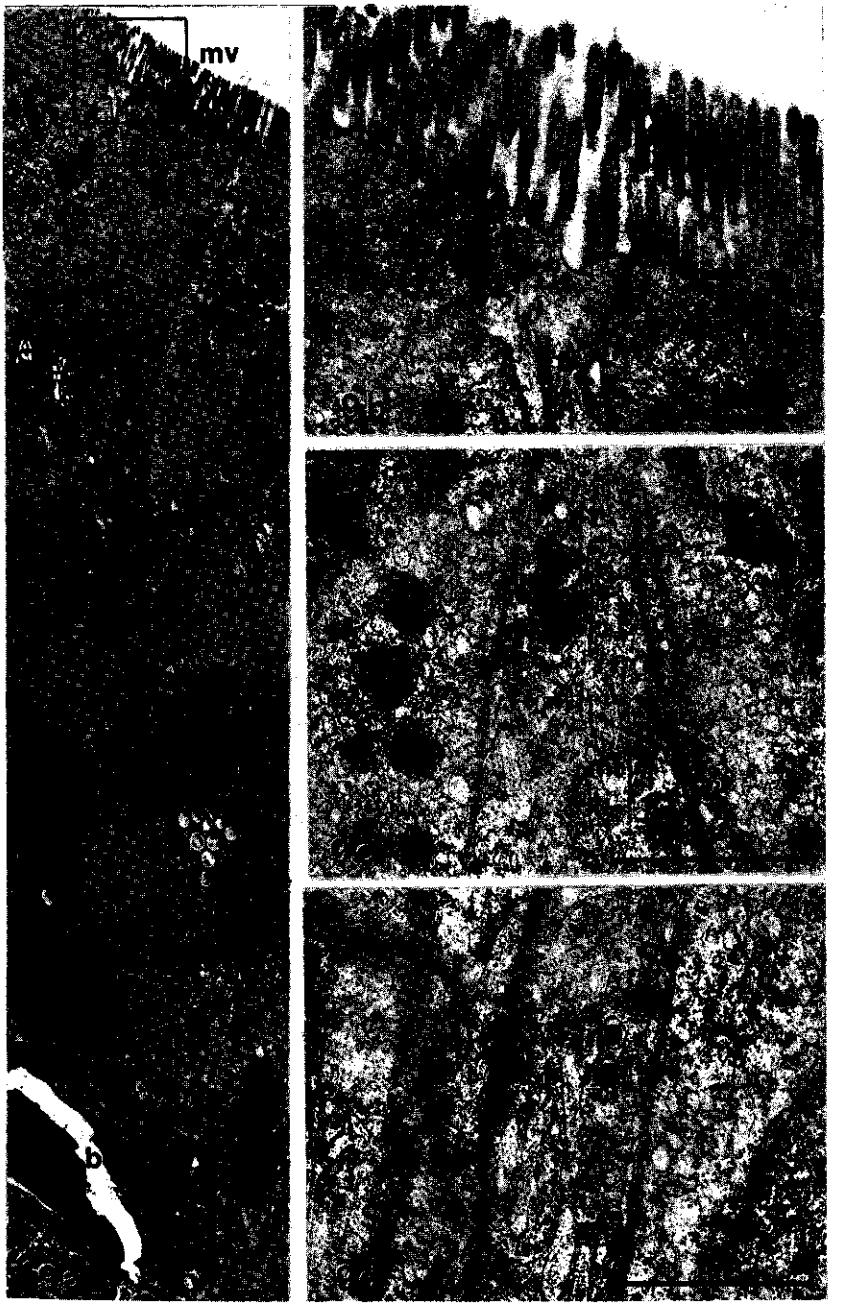

Fig. 9a–d. Electron micrograph of a type III cell. **a** Note the long and slender shape of the cell ×4500. **b** Apex of the same cell with microvilli and microtubules. ×20,700. **c** The subapical part of this cell with many microtubules and a few multivesicular bodies. ×26,100. **d** The supranuclear part of this cell with the tubular like structures (note the low electron dense contents), many ribosomes and polyribosomes and a few granules. ×26,100. *bc*, blood capillary; *bm*, basement membrane; *L*, lysosome; *mb*, multivesicular bodies; *mt*, microtubules; *mv*, microvilli

Electron Microscopy

Mainly based on the size and shape of the granules, four types of enteroendocrine cells can be distinguished in the intestine of *B. conchonius* (Table 2). The difference in size between the granules of the four cell types is illustrated with size distribution curves (Fig. 5). Nearly all granules have electron dense cores, visible by staining with lead citrate, and narrow electron lucent halos. The mitochondria of the enteroendocrine cells may be swollen, in contrast with those of the neighbouring epithelial cells and most of them are located in the basal part of the cell. A distinct Golgi apparatus is usually present above the nucleus. Frequently, lysosomes can be observed some of them containing one or a few granules.

The cells of type I have round to slightly oval granules with an average diameter of 123 nm (Fig. 4). They rest on the basement membrane between the basal part of the epithelial cells. The apical part is rounded, and a cytoplasmic process to the lumen was never observed ("closed type"). This type usually stains more lightly than the neighbouring epithelial cells.

Type II cells have round to slightly oval granules with an average diameter of 170 nm, mainly located below the oval nucleus (Fig. 6). Generally, this type contains a cytoplasmic process directed towards the lumen ("open type"). Probably due to their slender and elongate shape, these cells have not been found extending from basement membrane to lumen in one section. The apical part of probably a type II cell was observed to bear microvilli and to contain small vesicles and microtubules; a short cilium extended in the intercellular space below the desmosomes.

Type III cells contain round to slightly oval granules with an average diameter of 231 nm (Fig. 7). Usually, these cells have a cytoplasmic process directed towards the lumen. Only one cell was found extending from basement membrane to lumen (Fig. 9 a). The oval and generally lobated nucleus of this cell is situated below the level of the nuclei of the absorptive cells. Most of the granules are located in the basal part of the cell but some are perinuclear and supranuclear, close to the Golgi apparatus. The narrow subapical part of the cell, near the intestinal lumen, contains a poorly developed rough endoplasmic reticulum and many free ribosomes and polyribosomes. Many longitudinally oriented tubular-like-structures (probably smooth endoplasmic reticulum), with a diameter of 100–200 nm and a content of low electron density, are present in the subapical and supranuclear cytoplasm (Fig. 9 d). The narrow apical end bears microvilli of normal length. A clear terminal web has not been observed below these microvilli (Fig. 9 b), but many longitudinally oriented microtubules, small vesicles and multivesicular bodies are present in the apical cytoplasm (Fig. 9 c).

Type IV cells contain round to irregular granules with a longest axis of about 237 nm (Fig. 8). The size distribution curve for the granules is about the same as for type III cells. The cells of this type, which are the least abundant, may belong to the "open type".

Discussion

According to its feeding-habits, *B. conchonius* may be considered as an omnivorous teleost. Variability of the gut is generally found in omnivorous fish (Kapoor et al.,

1970). Cyprinids do not have a stomach, as is shown by the absence of gastric glands, the entrance of the bile duct and the pancreatic duct immediately after the oesophageal sphincter, and by the fat absorptive capabilities of epithelial cells of the first part of the digestive tract, the intestinal bulb (Gauthier and Landis, 1972; Stroband, 1977). The gut of *B. conchonius* fits the description of McVay and Kaan (1940, goldfish), who were the first to observe a regional differentiation in the intestinal tract of cyprinids. The histological and ultrastructural characteristics of the different zones are similar to those described by Gauthier and Landis (1972), Noaillac-Depeyre and Gas (1973 a, b) and Stroband (1977) for goldfish, carp and grasscarp respectively.

The presence of enteroendocrine cells between the fat absorptive cells in the digestive tract of cyprinids has not previously been described. On the basis of the present ultrastructural results four types of enteroendocrine cells can be distinguished in the intestine of *B. conchonius*. Most of these cells are of the "open type". Cells of this kind may be receptor-sensory, receiving specific chemical information at the apex that stimulates or inhibits secretion at the base (Fujita and Kobayashi, 1974). This hypothesis is supported by the presence of microvilli, small vesicles (pinocytosis?) and longitudinally oriented microtubules in the apical end of cell types III and probably II; these features are also described in receptor cells of taste buds (Murray, 1973). The presence of cilia on enteroendocrine cells of a frog (Kataoka, 1974) and in the dog G cell (Fujita and Kobayashi, 1971) may support this hypothesis. The present results and recent observations in larvae justify the view that cilia might be of general occurrence in one or more enteroendocrine cell types of *B. conchonius*. Enteroendocrine cells of the "closed type", in mammals located mainly in the stomach, are also present in the intestine of *B. conchonius*. Fujita and Kobayashi (1974) assumed this cell type to be stimulated by physical stimuli (pressure and tension, temperature). Both types may also receive humoral stimuli from the bloodstream that influence the release of granules.

In mammals, 6 to 11 types of enteroendocrine cells are known (Table 3). The table shows a strong variability in the size of the granules for a given cell type between different species. Therefore, only the location, and the different shape and electron density of the granules can be standard for comparison. According to the modified list of Wiesbaden (Solcia et al., 1973) and a comparative study of Ferreira (1971), the A and L cells probably represent the same type. It is also supposed that the D and M cell are variants of the S cell. Serotonin, gastrin and enteroglucagon are known to be enterohormones of EC, G and A cell respectively. In stomachless cyprinids the gastrin producing G cell, with granules of a very low electron density, are absent. None of the four cell types in *B. conchonius* shows any resemblance to the ECL cell, which is characterized by granules with a small irregular core and a large lucent halo. The granules of type IV show a great similarity to those of the EC cell. However, no serotonin fluorescence (hence no argentaffinity) can be demonstrated, which is in agreement with the observations of Erspamer (1954, 1958) and Fänge (1962) on the gastrointestinal tract of teleosts and cyclostomes. So the presence of EC cells in *B. conchonius* is questionable, but might be expected. According to biochemical studies of Nilsson (1970), Barrington and Dockray (1972) and Dockray (1974, 1975) on the gastrointestinal tract of teleosts, the enteroendocrine cells of *B. conchonius* are likely to be involved in the production of

Table 3. Types of enteroendocrine cells in mammals (based on literature)

Cell type	Location[b]	Postulated product	Granules[c]				
			Size (nm)				Electron density
			rabbit[1]	cat[2]	pig[8]	human[4, 16]	
EC	stomach intestine pancreas	serotonin[a] + polypeptide (motilin[13, 14, 19])	160–410 250–850	240 350	350 180–670 (450)	200 400	high
ECL	stomach	histamin[3] + polypeptide	110–400	300–350	110–570	300–800?	high; irregular core
G	stomach	gastrin[a]	160–450	150–400	120–390	180	very low to high in one cell
D	stomach intestine pancreas	secretin, pancreozymin or enterogastron[6, 16] somatostatin[18] vasoactive intestinal peptide[14, 15]	160–330	150–500	180–400	300–400	low
D_1	stomach pancreas	gastrin[9]			100–280		high?
A	stomach intestine pancreas	enteroglucagon[a]		200		150–250	high
L	mainly intestine	cholecystokinin[2] enteroglucagon[11]	250–350	350–400	150–540	350	high
S	mainly intestine	secretin[1, 2, 5, 7, 10]	100–250	200	150–450	100–150	intermediate
M (I)	intestine	pancreozymin[5] cholecystokinin[12, 17]			150–450	150–250	high

A T cell was described by Sasagawa et al. (1974) and a K cell (gastric inhibitory peptide?) by Buffa et al. (1975)

1. Capella et al. (1969) 2. Vassallo et al. (1969) 3. Håkanson et al. (1970) 4. Pearse et al. (1970) 5. Bussolati et al. (1971) 6. Fujita and Kobayashi (1971) 7. Polak et al. (1971) 8. Capella and Solcia (1972) 9. Greider et al. (1972) 10. Solcia et al. (1972) 11. Pearse (1973) 12. Solcia et al. (1973) 13. Pearse et al. (1974) 14. Polak et al. (1974a) 15. Polak et al. (1974b) 16. Sasagawa et al. (1974) 17. Solcia et al. (1974) 18. Polak et al. (1975a) 19. Polak et al. (1975b)

[a] These products are generally accepted

[b] Cells in the stomach are generally of the "closed type", whereas cells of the pyloric antrum and intestine are of the "open type"

[c] Except for the EC and ECL cell (irregular granules) all cells contain round granules

secretin (S cell?) and cholecystokinin-pancreozymin (D cell?). An enteroglucagon producing A cell may also be present. However, at present there is no evidence available and additional studies, including examination of the islets of Langerhans, must be made.

The difficult light microscopical demonstration of the enteroendocrine cells may account for the negative reports concerning their presence in teleosts. The positive reactions with the argyrophilic method, lead haematoxylin and Schmorl's ferricyanide are in agreement with the results of Gabe (1973) in *Protopterus annectens*, Gabe and Martoja (1971) in *Mugil auratus*, and of Van Noorden et al., (1972), Van Noorden and Pearse (1974) and Östberg et al. (1976) in some cyclostomes.

A specific amine precursor uptake has not been observed in the intestine of the adult of *B. conchonius*. The absence of the APUD-reaction is in accordance with the results obtained in adult cyclostomes by Van Noorden and Pearse (1974) and Östberg et al. (1976). The absence of serotonin in the intestinal epithelium of many fishes and the inability of the enteroendocrine cells of adult cyclostomes and *B. conchonius* to take up amine precursors is of phylogenetic interest. Only Read and Burnstock (1968) have described serotonin containing enterochromaffin cells in the digestive tract of some teleosts (*Anguilla occidentalis australia, Salmo trutta, S. irideus*). Argentaffin cells, probably producing serotonin, were also found in the intestinal epithelium of *Mugil auratus* (Gabe and Martoja, 1971) and even in the gut of *Amphioxus* (Gerzeli, 1961) and tunicates (Erspamer and Asero, 1952; Welsh and Loveland, 1968; Fritsch, 1976). The absence of an APUD-reaction in adult teleosts and cyclostomes does not support the hypothesis of neural crest origin of the enteroendocrine cells, as first supposed by Pearse (1968, 1969). However, an amine precursor uptake is present in the intestinal epithelium of larval lampreys (Van Noorden et al., 1972) and also of larvae of *B. conchonius* (Rombout et al., to be published).

The resemblance of enteroendocrine cells to (auto-)phagosomes in their reaction to selective stains is a remarkable phenomenon. The continuous presence of (auto-)phagosomes in epithelial cells of the gut has been described previously for the trout (Kimura, 1973) and appears to be normal in teleosts. Even the small scattered fluorescent lead haematoxylin-positive structures described by Östberg et al. (1976) in the intestine of a cyclostome might be (auto-)phagosomes. In mammals, however, (auto-)phagosomes are less common, but their number may greatly increase under certain conditions. Shelburne et al. (1973) found a glucagon-induced autophagocytosis in rat liver, and a conspicuous autophagocytosis when isolated flounder kidney tubules are incubated in vitro. The increase of (auto-) phagosomes in epithelial cells of the gut of mice can be induced through X-irradiation (Hugon and Borgers, 1966), after injection of a substantial dose of ^{3}H-thymidine (Throughton and Trier, 1969), and after administration of colchicine (Stolpmann and Merker, 1967). Hazel Cheng and Leblond (1974) described the phagocytosis of non-viable cells by crypt base columnar cells after injection of ^{3}H-thymidine in mice. The appearance of phagosomes was interpreted as resulting from ingestion of non-viable cells. The general presence of (auto-)phagosomes in the intestinal epithelial cells of fishes may be an indication of a continuous process of autophagocytosis but eventually also of a phagocytosis. The irregular shape of

some cells containing these phagosomes may be an indication for the process of phagocytosis.

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Chapter II

Pancreatic Endocrine Cells of *Barbus conchonius* (Teleostei, Cyprinidae), and Their Relation to the Enteroendocrine Cells

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Summary. The pancreatic endocrine cells of *Barbus conchonius* are concentrated in a large (principal) islet, located near the gall bladder, and in a number of smaller islets. Five types of endocrine cells can be distinguished in these pancreatic islets: B cells, A_1 (or D cells), 2 types of A_2 cells (A_{2r} cells with round granules; and A_{2fl} cells with flocculent granules) and a scarce 5th cell type. The hormones produced by B and A_{2fl} cells are probably insulin and glucagon respectively. The A_{2r} cell contains granules with the same diameter as the granules of the enteroendocrine type III cell of the gut. Both cell types may resemble the enteroglucagon-producing EG cell of mammals. The function of the A_1 cells, which are frequently found without secretory granules, and of the 5th cell type, will be discussed.

The pancreatic islets of *B. conchonius* are strongly innervated, which suggests the presence of a direct nervous control system.

Some intermediate or mixed cells containing exocrine and endocrine A_{2r} granules are found contiguous with the principal islet. The origin of pancreatic endocrine cells is also the subject of discussion.

Key words: Pancreatic endocrine cells – Enteroendocrine cells – Ultrastructure – Cyprinidae.

Many studies have been made on enteroendocrine cells of mammals, but information is scarce on these cells in fishes. Four types of enteroendocrine cells have been described in the digestive tract of a teleost, *Barbus conchonius* (Rombout,

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1977). These cells were thought to be involved in the production of secretin, cholecystokinin-pancreozymin and enteroglucagon, but evidence was not obtained for this assumption. No serotonin-producing EC cells were found and gastrin-producing cells (G and D_1 cells) cannot be expected in stomachless cyrinids. In mammals and birds, A and D cells are present in the pancreatic islets as well as in the digestive tract. In order to ascertain whether these cells are present in the gut of *B. conchonius*, an investigation of the pancreatic islets in this fish is required.

In the pancreas of teleosts the endocrine cells are generally arranged in organ-like structures, the principal islets or Brockmann bodies (Brinn, 1973), in which at least three cell types, A_1 (= D), A_2 and B, can be recognized. However, there are differences in the location and frequency of the cell types, and in the shape and electron densities of their secretory granules (see Falkmer and Patent, 1972; Kobayashi et al., 1976; Klein and Lange, 1977). For the present study, the pancreatic islets of *B. conchonius* were examined with histochemical and electron microscopical methods, and the development of the endocrine pancreas was studied.

Materials and Methods

B. conchonius, reared in the laboratory at 23°C and fed with Trouvit pellets (Trouw &Co, Putten, Holland), were decapitated at different ages. The complete digestive tract with hepatopancreas was dissected out and fixed in Bouin. Vacuum embedding in Paraplast Plus (Sherwood) was followed by sectioning at 2–4 µm.

Histochemical Staining Methods

Staining Methods for B Cells: aldehyde-fuchsin method (Gabe, 1953) or crotonaldehyde method after permanganate oxidation (Gomori, 1939).

Silver Impregnation Methods: according to Hellman and Hellerström (1960) for A_1 (or D) cells and to Grimelius (1968) for A_2 cells.

These staining methods were applied to adjacent 2 µm sections and also sequentially to the same sections as follows: 1. Silver impregnation according to Grimelius; 2. Silver staining with the method of Hellman and Hellerström; 3. Crotonaldehyde staining. Between steps 1 and 2 the silver grains were removed by permanganate oxidation (Gomori, 1939).

Immunohistochemical Methods

Glucagon. Incubation with rabbit-anti-bovine glucagon serum (first step) was followed by incubation with goat-anti-rabbit-peroxidase serum (second step). Peroxidase was demonstrated with the DAB-method (Pearse, 1972). Adjacent sections were stained with the silver impregnation method. The following controls were carried out and found negative: 1) normal rabbit serum instead of the first step; 2) only the second step and DAB-method; 3) DAB-method only.

Insulin. After incubation with Guinea pig-anti-bovine-insulin-FITC serum (Nordic, Tilburg, Holland), sections were examined under a Zeiss fluorescence standard microscope with a HBO-50 superpressure mercury lamp, selection filters KP 490 and KP 500 and barrier filter LP 528 (epi-illumination). The sections were sequentially stained with crotonaldehyde.

Correlative Light Microscopical and Electron Microscopical Examinations

Larvae of several ages and small parts of gut and pancreas of adult specimens were fixed for 2 h at 0° C in a mixture of 2% OsO_4 and 2% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.2) or in 2% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.2). After embedding in Epon 812, semi-thin or ultra-thin sections were cut on a Reichert OMU-IV microtome. Ultra-thin sections were collected on copper grids, stained with saturated uranyl acetate and then with lead citrate. Adjacent semi-thin sections were stained with crotonaldehyde or according to Grimelius after removal of Epon (Lane and Europa, 1965). Grimelius' silver impregnation was also applied to ultra-thin sections before these were mounted on copper grids.

Results

Pancreatic Islets

Light Microscopy. The endocrine cells of the pancreas of the adult *B. conchonius* are concentrated in a large islet (principal islet or Brockmann body), located between gall bladder and intestinal bulb, and in many small islets located caudal to this principal islet. None of the islets is enclosed by a capsule of connective tissue, as in the case of some other fish species (Brinn, 1973). The principal islet is generally surrounded only by a thin layer of exocrine pancreas. With histochemical methods, 4 different cell types can be distinguished in the pancreatic islets:

B cells, round to triangular cells, stained by aldehyde-fuchsin, crotonaldehyde and anti-insulin-FITC (Fig. 2). These insulin-producing cells are located in groups or strands in the central part of the islet. The extent of staining with aldehyde-fuchsin or crotonaldehyde varies considerably in different specimens.

A_2 cells, triangular to spindle-shaped cells, stained with Grimelius' method, are generally located in the peripheral part of the islet. Some cells at the outer edge of the islet are stained more intensely (black) than other A_2 cells (brown). The former (black) cells are stained more distinctly with toluidine blue in semi-thin Epon sections. Comparison of the Grimelius method with the anti-glucagon peroxidase method applied to adjacent sections, suggests that the A_2 cells contain glucagon (Fig. 3).

A_1 (= D) cells, spindle-shaped cells, positive with the Hellmann and Hellerström method, are mainly located between groups or strands of B cells, but also between the A_2 cells (Fig. 1). Many of the A_1 cells are also stained with the Grimelius method.

Clear cells, found after staining of adjacent sections (Fig. 1) or sequential staining on the same section, are negative with any of the methods used. The number of clear cells is variable (0–50%) depending on the specimen.

Electron Microscopy. Based on size and shape of the secretory granules, 5 distinct cell types can be distinguished (Figs. 5, 7):

B cells are recognized by their clustering, their central location and by comparing them in crotonaldehyde stained semi-thin Epon sections with adjacent ultra-thin sections. B-cells contain large round granules with a variable electron density. However, B cells with empty vesicles (granules?) have also been observed.

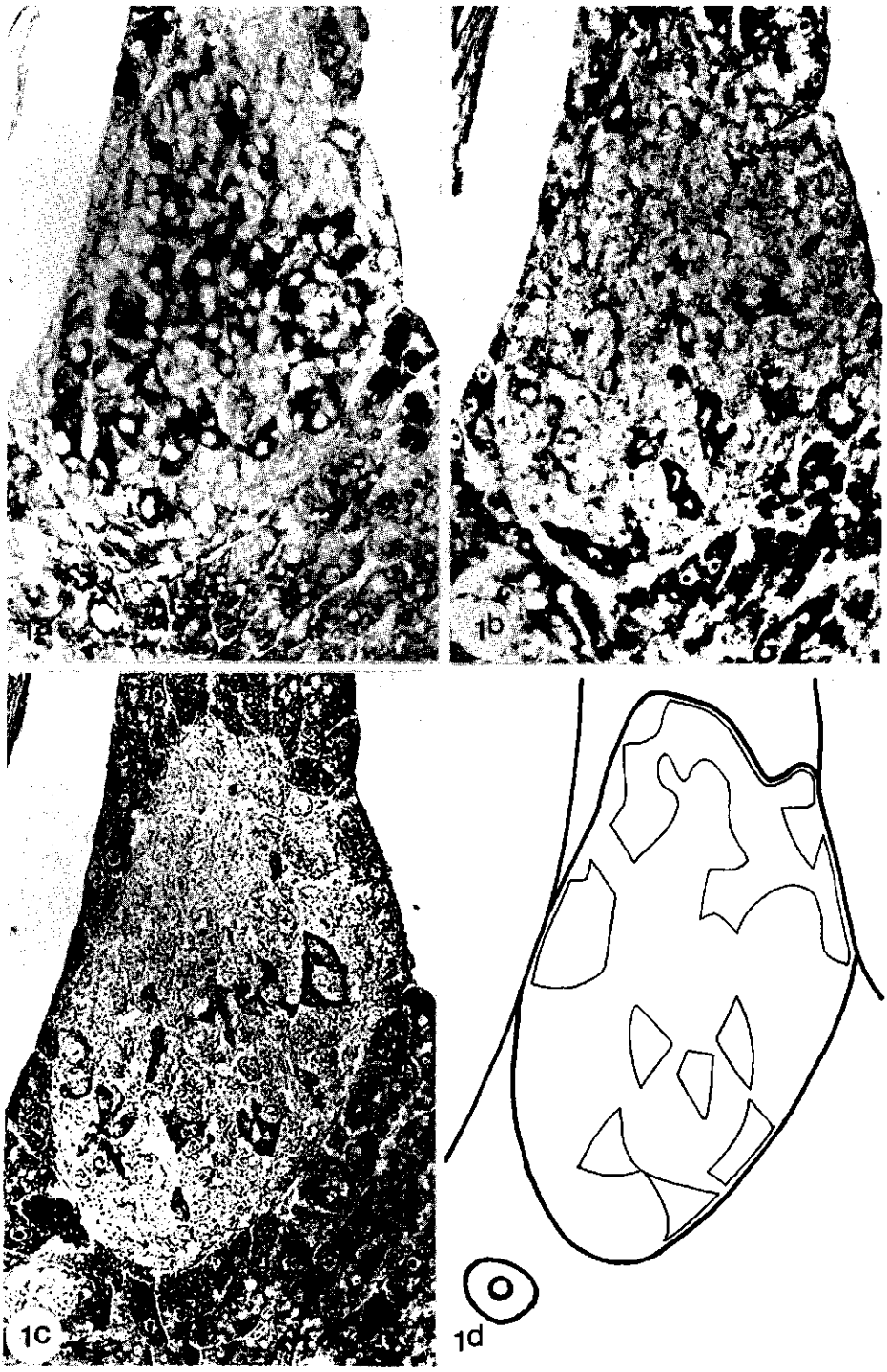
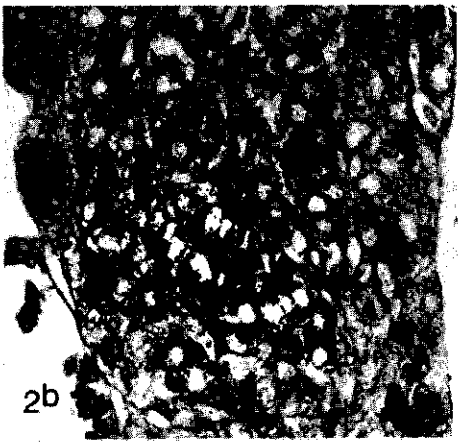
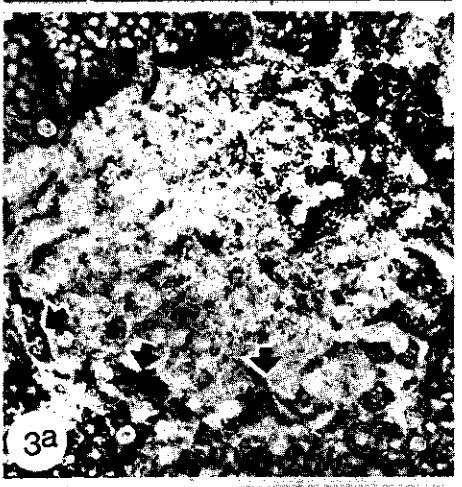
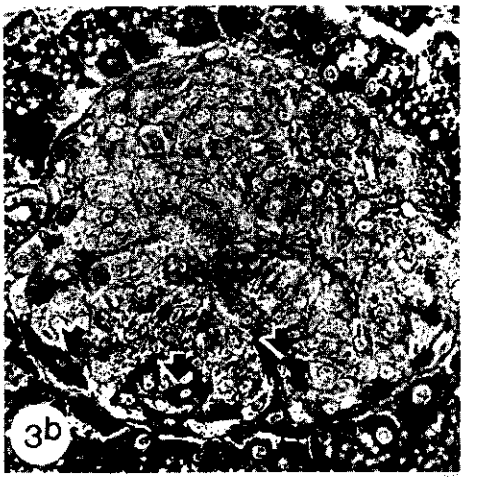
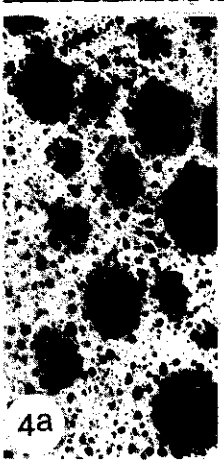


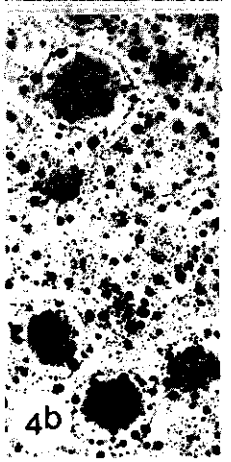
Fig. 1 a–d. Adjacent paraplast sections (2 μm) of a principal islet. × 700. **a** Crotonaldehyde staining for B cells. **b** Grimelius' method for A_2 cells. **c** Hellman and Hellerström method for A_1 cells. **d** The areas with clear cells are outlined. Note the different locations of the four cell types

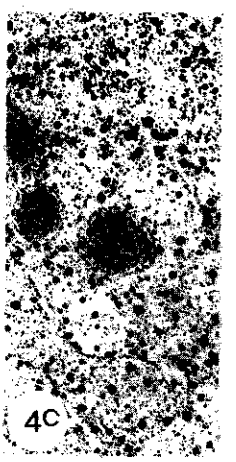












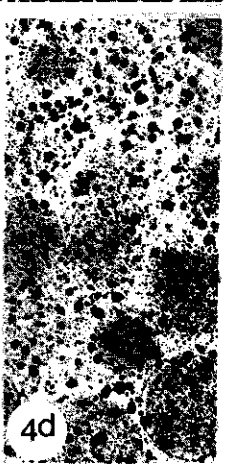


Fig. 2a–b. Paraplast section (2 μm) of a principal islet. × 700. **a** fluorescence after incubation with anti-insulin-FITC. **b** The same section after crotonaldehyde staining

Fig. 3a–b. Paraplast section (4 μm) of a principal islet. × 550. **a** Anti-glucagon-peroxidase reaction. **b** Adjacent section after applying Grimelius' method. Note the similar location of the positive cells (*arrows*)

Fig. 4a–d. Electron micrographs of the granules of the 4 common cell types after Grimelius staining on ultra-thin sections. × 45,000. **a** A_{2r} granules; **b** A_{2fl} granules; **c** A_1 granules; **d** B granules. Note the strong positive A_{2r} granules and the weak positive A_{2fl} granules

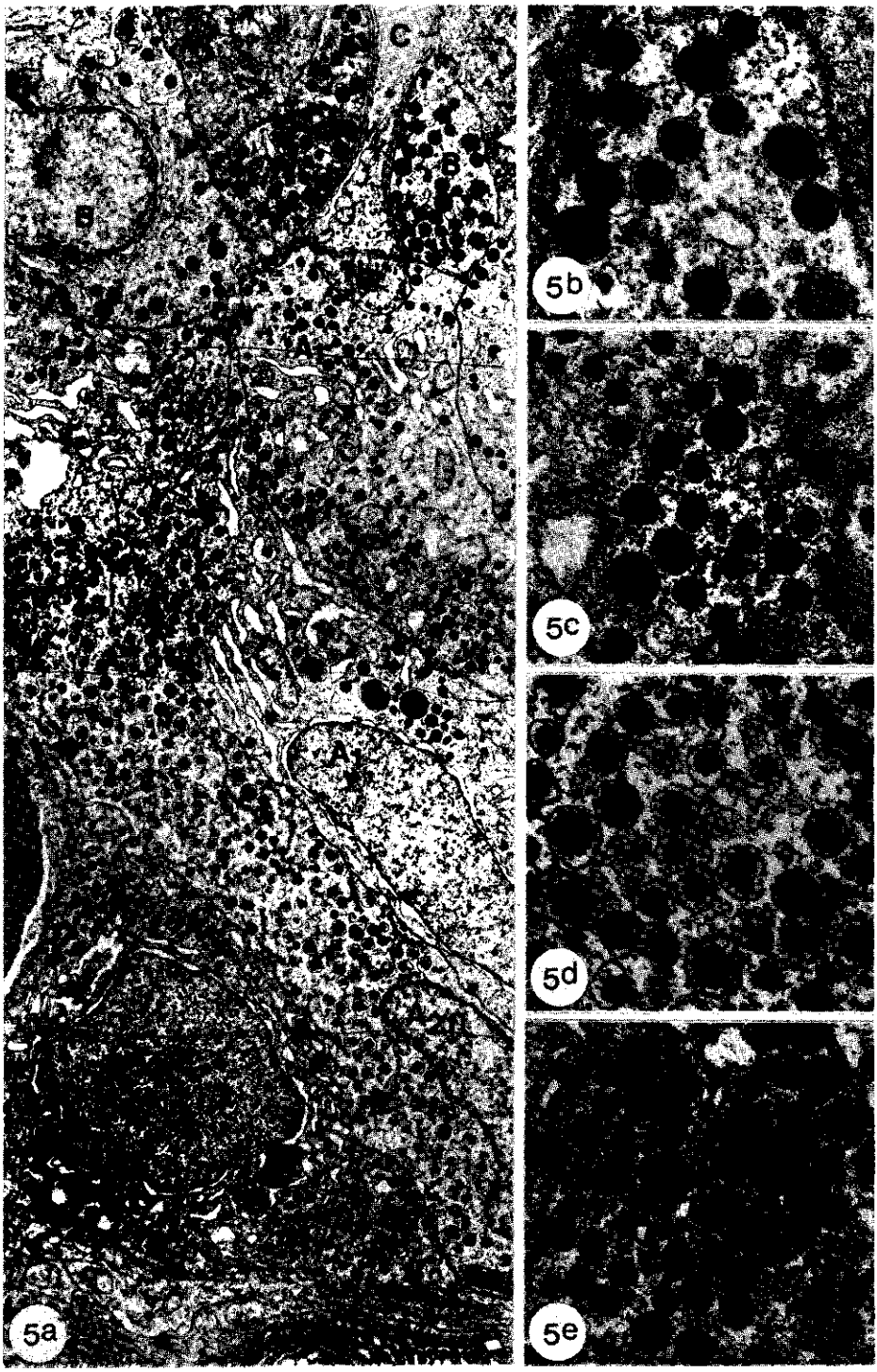


Fig. 5a–e. **a** Electron micrograph of an area of the principal islet. ×8,000. **b–d.** details of secretory granules. ×27,500. **b** B cell; **c** A_1 cell; **d** A_2 cell with flocculent granules (A_{2fl}); **e** A_2 cell with round granules (A_{2r}). *C:* blood capillary; *N* = nerve ending; *P* = exocrine pancreatic cell

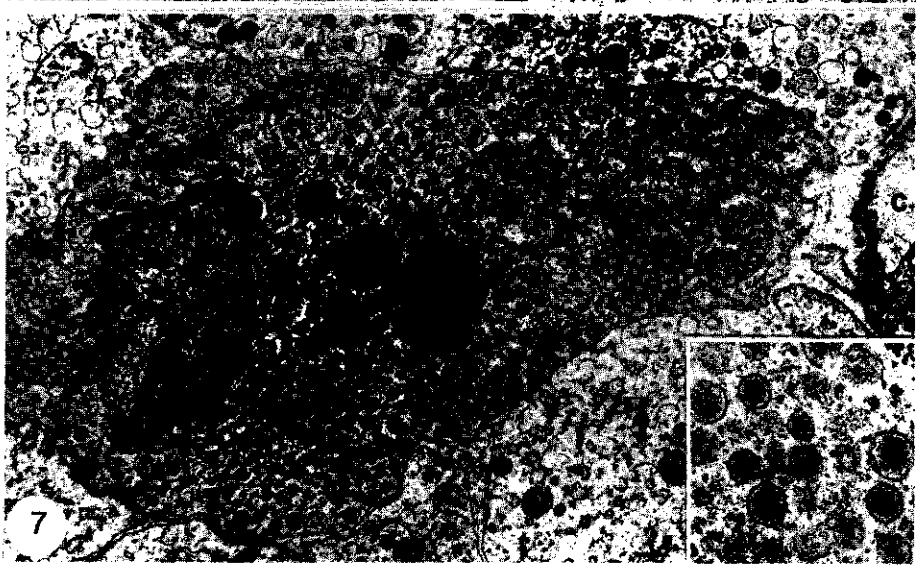


Fig. 6a and b. **a** Electron micrograph of part of a principal islet showing several cells devoid of granules. ×4,100. **b** Detail of some of these cells containing a few small granules with an electron dense core (*arrows*). ×10,500. As A_1 granules are absent in this islet, these cells probably represent the A_1 cells. A_1: A_1 cell; A_{2fl}: A_2 cell with flocculent granules; A_{2r}: A_2 cell with round granules; *c* blood capillary; *p* exocrine pancreas; *pd* pancreatic duct

Fig. 7. Electron micrograph of a 5th cell type. ×12,000. *Inset:* Note the small granules with a moderate electron density. ×33,500. *c* blood capillary; *l* lysosome

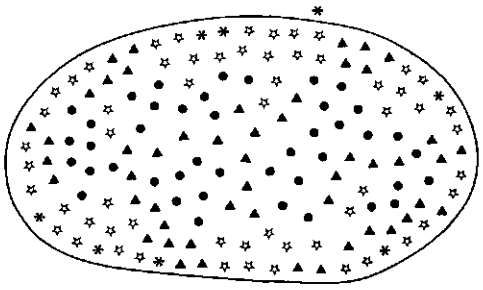


Fig. 8. Average distribution of the 4 common endocrine cell types of the pancreatic islets, as observed with the electron microscope in a number of pancreatic islets. The rare 5th cell type is not shown in this figure. ▲ A_1 cell; ✲ A_{2fl} cell; ✱ A_{2r} cell; ● B cell

A_2 cells are recognized by their peripheral location and by their positive staining with the Grimelius method in semi-thin and ultra-thin sections (Fig. 4). With the electron microscope two A_2 cell types can be distinguished. The strongly Grimelius-positive cells, most of which are situated at the periphery of the islets, but some which lie between the exocrine cells outside the islets, contain many electron-dense round granules and are named A_2 round (A_{2r}) cells. The second type of Grimelius-positive cell is large and located more centrally though still in the peripheral zone. This type contains many granules with a flocculent core of variable electron density, a large irregular halo and generally a wrinkled granule membrane; cells of this type are named A_2 flocculent (A_{2fl}) cells. *A_1 cells* are recognized by their position between B and A_{2fl} cells and by their weak Grimelius staining in ultra-thin sections.

The granules of these cells are similar to the A_{2r} granules, but many small electron dense granules (about 120 nm) are also present, which is evident from the histogram of granule size (Fig. 9). The A_1 cells differ from the A_{2r} cells in that they have swollen endoplasmic reticulum and peripherally located granules (near the capillaries). In several specimens the A_1 cells were completely degranulated or contained only a few granules (Fig. 6).

Some endocrine pancreatic cells (A_2 and A_1) bear a cilium (Fig. 10).

The 5th cell type is characterized by small granules (average largest diameter about 165 nm) with a moderately electron dense core (Fig. 7).

As this type is scarce in the principal islets, only the average distribution of the 4 other cell types, observed with the electron microscope, is given in Fig. 8.

Some specimens contain intermediate or mixed cells with both zymogen granules (diameter up to 2.7 μm) and small granules resembling the granules of the A_{2r} cells (Fig. 11).

Innervation

The pancreatic islets are heavily innervated (Figs. 5, 12).

Two types of nerve endings are observed: 1. those containing electron-lucent vesicles with an average diameter of 140 nm; 2. endings containing vesicles of about 100 nm with a dense core and very small empty vesicles (about 60 nm). Synaptic contacts with endocrine cells are occasionally observed (Fig. 12).

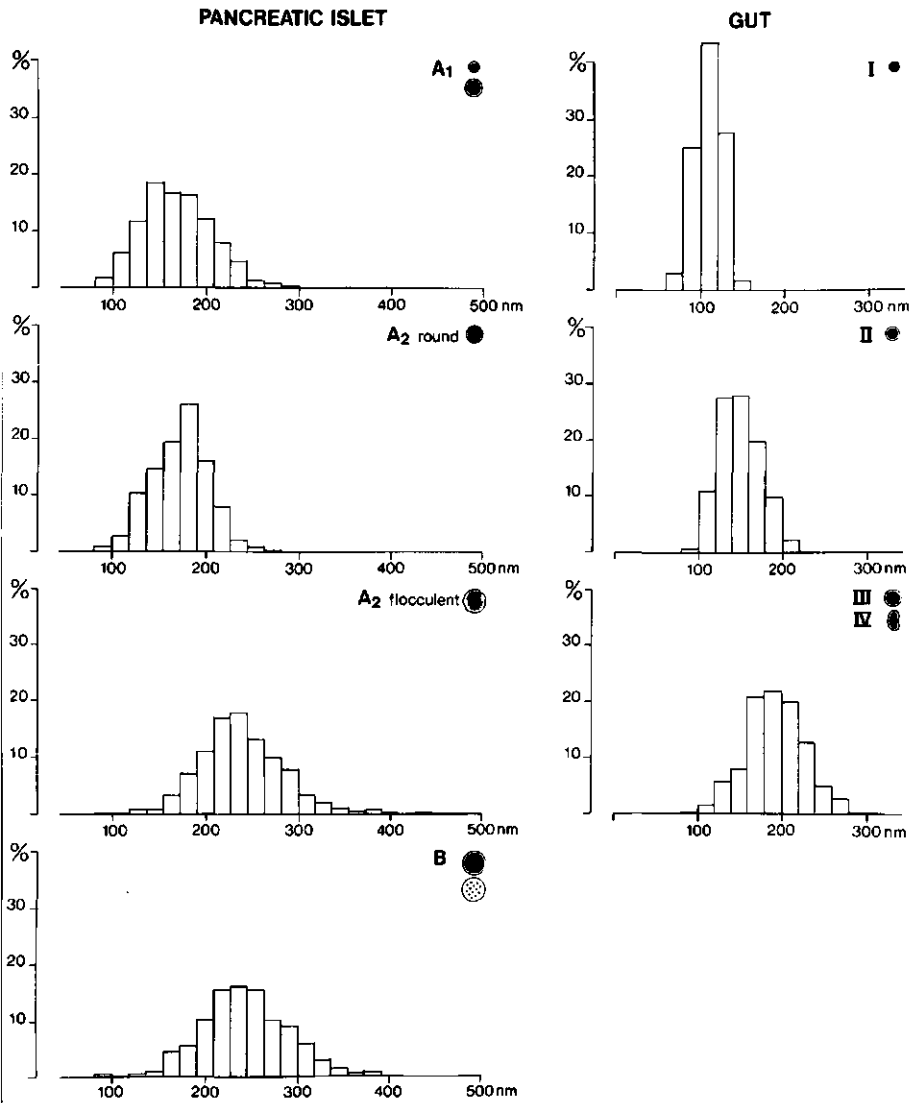
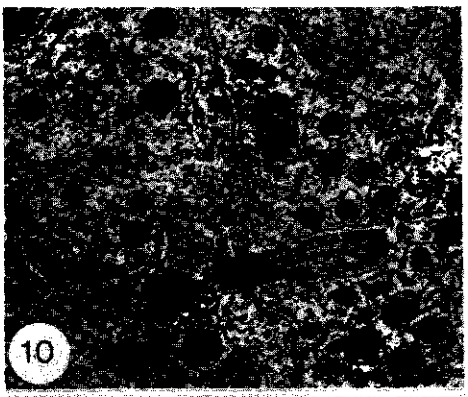


Fig. 9. Histograms for the diameter of the secretory granules of the endocrine cell types in pancreas and gut

Development

From the 3rd day to 3 weeks after fertilization, only the principal islet is found near the gall bladder. From the 4th week, many small islets appear in the pancreas, caudal to the first islet. After the first appearance of the principal islet, B, A_1 and A_{2fl} cells can be recognized, but they contain only a few secretory granules. A_{2r} cells, however, are not found until 1 month after fertilization.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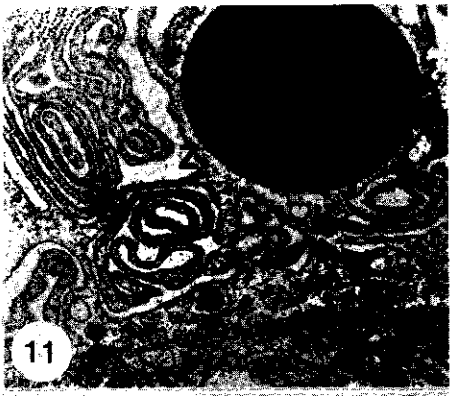
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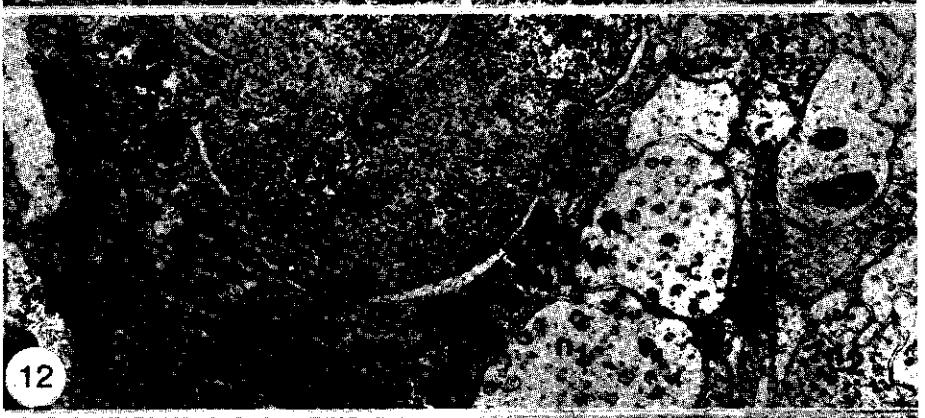
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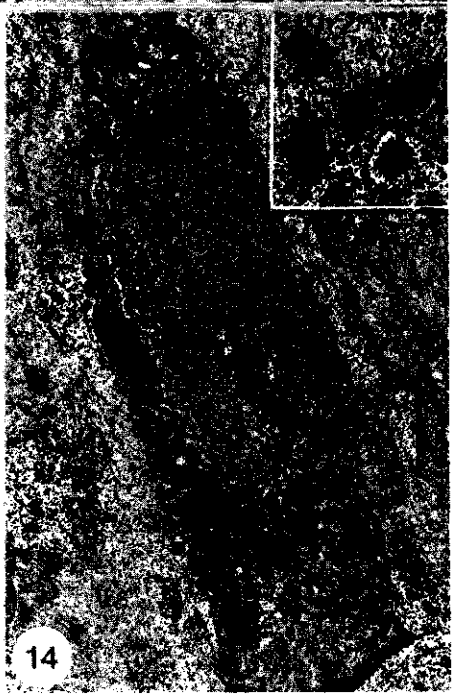
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Table 1. Staining properties of pancreatic islet cells and enteroendocrine cells

cell type \ staining method	aldehyde-fuchsin crotonaldehyde, anti-insulin-FITC	Hellman and Hellerström	Grimelius L.M.	Grimelius E.M.	anti-glucagon/ PO
A_1 (= D)	−	++	−/++	±	−
A_{2r}	−	−	++	++	+?
A_{2fl}	−	−	+	+	+
B	++	−	−	±	−
EEC II, III	−	−	++	++	−

Cilia are more often found on pancreatic endocrine cells in larval specimens than in adult specimens.

Enteroendocrine Cells

As already reported (Rombout, 1977) 4 types of enteroendocrine cells can be distinguished in the digestive tract of *B. conchonius*. Of the various (immuno-) histochemical methods applied to light microscopical sections, only the Grimelius stain was found to be positive for the enteroendocrine cells (Fig. 13). Grimelius staining of ultra-thin sections showed cell types II and III to be positive (Fig. 14). The histochemical results for the different endocrine cell types of pancreas and gut are summarized in Table 1.

The ultrastructure and size distribution curves of the secretory granules indicate that A_{2r} cells and type III enteroendocrine cells contain similar secretory granules (Fig. 9).

Discussion

The pancreatic endocrine cells of B. conchonius are concentrated in one large principal islet and many small accessory islets, as already reported for other teleosts (Brinn, 1973). Light and electron microscopical studies on pancreatic islets of

Fig. 10. Two types of A_2 cells. The A_{2r} cell contains a cilium extending in the intercellular space. ×18,000

Fig. 11. Intermediate cell in the exocrine pancreas, containing A_{2r} granules (*arrows*) and zymogen (*Z*) granules. ×13,000

Fig. 12. Principal islet showing the strong innervation of the pancreatic islets. Note the two types of nerve endings (n_1 with large vesicles and n_2 with two kinds of small vesicles) and a synaptic contact of a n_1-nerve ending on an A_{2r} cell (*arrows*). ×15,000

Fig. 13. Basally located argyrophilic cells (*arrows*) in the gut after Grimelius staining in a 4 µm section. ×420

Fig. 14. Electron micrograph of the basal part of the intestinal epithelium after Grimelius staining. Enteroendocrine type III cell, ×8,000. *Inset:* Detail: Note the Grimelius-positive granules. ×55,000

teleosts (see: Kobayashi et al., 1976) revealed many differences between the different species, especially at the ultrastructural level. These differences may well be due to the different fixatives used. Insulin-producing B cells, glucagon-producing A_2 cells and A_1 cells are common types in the pancreatic islets of fishes. Our results show two types of A_2 cells, which were also found by Klein and Lange (1977) in *Xiphophorus helleri*. Apart from our finding that the A_{2r} cells are Grimelius positive in *B. conchonius* whereas they are negative in *X. helleri*, our results are in agreement with those of Klein and Lange. The ultrastructure of pancreatic endocrine cells of closely related species such as *Carassius carassius* (Kobayashi and Takahashi, 1970; Kudo and Takahashi, 1973) and *Cyprinus carpio* (Nakamura and Yokote, 1971) is less similar, but the distribution pattern of the different cell types is broadly the same.

The clear cells of fish (Brinn, 1973) were also found in the pancreatic islets of *B. conchonius*. Several suggestions have been made as to their nature, e.g., a variety of B cells (Kobayashi and Takahashi, 1974) or D-cells (Qureshi and Matty, 1969; Kobayashi and Takahashi, 1970), a distinct cell type with unknown function (Bencosme et al., 1965) and young immature cells (Falkmer et al., 1964). From our results it is evident that most of these cells are A_1 cells, because electron-microscopically they are frequently devoid of granules, although it cannot be excluded that some of them may be functional stages of B or A_2 cells.

The function of the A_1 or D cells in fish is not clear. Some authors suggested that they produce a gastrin-like peptide, analogous to the D_1 cells of mammalian islets of Langerhans (Greider and McGuigan, 1971). However, most probably this hormone cannot be expected in stomachless cyprinids. Other authors suggested that they produce somatostatin, the release-inhibiting factor for the growth-hormone, as has been demonstrated with the immunofluorescence technique for mammalian D cells (Polak et al., 1975; Erlandsen et al., 1976). Recently, somatostatin-like immuno-reactivity was also found in two species of teleosts (Johnson et al., 1976) and in two species of cyclostomes (Van Noorden et al., 1977). Probably somatostatin inhibits also the release of insulin and glucagon (see: Erlandsen et al., 1976; Johnson et al., 1976). The hormone may be of particular interest for regulating pancreatic hormones of teleosts, because the number of A_1 (or D) cells is much larger in teleosts than in mammals.

The function of the 5th cell type, which is scarce, is not known. In addition to the B, A_1 and A_2 cells, one or two cell types with unknown function have been described in several teleosts: *Gadus callarias* (Thomas, 1970, type IV), *Anguilla rostrata* (Brinn and Epple, 1972, type IV), *Ictalurus catus* (Brinn, 1973, type IV), *Carassius carassius* (Kudo and Takahashi, 1973, type IV, V), *Conger japonicus* (Kobayashi and Takahashi, 1974, type IV), *Limanda herzensteini* (Kobayashi et al., 1975, type EC(?), V, VI), *Limanda limanda* (Thomas, 1975, type IV) and *Fugu rubripes* (Kobayashi et al., 1976, type F_f). With the exception of the type IV cells of *Ictalurus catus* and type VI cells of *Limanda herzensteini*' none of these cell types resembles our 5th type.

Our results show a glucagon-like immunoreactivity of the A_2 cells. The location and number of these anti-glucagon positive cells can be correlated with the location and number of A_{2f} cells. No clear indication was found for a glucagon-like peptide in the A_{2r} cells of *B. conchonius* (due to the small number of A_{2r} cells in this fish

species). The A_{2r} cells of *Xiphophorus helleri,* however, contain a glucagon-like peptide (Klein and Lange, 1977). These observations, and the presence of an A_{2r}-like cell in the gut of *B. conchonius* (the enteroendocrine type III cell), might indicate that both types of cells produce enteroglucagon, just as the EG cells in mammals. The absence of anti-glucagon positive cells in the digestive tract of *B. conchonius* may be explained by the difference in specificity of the anti-bovine-glucagon serum (positive for glucagon-containing cells and negative for enteroglucagon-containing cells).

The absence or scarcity of both A_{2r} and enteroendocrine type III cells in pancreas and gut of larval *B. conchonius,* while all the other endocrine cell types are present (see: Rombout et al., 1978) cannot be explained at present.

The heavy innervation of the pancreatic islets, observed in *B. conchonius*, was also found in several other teleosts (see: Brinn, 1975). The nerve endings with two types of small vesicles (n_2) are comparable with those of *Xiphophorus helleri* (Klein, 1971). Recently, Klein and Streicher (1976) have excluded the presence of nerve endings of the adrenergic type in this fish. Brinn (1975) even excluded the presence of cholinergic innervation and suggested some sort of islet innervation unique for teleosts, possibly a purinergic innervation.

The presence of cilia extending in the intercellular space between the pancreatic endocrine cells of fish has only been reported for A_2 cells of *Ictalurus nebulosus* (Bencosme et al., 1965). These cilia are more frequently present between pancreatic endocrine cells (A_1 and A_2) of larval *B. conchonius* than in adult specimens; the same was observed for enteroendocrine cells (Rombout et al., 1978). This decrease in the number of cilia during development was also observed for pancreatic endocrine cells of rats (Pictet and Rutter, 1972) and mice (Munger, 1958); the authors suggested a chemoreceptor function for these cilia.

A neuroctodermal or placodal ectodermal origin has been suggested for endocrine cells of pancreas and gut of birds and mammals, because of their APUD characteristics (Pearse and Takor Takor, 1976). Several authors, however have ruled out the neural crest (see Andrew, 1976) or even the neuroectoderm (Fontaine and Le Douarin, 1977) as possible sources of endocrine cells in pancreas and gut. Contrary to birds and mammals, these endocrine cells in adult fish do not have APUD characteristics. During development the enteroendocrine cells of larval *B. conchonius* show APUD characteristics during a short period only (when 3–6 days old) but the pancreatic endocrine cells do not (Rombout et al., 1978).

A neurectodermal or placodal ectodermal origin of the enteroendocrine cells of fish cannot be excluded, but no indication for this origin is available for the pancreatic endocrine cells. The presence of intermediate cells in the exocrine pancreas of *B. conchonius* with zymogen granules and granules resembling those of the A_{2r} cells, and possibly the presence of isolated A_{2r} cells between the exocrine cells, even support a common endodermal origin of pancreatic A_{2r} cells and exocrine cells. Consequently, an endodermal origin can also be suggested for enterendocrine type III cells. Many authors have already described intermediate cell types (with granules of A, B and rarely D cells) for various species of vertebrates (see: Melmed et al., 1972), which are also considered either as artifacts or more likely as a product of fusion between two or more cells (see: Pearse and Takor Takor, 1976).

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Chapter III

Distribution, structure, and possible function of enteroendocrine cells of *Barbus conchonius* (Cyprinidae).

J.H.W.M. Rombout

SUMMARY

On the basis of size and shape of the secretory granules, four different enteroendocrine cell types can be distinguished in *B. conchonius*. Cell type I (small granules) is located throughout the intestine, but most frequently present in the third segment; cell type II (intermediate granules) is regularly distributed in the first segment and is scarce in the second segment; cell type III (large granules) and cell type IV (size of type III granules, but shape irregular) are only present in the first segment and mainly confined to the intestinal bulb.

The different distributions and data from literature are reasons to suppose various functions for the mentioned cell types. Cell type I may have a regulatory function in gut motility, possibly to be ascribed to motilin, VIP or neurotensin. Cell type II may be involved in CCK-PZ secretion, just as mammalian type "I" cells. Cell type III found in both pancreas and gut, may be involved in producing a pancreatic hormone: a glucagon-like peptide or PP. The irregular shape of the granules of cell type IV may be due to fixation and the function may be similar to that of cell type III.

Most if not all enteroendocrine cells are of the "open" type, and they have probably a chemoreceptory function. As no nerve terminals have been found near these cells they seem to be self-sufficing.

INTRODUCTION

Four types of enteroendocrine cells, mainly recognized by the size and shape of the secretory granules, have been described for the digestive tract of

ABBREVATIONS: CCK-PZ: cholecystokinin-pancreozymin; EC: enterochromaffin; EG: enteroglucagon; GEP: gastro-entero-pancreatic; GIP: gastric inhibitory peptide; GLI: glucagon-like immunoreactive; I: intermediate granule-containing; L: large granule-containing; PP: pancreatic polypetide; VIP: vasoactive intestinal peptide

B. conchonius (Rombout, 1977). However, a difficulty in GEP endocrine cell type identification and homologization is that neither in light microscopy nor in electron microscopy, specific cell type characteristics are the same for all vertebrate species (Lange & Kleine, 1976; Rombout, 1977). The characterization of GEP endocrine cells in fish requires a strictly correlated light and electron microscopical technique; especially immunohistochemistry is indispensable for identifying the function of different cell types. As antisera against fish enterohormones are not available and because several current antisera against mammalian enterohormones fail to show a cross-reactivity with fish enteroendocrine cells (Pearse, personal communication), other criteria must be found for a provisional identification of the function of these cells. Examination of pancreatic islets of *B. conchonius* revealed a cell type common to gut and pancreas and probably resembling the EG (=L) cell of mammals (Rombout et al., 1979). The function of other cell types remains questionable. As the distribution of the distinct GEP endocrine cell types in the digestive tract is more or less similar for several mammalian species (Fujita & Kobayashi, 1974; Sasagawa et al., 1974; Forssmann, 1976; Capella et al., 1976; Solcia et al., 1978), examination of the distribution of enteroendocrine cell types in *B. conchonius* may give an indication of their possible functions.

Special attention will be paid to the very short third segment of the gut (< 5%) as the enteroendocrine cells in this part of the gut had not yet been examined with the electron microscope.

MATERIALS AND METHODS

Specimens and different parts of the gut of adult specimen were fixed for 2 h at 0°C in a mixture of 1% OsO_4, 2% glutaraldehyde and 1% potassium dichromate buffered in 0.1 M cacodylate (pH 7.2). After embedding in Epon 812, ultra-thin sections were cut on a Reichert OMU-IV microtome, collected on coppergrids, stained with saturated uranyl acetate and subsequently with lead citrate. The distribution of the enteroendocrine cell types in different parts of the gut has been examined with a Philips EM 300 electron microscope.

RESULTS

Based on electron microscopical observations, a differentiation can be made in the distribution of the different enteroendocrine cell types in *B. conchonius*

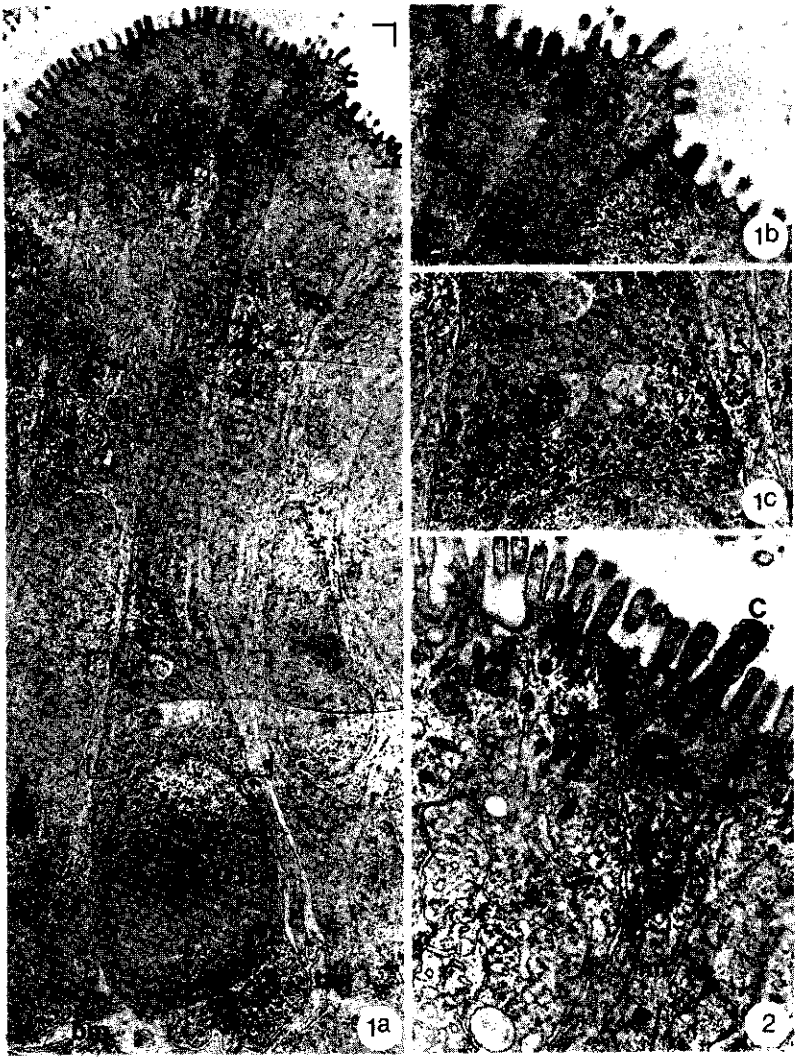

Fig. 1a-c. Electron micrograph of an enteroendocrine type I cell in the third segment of the gut of a 6 days old larva. a Note the broad secretory granule-containing base and the long narrow process to the lumen. x 8,000 b Detail of the outlined apical part with microtubules and small microvilli. x 18,000 c Detail of the outlined basal part with many small secretory granules. x 18,000

Fig. 2. Electronmicrograph of the apical part of an enteroendocrine type I cell in the second segment of a 6 days old larva. Note the presence of a cilium, microtubules and a single small secretory granule (arrow). The apical part of the neighbouring enterocyte shows pinocytotic activity. x 26,000
bb: basal body; bm: basement membrane; c: cilium; ce: centriole; mt: microtubules; N: nucleus; pv: pinocytotic vesicles

Table: Enteroendocrine cells in *Barbus conchonius* (electron microscopy)

Type	Granules			Cell shape	location[a]			
	size (nm)	shape	density		1	2	3	P
I	123	round	high	open	+	+	++	-
II	170	round	high	open	++	±	-	-
III[b]	231	round	high	open	++	-	-	+
IV[b]	237	irregular	high	open	-/++	-	-	±

[a]) 1, 2, 3: first, second and third segment; P: pancreas (Rombout et al., 1979)
[b]) Cell types III and IV are absent in gut and pancreas of early larval stages.

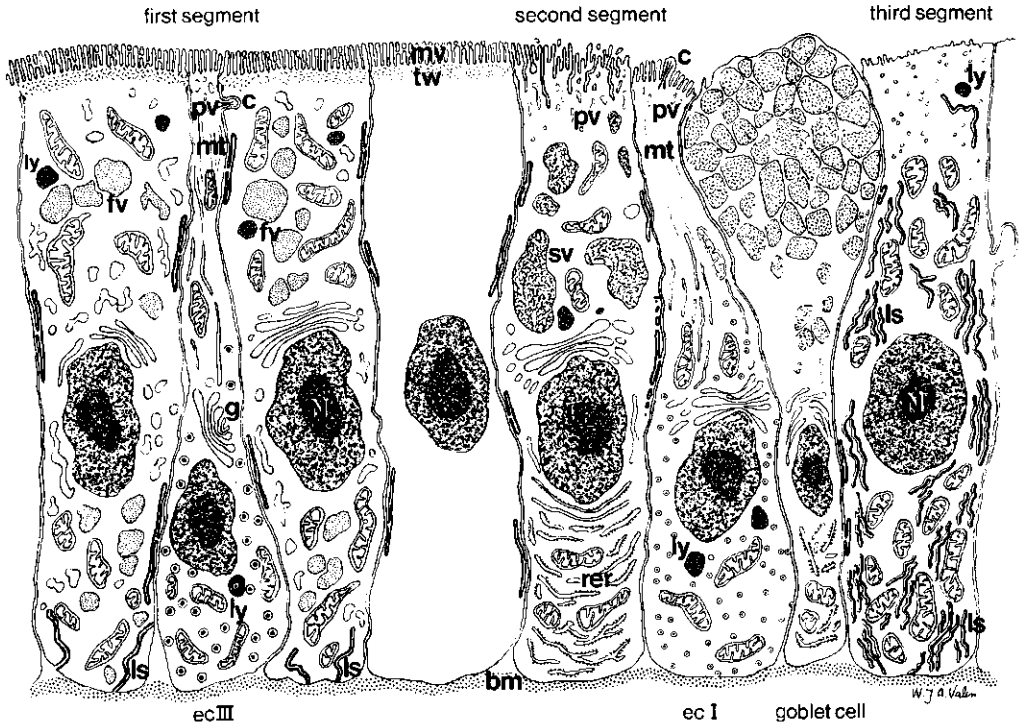

Fig. 3. Schematic drawing of the intestinal epithelium of different segments; containing enterocytes of the first, second and third segments, enteroendocrine cell types I and III and a goblet cell. Note the characteristic "open" shape of the enteroendocrine cells; the broad secretory granule-containing base, the microtubules in the long narrow extension towards the lumen and the presence of microvilli, a cilium, and pinocytotic vesic in the apical end.
bm: basement membrane; c: cilium; fv: fat vacuole; ls: lamellar structure ly: lysosome; sv: supranuclear vacuole; tw: terminal web.

Cell type I is found in all parts of the gut, but is frequently present in the third segment near the anus (fig. 1). Cell type II is found regularly distributed in the first segment and scarce in the second segment. Cell type III is mainly found in the first segment, particularly in the intestinal bulb. Cell type IV, recognized by its more or less irregular granules, has the same distribution as cell type III, but there is a strong variation in number in different specimens. All cell types are mainly represented as the "open" type. However, the presence of some cells of a "closed" type may not be excluded after electron microscopical examination only. Enteroendocrine cells of the "open" type have a broad secretory granule-containing base and a long narrow extension towards the lumen (fig. 3). This dendrite-like cytoplasmic process contains longitudinally oriented microtubules. The often more or less "swollen" apical end of the "open" cell type bears microvilli and in some cases a cilium (apically or laterally; figs. 2, 3). The terminal web is absent in the apical cytoplasm and pinocytotic activity can be observed between the microvilli. Nerve endings are not present in the vicinity of the enteroendocrine cells.

DISCUSSION

In the digestive tract of teleosts only a few hormones or hormone-like substances can be found with biochemical methods: secretin, CCK-PZ, substance P and histamin (cf. Fänge & Grove, 1979). By means of immunocytochemical methods, a caerulein-like peptide (Larsson & Rehfeld, 1977: caerulein in teleosts possibly functions as gastrin), PP (Falkmer & Stefan, 1978), VIP (Larsson et al., 1979) and a somatostatin-like peptide (Dubois et al., 1979) can be demonstrated in the gastrointestinal epithelium of teleosts. However, recently Buffa et al. (1979) and Grube & Weber (1980) observed unspecific, dilution dependent, binding of immunoglobulins to some enteroendocrine cells. Therefore, the immunocytochemical results must be considered with caution. Moreover, as suitable antisera against enterohormones in fishes are not available, other criteria must be applied for identification.

The present study has shown clear differences in the location of enteroendocrine cell types in *B. conchonius*.

Cell type I is found throughout the gut. In mammals only the EC cell is present in all parts of the digestive tract (Sasagawa et al., 1974; Forssmann, 1976; Solcia et al., 1978). Recently two types of EC cells were found in

mammals; an EC_1 cell containing serotonin and substance P, and an EC_2 cell containing motilin (Polak et al., 1976; Pearse & Takor Takor, 1979). Both cell types are involved in regulating muscular contraction of the digestive tract. As this kind of regulation may also be expected in fish, cell type I is the first to be considered as active in that respect. Like other teleosts (Erspamer, 1954, 1958; Fänge, 1962), *B. conchonius* does not have serotonin in the enteroendocrine cells (Rombout, 1977). Therefore motilin or a motilin-like peptide may possibly be present in type I cells, but the negative immunocytochemical results in the mudsucker do no support this assumption (Seino et al., 1979). In addition to motilin, VIP (cf Larsson, 1977) and neurotensin (Reinecke et al., 1980) may also affect contraction of the intestinal smooth musculature. As recently VIP-(Larsson et al., 1979) and neurotensin-(Reinecke et al., 1980) immunoreactive cells have been found in the intestinal epithelium of the cod and goldfish respectively, these hormones may also be present in cell type I.

Cell type II is mainly located in the first segment of the intestine. In cyprinids there is no peptic digestion (Jany, 1976); consequently hormones directly or indirectly involved in peptic digestion, e.g. gastrin, histamin, GIP and secretin, cannot be expected to be found. As cell type II does not occur in the pancreas, the production of a pancreatic hormone seems to be unlikely too. So only the following hormones may be expected in cell type II: somatostatin, CCK-PZ and, if not present in type I cells, neurotensin and VIP. Somatostatin-immunoreactive cells (A_1 = D cells) have recently been demonstrated in the pancreatic islets of teleosts (Johnson et al., 1976; Falkmer et al., 1978; Klein & Van Noorden, 1978; Falkmer, 1979; Dubois et al., 1979). With the exception of the gastric mucosa of the trout (Dubois et al., 1979), somatostatin-containing A_1 cells have not been found in the digestive tract of teleosts (Seino et al., 1979). Falkmer et al. (1978) even concluded that in fish "almost all D cells have left the gastrointestinal mucosa", which might explain the great number of A_1 cells in the pancreatic islets of fish. As no D cells can be found in the gut of *B. conchonius* (Rombout et al., 1979), somatostatin cannot be expected in the intestinal epithelium of this species. CCK-PZ was demonstrated in several fish species (Fänge & Grove, 1979; Vigna, 1979). This hormone seems to be indispensable for the endocrine regulation of digestion, and may be expected in type II cells, the more so by the presence mainly in the first segment of the gut, which is in agreement with the location of CCK-PZ producing "I" cells in the digestive tract of mammals

(Fujita & Kobayashi, 1974; Sasagawa et al., 1974; Forssmann, 1976; Polak, 1976; Solcia et al., 1978; Buchan & Polak, 1980).

Cell type III resembles the A_{2r} cell of the pancreas and thus seems to be involved in the secretion of a pancreatic hormone. Rombout et al., (1979) suggested enteroglucagon as a possible hormone in this cell type, which is homologous to mammalian L (=EG) cells. However, recent information indicates that the role of enteroglucagon is rather obscure (cf. Fujita & Kabayashi, 1977) and it seems better to use the name GLI for this hormone. Since Van Noorden & Patent (1978) and Klein & Van Noorden (1980) demonstrated PP-immunoreactivity in the pancreatic A_2 cells with round granules of several teleosts, this hormone can be expected in A_{2r} cells, and hence in enteroendocrine type III cells; the more so because PP-like immunoreactive cells were demonstrated in the gastrointestinal mucosa of some teleosts (Falkmer & Stefan, 1978) and PP seems to be involved in regulatory processes for food-intake and digestion (Falkmer, 1979).

The cell types III and IV are similar as to distribution and size of granules, and the only difference between these cells is the irregular shape of the granules in cell type IV. As pancreatic A_{2r} cells may also contain irregular granules, depending on the applied method of fixation (Rombout, unpublished results), and as several specimens hardly possess type IV cells in the gut, it is possibly not justified to make a differentiation between the cell types III and IV.

On the basis of the size of the granules no more than 3 or 4 enteroendocrine cells can be recognized in *B. conchonius*, but theoretically it may be possible that two or even more cell types contain similar secretory granules. In fact, this has been noticed for several cell types of mammals; at present three types of D cells and two types of EC and G cells are known (cf. general introduction), whereas the neurotensin-producing N cell is distinguished from the population of L cells (Frigerio et al., 1977; Helmstaedter et al., 1977; Polak et al., 1977; Sundler et al., 1977).
Moreover, in fish the presence of "primitive hormones" resembling two or more mammalian hormones may also be possible. This was already suggested for the lamprey, which contains a single enteroendocrine cell type with gastrin, caerulein and glucagon immunoreactivity (Van Noorden & Pearse, 1974). Recently Larsson & Rehfeld (1977) and Vigna (1979) suggested the same for gastrin as evolved from CCK-like hormones. In lower vertebrates, only a CCK-like hormone (possibly caerulein) may have both CCK- and gastrin-like activities.

In summary the mammalian nomenclature for CCK-PZ-producing cells (I: intermediate granules) and GLI cells (L: large granules) may be applied to *B. conchonius* for cell types II and III respectively. Future studies based on immunocytochemical methods and physiological experiments may find conclusive evidence for the exact functions of fish enteroendocrine cells.

Most if not all enteroendocrine cells are of the "open" type, and have a long dendrite-like extension towards the lumen; this indicates that most of the stimuli originate in the intestinal lumen. An earlier suggestion that enteroendocrine type I cells are of the "closed" type (Rombout et al., 1977) is therefore withdrawn. The apical end of these cells probably receives adequate chemical stimuli from the lumen (probably specific for each cell type) which may activate or inhibit basal secretion. Such a chemoreceptory-secretory function was already suggested for mammalian enteroendocrine cells by Fujita & Kobayashi (1974). As no nerve endings can be observed near the enteroendocrine cells, these seem to be self-sufficing, but a paracrine impact from one cell to another may not be excluded.

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Chapter IV

Enteroendocrine APUD cells in the digestive tract of larval *Barbus conchonius* (Teleostei, Cyprinidae)

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SUMMARY

The development of *Barbus conchonius* is described with special attention to the differentiation of the gut.

Amine precursor uptake and decarboxylation (APUD)* are present in enteroendocrine cells during development, whereas these processes are lacking in adult specimens. The first APUD cells originate on the fourth day of development in the anterior part of the gut and on the fifth day in the caudal areas. The APUD facility of the cells disappears within 2 days, and after the 6th day APUD cells can no longer be distinguished in the intestinal epithelium.

The first APUD cells were observed when four types of enteroendocrine cells were recognized with the electron microscope. These enteroendocrine cells contain granules of different electron densities, and microtubules and cilia can be observed. Some enteroendocrine-like cells are found below the basement membrane of the intestinal epithelium, indicating a possible extra-endodermal origin.

APUD cells, except melanoblasts, have not been found migrating from the neural crest in ventral direction. The origin of the enteroendocrine cells of *B. conchonius* is discussed.

* Abbreviations: APUD, amine precursor uptake and decarboxylation; FIF, formalin-induced fluorescence; L-DOPA, L-dihydroxyphenylalanine; 5-HTP, 5-hydroxytryptophan.

INTRODUCTION

Enteroendocrine cells and pancreatic islet cells of birds and mammals together with several other endocrine cells belong to the APUD series (reviews: Pearse, 1973; Andrew, 1976*a*).

APUD cells take up and decarboxylate amine precursors, and most of these cells produce polypeptide hormones. Pearse (1966, 1969) was the first to propose a neural crest origin for the APUD cells; this was based on the ability to store amines and on the regular presence of cholinesterase. These cells have been shown to have a common origin with melanocytes, adrenal medullary cells, ultimobranchial and thyroid C cells, carotid body type I and probably type II cells, and possibly with pituitary ACTH cells (reviews: Pearse, 1973; Andrew,

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1976*a*). Pearse & Polak (1971) described APUD-FIF cells in mouse embryos migrating from neural crest to the future stomach and duodenum and to the dorsal pancreatic bud. For this reason they considered the neural crest as a source of the APUD cells (possibly not of all cell types) in these mentioned places. The presence of polypeptides of hypothalamic origin, e.g. of substance P (Polak, Hertz & Pearse, 1976), somatostatin (Polak *et al.* 1975), neurotensin (Helmstaedter, Taugner, Feurle & Forssmann, 1977; Sundler *et al.* 1977) and vasoactive intestinal peptide (Polak, Pearse, Garaud & Bloom, 1974; Buffa *et al.* 1977) in some enteroendocrine cells is in support of the theory of the neurectoderm origin of these cells. The presence of a gastrin-like peptide in the vertebrate brain (Vanderhaeghen, Signeau & Gepts, 1975) may be additional evidence for this theory. However, Andrew (1963, 1974) cultured parts of blastoderm and primitive gut of chick embryos as chorio-allantoic grafts, and Le Douarin & Teillet (1973) and Fontaine & Le Douarin (1977) transplanted neural primordia or germ layers from quail onto chick embryos (structural differences in the interphase nuclei); they concluded that enteroendocrine cells of birds do not originate in the neural crest. The same was found for pancreatic APUD cells by Andrew (1976*b*) by applying transplantation techniques.

Enteroendocrine cells are also found in the digestive tract of fish. Recently, four types of enteroendocrine cells have been distinguished in the adult *B. conchonius* (Rombout, 1977). In this cyprinid, as in other teleosts and cyclostomes, serotonin (Erspamer, 1952) or uptake of L-DOPA (Östberg, Van Noorden, Pearse & Thomas, 1976) was not observed in the digestive tracts of adult specimens. However, in a larval cyclostome (Lampetra) intestinal APUD cells were reported by Van Noorden, Greenberg & Pearse (1972). In order to test the hypothesis of the neural crest origin of the enteroendocrine cells, the distribution of APUD cells has been studied during the early development of *B. conchonius*, a species in which spawning can easily be induced.

MATERIALS AND METHODS

Fertilized eggs of *B. conchonius* were kept in Petri dishes at 25 °C. Embryos and larvae were fixed in Bouin and Zenker at various developmental stages. After vacuum-embedding in Paraplast plus (Sherwood) the material was serially cross-sectioned at 5 μm and stained with haemalum and eosin. The larvae were fed artemia-nauplii from the 5th day.

Amine precursor uptake and decarboxylation (APUD)

Formalin-induced fluorescence (FIF)

Embryos and larvae were incubated in 25 mg/l L-DOPA continuously or during 24 h at different developmental stages and fixed in 4% neutral formalin. Several stages were freeze-dried followed by fixation in formaldehyde vapour.

However, as with respect to fluorescence no difference was found with fixation in formalin solution; the latter method was used routinely.

Cross-sections and sagittal sections of 5 μm were examined with a Zeiss fluorescence Standard microscope with an HBO-50 superpressure mercury lamp, and selection filters BP 405:5, BP 405:14, and barrier filter 418 (epi-illumination).

Radioautography

Embryos and larvae were incubated in 50 or 100 μCi/ml L-3,4-dihydroxy (ring-2,5,6-^{3}H) phenylalanine (spec. act. 30 Ci/mmol) or in 20 or 100 μCi/ml DL-5-hydroxy (G-^{3}H) tryptophan (spec. act. 1·37 Ci/mmol) obtained from the Radiochemical Centre, Amersham, England. After an incubation of at least 4 h, the animals were fixed in 4% neutral formalin or freeze-dried followed by formaldehyde vapour fixation. Cross-sections and sagittal sections were prepared for radioautography with a Kodak NTB-2-emulsion. After an exposure time of 9 (DOPA) or 19 (5-HTP) weeks, the slides were developed with Kodak D-19 developer.

Electron microscopy

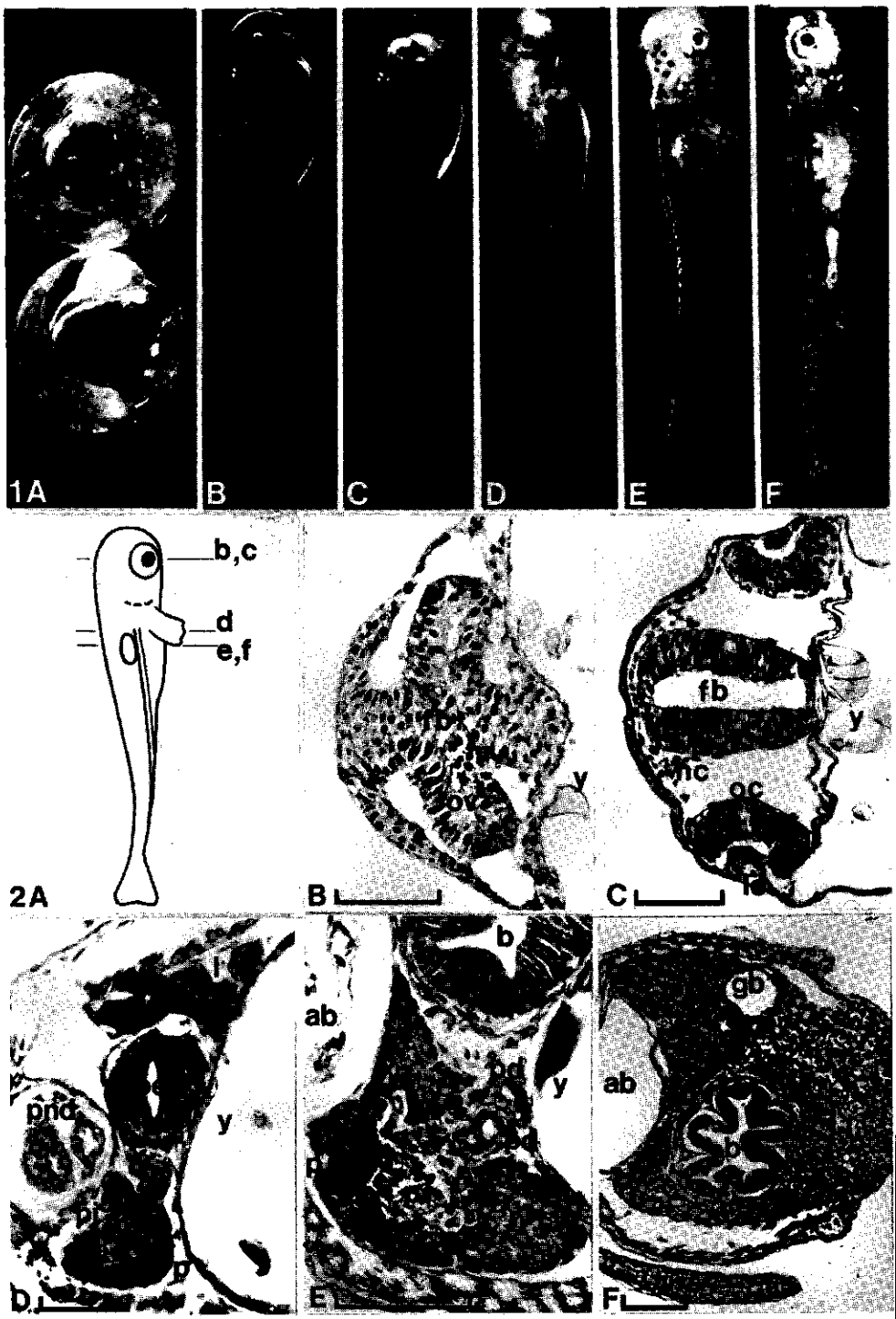
Specimens of 3, 4, 5 and 6 days old were fixed for 15 min at 0 °C in a mixture containing 1% OsO_4 and 2% glutaraldehyde buffered with 0·1 M cacodylate, pH 7·2, and postfixed for 1 h at 0 °C in a mixture containing 1% OsO_4, 2% glutaraldehyde and 1% potassium dichromate. Subsequently, the animals were dehydrated and embedded in Epon 812. Ultrathin sections were mounted on pioloform-coated copper grids and stained with saturated uranyl acetate and lead citrate. Photographs were made with a Philips EM 300 electron microscope.

RESULTS

Development

Several developmental stages are shown in Fig. 1.

First day. Within 8 h after fertilization a blastula is formed that consists of blastoderm, blastocoel and periblast. During gastrulation and at 11 h after fertilization, neurulation starts in the anterior area of the embryo. The ectoderm becomes considerably thicker along the central line and forms a wedge-shaped mass of stratified cells, the neural keel. The anterior part of the neural keel forms an enlarged solid mass, the *anlage* of the fore-brain with lateral enlargements, the prospective optic vesicles (Fig. 2B). The neurulation in the caudal area of the embryo is completed after 19 h. Then the neural crest cells can be recognized (Fig. 2C). At the end of neurulation a cavity is formed in the rostral part of the neural cord (Fig. 2C). During the second day this cavity extends in the caudal direction. During neurulation the notochord, the mesoderm and a thin layer of endoderm become separated from an undifferentiated mass of endochordame-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soderm, situated above the periblast and yolk. At the end of the first day the rostral part of the notochord becomes vacuolized. At this stage (24 h) the optic cups, lenses, otic vesicles, primary nephric ducts, hearts, blood vessels and all the myotomes are present, and the tail has become separated from the yolk sac (Fig. 1A).

Second day. In the first hours of the second day the heart starts to beat and a few hours later colourless blood begins to circulate. Between 27 and 32 h nearly all larvae have hatched and remain inactive at the bottom of the Petri dishes. Around the time of hatching, pigmentation of the eyes begins, and at the end of the second day the first melanocytes become visible. The anterior endoderm becomes thicker and is elevated at its lateral sides, thus forming the pharyngeal *anlage*. At the end of the second day the first part of the gut, presumably the bulbus, becomes thicker and a liver diverticulum can be noticed on its lateral side.

The paired primary nephric ducts originate from a glomerular structure immediately behind the heart, between endoderm and myotomes, and join at the caudal end near the presumed anus.

Third day. During the third day a lumen is formed in the digestive tract except in the presumed oesophagus and hindgut. A prospective airbladder originates on the dorsal side of the oesophagus and a pancreas diverticulum is separated from the lateral side of the bulbus immediately caudal to the liver. At the end of the third day a gall-bladder is formed and the first islet of Langerhans can be observed. The head is lifted from the yolk sac and the first extremities become visible.

Fourth day. Gills and pharyngeal teeth are formed in the pharynx and irregular respiration sets in. The whole digestive tract becomes open from mouth to anus. The bulbus becomes very wide while the liver and pancreas proliferate strongly. The first islet of Langerhans is always located near the gall-bladder and close to the place where bile duct and pancreatic duct join and

FIGURES 1 AND 2

Fig. 1. Larvae of *B. conchonius*. (A) 24 h (diameter, 1 mm); (B) 30 h, just hatched (length, 2·5 mm); (C) 2 days (3 mm); (D) 3 days (4 mm; note the decreasing yolk content); (E) 4 days (4 mm; airbladder is filled with air); (F) 5 days (5 mm; after first feeding).

Fig. 2. Cross-sections of larvae of *B. conchonius* as indicated in (A); (B) 13 h, cranial part of the head. Note the neurulation and the prospective optic vesicles; (C) 19 h, same region after neurulation. Neural crest, optic cup, lenses and cavity in the brain are formed; (D) 88 h, the oesophagus is shown near the transition to the bulbus. Note the liver, the pancreas with the principal islet, and the pneumatic duct; (E) 4 days; note the position of the islet of Langerhans (principal islet); (F) 5 days; note the strong proliferation of liver and pancreas, the enlarged airbladder, the position of the spleen, and the pancreatic duct; bar 50 μm. *ab*, airbladder; *b*, bulbus; *bd*, bile duct; *fb*, forebrain; *gb*, gall-bladder; l, liver; *le*, lens; *nc*, neural crest; *oc*, optic cup; *oe*, oesophagus; *ov*, optic vesicle; *p*, pancreas; *pd*, pancreatic duct; *pnd*, pneumatic duct; *pi*, principal islet; *s*, spleen; *y*, yolk.

open into the bulbus (Fig. 2E). At the end of the fourth day a spleen is present and contains mainly erythrocytes. The primary nephric ducts join with the narrow hindgut near the anus. The airbladder enlarges and fills with air (Figs. 1E, 2F).

Fifth day. At the end of the fifth day, food uptake starts (Fig. 1F), the gut becomes functionally active, and the yolk has almost disappeared. Three zones can now be distinguished in the digestive tract: a fat absorptive zone (75%) is followed by a protein absorptive zone (20%) and a third zone, possibly a water and ion absorptive zone (5%).

At the end of the fifth day the intestinal epithelial cells vary in height, but intestinal folds cannot be observed until the eighth day.

Amine precursor uptake and decarboxylation

Incubation with [^{3}H]L-DOPA

Around the time of hatching ventrally migrating melanoblasts can be recognized as APUD cells with radioautography; during migration they start to form pigment. Formalin-induced fluorescence, also observed at this developmental stage, must be attributed to the autofluorescence of the blood plasma. With these techniques it was not possible to recognize APUD cells migrating from neural crest to digestive tract. As shown in Table 1, weak greenish-yellow fluorescent or ^{3}H-labelled APUD cells appear in the bulbus after 3 days. The amine content of these cells increases on the fourth day and again increases after the administration of L-DOPA. After 4 days, APUD cells are present in the first 75% of the gut, the prospective fat absorptive zone (Fig. 3, 4). On the fifth day, APUD cells originate also in the caudal part of the gut, while the APUD facility of these cells disappears in the rostral parts of the gut (Fig. 5). Around the sixth day, APUD cells can be observed only near the anus. As from the seventh day, cells with APUD characteristics are not present anymore. Application of FIF technique and radioautography together to the same sections showed that nearly all the ^{3}H-labelled cells correspond to fluorescent cells. The weakly fluorescent cells cannot be detected with radioautography and this may be attributed to insufficient exposure time. Radioautographic examination becomes difficult when feeding starts (after 5 days), probably because [^{3}H]L-DOPA is absorbed by the epithelial cells. The APUD cells are distributed between the epithelial cells of the intestine, but most of them are found in the first part of the gut, where the APUD cells are generally of the 'open type' (extending from basement membrane to the lumen), whereas the last part of the gut contains also cells of the 'closed type' (not extending to the lumen; Figs. 3, 4).

Most of the cells of the 'open type' have a flask-like shape (Figs. 3B, 6, 7). After fixation with formalin, many epithelial cells lose the contact with the basement membrane, while APUD cells regularly remain attached to this membrane (Fig. 3B), to which they appear to have a greater affinity. Radio-

Table 1. *Amine precursor uptake of enteroendocrine cells in larvae of* B. conchonius *at different developmental stages*

Developmental stage	Location*	FIF (5)	FIF after incubation in L-DOPA (10–20)	Labelling with [^{3}H]L-DOPA (6)
2 days	I	−	−	−
	II	−	−	−
3 days	I	−/+	−/±	±
	II	−	−	−
4 days	I	±/+	+/+++	+/+++
	II	−	−	±
5 days	I	−	−	−/+
	II	±	±/++	±/++
6 days	I	−	−	−†
	II	−/±	−/±	−/+
7 days	I	−	−	−†
	II	−	−	−
8–16 days	I	−	−	−†
	II	−	−	−

The number of larvae is given in parentheses. −, negative; ±, dubious; +, weak positive ++, positive; +++, strong positive.

* I, first 75 % of the intestine (fat absorptive zone). II, last 25 % of the intestine (protein + water and ion absorptive zone).

† High background, due the absorption of [^{3}H]L-DOPA.

autographically several APUD cells can be observed in the submucosa below the basement membrane (Fig. 7).

With the FIF technique these extra-epithelial APUD cells are difficult to observe, because of the autofluorescence of blood plasma (Figs. 3A, 6).

APUD cells cannot be detected in the islets of Langerhans.

Incubation with [^{3}*H*]5-*HTP*

No APUD cells have been found by this method in the digestive tract of larval *B. conchonius.*

Electron microscopy of the intestine

3-day-old larvae. In the bulbus, which has just acquired a lumen, enteroendocrine cells cannot be observed. The epithelial cells (height about 7 μm) contain a basally located nucleus, a small Golgi apparatus, a poorly developed smooth endoplasmic reticulum, some large mitochondria, many ribosomes and polysomes, and apically some very short microvilli (up to 0·3 μm).

4-day-old larvae. The epithelial cells of the first part of the gut (height about 15 μm) contain microvilli up to 0·7 μm. These cells contain a distinct Golgi apparatus, a swollen smooth endoplasmic reticulum and a clear terminal web. At this stage goblet cells and enteroendocrine cells of the four types (Rombout,


3A
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y
bv
y
a
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4B
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4A
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e

1977) are present in the rostral part of the gut. Most of the enteroendocrine cells are of type I and II whereas type III and IV are scarce at this stage. The basal part of these cells contain a few too many granules of different electron densities (Fig. 9). The observed narrow apical parts contain microtubules, some cilia, some small pinocytotic (?) vesicles and a few granules (Figs. 10, 12). Cilia are present on the apical side of these cells between the microvilli (Fig. 12) or extending in the intercellular space beneath the junctional complex (Fig. 11).

5- and 6-day-old larvae. The intestinal epithelium seems to be completely differentiated in 5-day larvae, when the absorptive cells have almost reached their maximal size (length about 23 μm; microvilli 1·5 μm).

Enteroendocrine cells are more numerous and contain more electron-dense granules. In the second and third zone nearly all the endocrine cells are of type I. At this stage the food uptake starts and the absorptive cells become functionally active. The absorptive cells of the first zone contain several large lipid vacuoles and many chylomicrons. The absorptive cells of the second zone contain several 'supranuclear bodies' and many pinocytotic vesicles. From the fourth day, cells with granules with an average diameter of 170 nm are found in the submucosa below the basement membrane of the intestinal epithelium (Fig. 8). Possibly these cells represent the enteroendocrine type II cell (Rombout, 1977), as no other cells with granules of this size are known in the digestive tract of *B. conchonius*.

DISCUSSION

The early development of *B. conchonius* is very similar to the development of other cyprinids: *Cyprinus carpio* (Verma, 1971), *Labeo gonius* (Khan, 1925) and *Carassius auratus* (Khan, 1929). Differences are only found in the time schedule and the sequence of developmental processes.

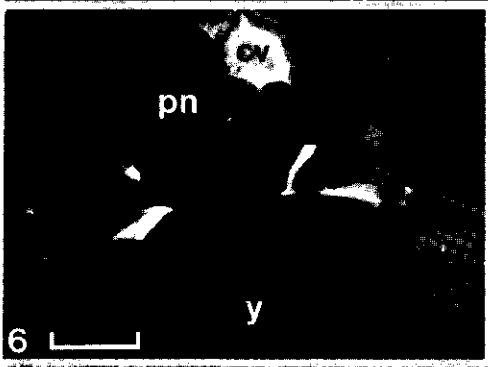
During development, APUD cells migrating from neural crest to prospective gut were not observed, contrary to observations in mouse embryos (Pearse & Polak, 1971). Only after administration of [^{3}H]L-DOPA were ventrally migrating ^{3}H-labelled melanoblasts noticed around hatching time.

FIGURES 3 AND 4

Fig. 3. Sagittal section of a 4-day-old larva after L-DOPA administration. (A) The gut is shown from airbladder up to anus. Many APUD cells are seen in the prospective fat absorptive zone, and only a few in the caudal 25 % of the gut. The blood vessels dorsal to the gut are also strongly fluorescent. (B) Detail of the rostral part of the gut; bar, 100 μm. *a*, anus; *ab*, airbladder; *bv*, blood vessel; *y*, yolk.

Fig. 4. Radioautograph of a sagittal section of a 4½-day-old larva after [^{3}H]L-DOPA administration. (A) About 70 % of the whole gut is shown. Note the black melanocytes and blood vessels dorsal to the gut. (B) Detail of the rostral part with some clear APUD cells. (C) Detail of the caudal part with some APUD cells of the 'closed type'; bar 100 μm. *a*, anus; *bv*, blood vessel; *m*, musculature; *mc*, melanocyte; *y*, yolk.


a
5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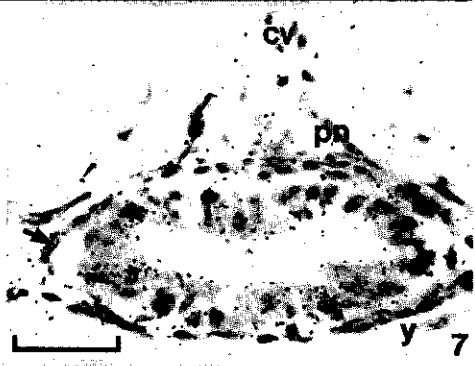
6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8

These melanoblasts can also be recognized by their pigment formation during migration, previously reported by Orton (1953) for fishes. The first APUD cells appear in the intestinal epithelium during the fourth day, when development is rather far advanced.

In contrast to the APUD cells of mammals, the cells of this species lose their characteristics after a few days and these characteristics do not return in later stages. This might be attributed to an increasing monoamine-oxidase activity causing a breakdown of the fluorescent amine. Inhibition of L-DOPA-decarboxylase, preventing the formation of biogenic amines, might be another cause.

Andrew (1975) found APUD cells in the intestinal groove of the chick, concentrated at the site of evagination of the pancreatic bud, at the end of the second day (16–18 somites), disappearing the third day and reappearing in the gut from 12 days onwards. Although she considers the possibility of a temporary loss of biogenic amine-synthesizing ability, she supposes that these early arising APUD cells are the precursors of one or more islet cell types, because APUD cells are found in the pancreas from its formation (27 somites) onwards. In the present study APUD cells have not been observed in the islet of Langerhans (principal islet) of *B. conchonius*.

The greenish-yellow fluorescence of the intestinal APUD cells of *B. conchonius* indicates the presence of a catecholamine, possibly of dopamine. This colour, however, does not mean that other catecholamines are absent. The presence of serotonin (yellow fluorescence) in the APUD cells can be excluded, as no uptake of [^{3}H]5-HTP was found. It is very possible that the APUD cells represent the enteroendocrine cells, as their amine storage mechanism is noticeable when four types of enteroendocrine cells can be observed with the electron microscope; moreover, their flask-like shape ('open type') and their occurrence as single cells throughout the intestinal epithelium are normal features of enteroendocrine cells. The amine storage mechanism might be required for the differentiation of the enteroendocrine cells. However, entero-

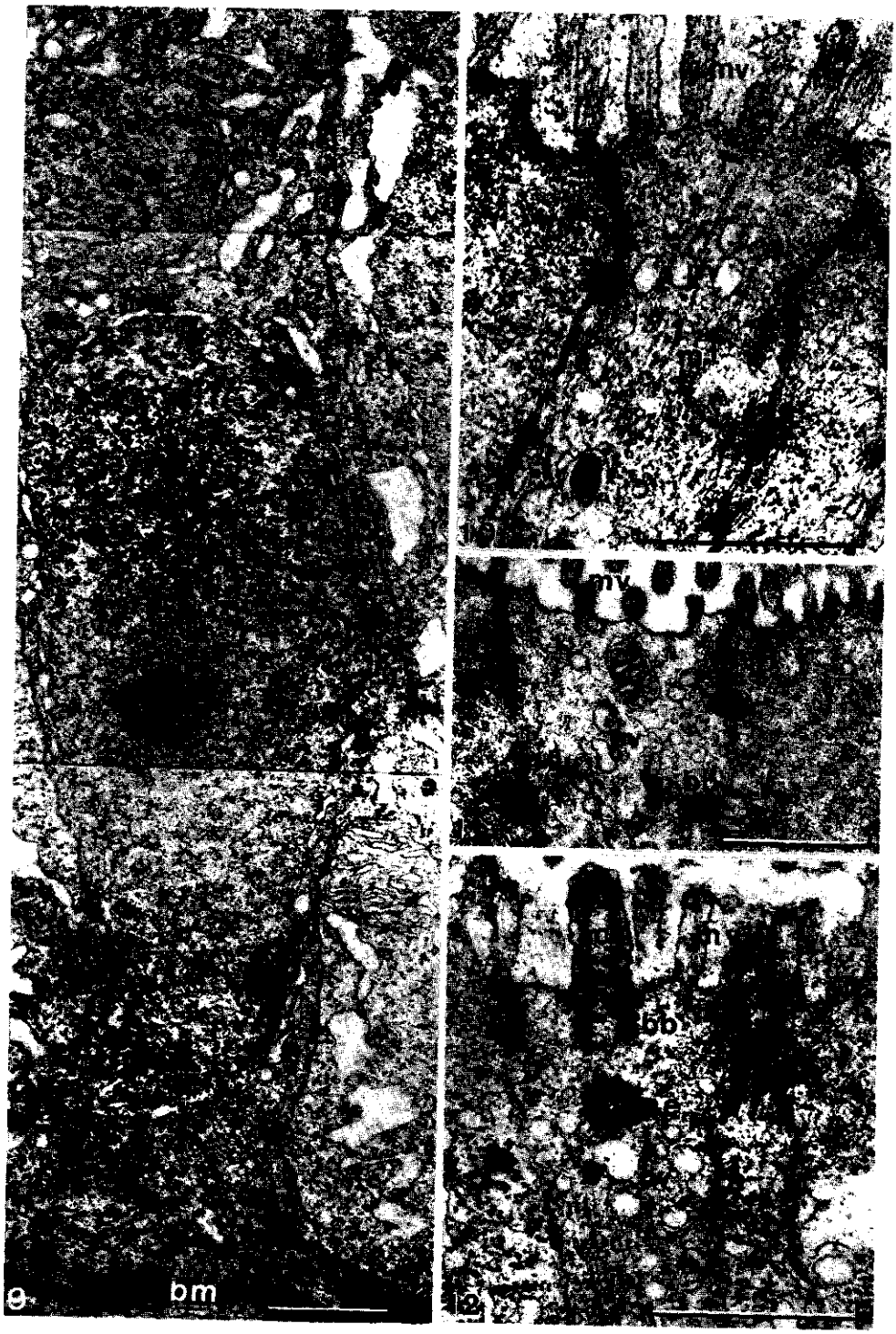
FIGURES 5–8

Fig. 5. Sagittal section of the gut of a 5½-day-old larva after L-DOPA administration. APUD cells in the gut only near the anus; bar 20 μm.

Fig. 6. Cross-section of a 4-day-old larva after L-DOPA administration. Flask-like APUD cells in the larval gut; bar 20 μm.

Fig. 7. Radioautograph of a cross-section of the same region of a 4-day-old larva after [^{3}H]L-DOPA administration. Some flask-like APUD cells and a weak APUD cell outside the intestinal epithelium (arrow); bar 20 μm.

Fig. 8. Electron micrograph of an enteroendocrine-like cell with granules of about 170 nm just below the basement membrane in the bulbus of a 5-day-old larva; bar 1 μm. *a*, anus; *bm*, basement membrane; *cv*, cardinal vein; *ec*, enteroendocrine-like cell; *pn*, primary nephric duct; *y*, yolk.


mv
mv
bm
n
bb

endocrine cells must also differentiate at later stages and in the gut of adult fishes where amines have not been found in appreciable amounts (Rombout, 1977).

More likely, the amine storage mechanism in the enteroendocrine cells of *B. conchonius* may be a remnant of their neurectodermal origin, in accordance with the hypothesis of Pearse (1966, 1969). The extra-endodermal origin of the enteroendocrine cells is also supported by the presence of APUD cells and enteroendocrine-like cells in the submucosa below the basement membrane. Enterochromaffin cells were also found outside the intestinal epithelium by Kull (1925) and Dias-Amado (1925), who considered them as of mesodermal origin, and by Simard & Van Campenhout (1938) and Monesi (1960), who suggested a migration of these cells from epithelium to connective tissue (endodermal origin).

Contrary to the indication of the neural crest origin of enteroendocrine cells and pancreatic islet cells in mice by Pearse & Polak (1971) are the negative results with the chick obtained by Andrew (1963, 1974, 1976*b*) and Le Douarin & Teillet (1973). Fontaine & Le Douarin (1977), who combined at an early stage lower and upper germ layers from chick and quail embryos (chimaeras), even excluded the neurectoderm as possible origin for enteroendocrine cells and pancreatic islet cells. Consequently, in chick and quail it seems hardly possible that the neural crest or even the neurectoderm is the source of these cells.

Andrew (1976*a*) suggested that 'the common factor sought in the genesis of the APUD cells may be the biochemical rather than the embryological relationship of the progenitor cell types'. Our results show, however, that the amine storage mechanism in fish is an embryological feature that is lacking in adult specimens. This was also found by Fontaine (1974) for the metanephritic FIF cells of the chick, which originated in the neural crest. A disappearance of the APUD facility was also suggested by Pearse & Takor Takor (1976) for endo-

FIGURES 9–12

Fig. 9. Electron micrograph of an enteroendocrine type II cell of larval *B. conchonius* containing granules of about 170 nm; bar 1 μm.

Fig. 10. Electron micrograph of the apical side of an enteroendocrine type III cell of larval *B. conchonius*, containing granules of about 230 nm. Note the presence of pinocytotic vesicles and microtubules; bar 1 μm.

Fig. 11. Electron micrograph of the apical side of an enteroendocrine type II cell of an adult *B. conchonius*. Note the presence of a short cilium, extending in the intercellular space below the junctional complex (arrow), pinocytotic vesicles and microtubules. This type of cilium is also present in the type II cell of fig. 9 (not shown in figure); bar 1μm.

Fig. 12. Electron micrograph of the apical side of an enteroendocrine cell of larval *B. conchonius*. Note the cilium between the microvilli and the presence of pinocytotic vesicles and microtubules; bar 1 μm. *bm*, basement membrane; *bb*, basal body; *c*, cilium; *ce*, centriole; *g*, Golgi apparatus; *mt*, microtubules; *mv*, microvilli; *n*, nucleus; *pv*, pinocytotic vesicle.

crine cells outside the APUD series, e.g. gonadotrophs, thyrotrophs and parathyroid chief cells, which possibly also originate in the neurectoderm or placodal ectoderm. Our results fit the hypothesis of these authors who consider the polypeptide hormone-producing cells as 'nerve cells whose essential function (neurotransmission) has been transposed into an endocrine one', with or without loss of the APUD facility. In summary, it may be possible that enteroendocrine cells of *B. conchonius* have a neurectoderm or placodal ectoderm origin.

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Chapter V

An experimental study on neural crest migration in *Barbus conchonius* (Teleostei, Cyprinidae), with special reference to the origin of the enteroendocrine cells.

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SUMMARY

A neural crest transplantation technique is described for fish. As in other classes of vertebrates, two pathways of neural crest migration can be distinguished: a latero-ventral pathway between somites and ectoderm, and a medio-ventral pathway between somites and neural tube/notochord. In this paper evidence is presented for a neural crest origin of spinal ganglion cells and pigment cells, and indication for such an origin is obtained for sympathetic and enteric ganglion cells and for cells that are probably homologues to adrenomedullary and paraganglion cells in the future kidney area. The destiny of neural crest cells near the developing lateral line sense organs is discussed. When grafted into the yolk, neural crest cells or neural tube cells appear to differentiate into "periblast cells"; this suggests a highly activating influence of the yolk. Most of the neural crest cells are found around the urinary ducts and, when grafted below the notochord, even within the urinary duct epithelium. These neural crest cells do not invade the gut epithelium, even not after grafting neural crest near the future gut. Consequently enteroendocrine cells in fish are not likely to have a trunk- or rhombencephalic neural crest origin. Another possible origin of these cells will be proposed.

INTRODUCTION

In all vertebrate embryos the neural crest appears directly after neurulation in an anterior-posterior wave-like movement along the embryonic axis. Almost immediately after its development the neural crest cells begin to disperse throughout the embryo. However, most of the early intra-embryonic cell movements escape observation as the cells are undifferentiated and cannot

be distinguished from the tissues in which they move. Therefore, several experimental techniques have been developed, mainly based on extirpation, explantation and transplantation (c.f.: Weston, 1970; Le Douarin, 1976) to study the destiny and the pathways of migration of neural crest cells. In fish, migration pathways of pigment cells have been extensively studied, for pigment formation takes place in an early stage of migration (Orton, 1953; Shepard, 1961). The relatively few experimental studies on other neural crest cells in fishes were restricted to extirpation and explantation experiments (Lopashov, 1944; Damas, 1951; Newth, 1951, 1956) Except the pigment cells and cells of the spinal ganglia, no other cells or structures are known to be of neural crest origin, but this origin has been assumed for ganglia and arteries in the head region and for some parts of the visceral and head skeleto: The scarce information on the destiny of the neural crest has been the main mot ve for developing a technique to study migration of neural crest cells in fishe.

Recently, enteroendocrine cells and their relation to the APUD-system (acronym stands for *A*mine *P*recursor *U*ptake and *D*ecarboxylation) have been described for *B. conchonius* (Rombout, 1977; Rombout et al., 1978). The cells of the gastro-entero-pancreatic (GEP) endocrine system, together with many other endocrine cells, belong to the APUD-series, which is presumed to be derived from a common neural ancestor (Pearse, 1969; Pearse & Takor Takor, 1979); such an origin was proved for some cell types of this series (c.f. Andrew, 1976), but not for cells of the GEP endocrine system (Pearse, 1979). Therefore, special attention will be paid to a possible contribution of the neural crest cells to the gut. Our experiments were restricted to the trunk and the late rhombencephalic region.

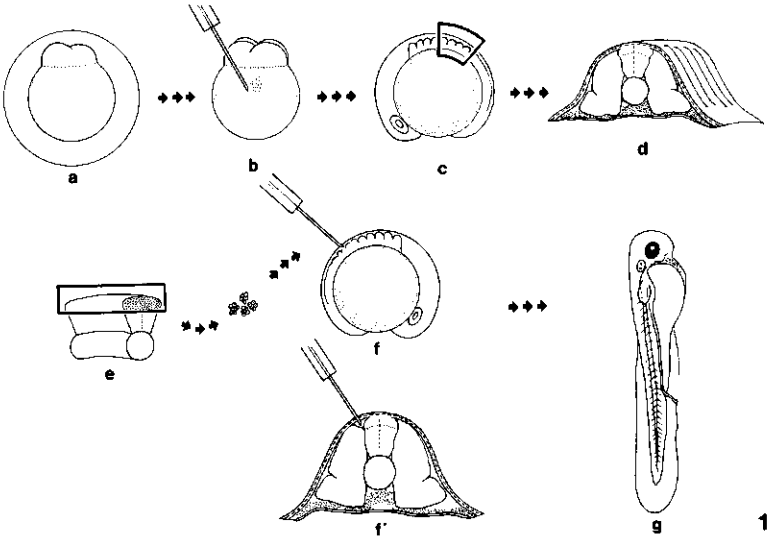
A stable cell labeling technique as applied in birds (Le Douarin, 1973) was not yet available for fish. So ^{3}H-thymidin, which had been used in avian and amphibian embryos (Weston, 1963; Chibon, 1966; Johnston, 1966) has been used as a marker for studying migration of neural crest cells.

MATERIALS AND METHODS

Embryos of *Barbus conchonius* have been used for this investigation. Early developmental stages were determined by counting the somites, and later stages were determined by normal developmental stages at 25°C. The small size of the embryo (Ø $\pm$ 0.8 mm), the convex shape and the pressure in the yolk prevented the transplantation of parts of the neural tube. Therefore, the transplantation

technique was modified as will be described. H^3-thymidin was used as nuclear marker (spec. act. 24 Ci/mmol; Radiochemical Centre, Amersham, England).

Experimental procedures (fig. 1a-g):



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After fertilization the chorion of the eggs of *B. conchonius* was removed (fig. 1a) with 0.5% protease (Sigma, chem. comp. P 5130). The eggs were then rinsed and cultured in 10% Holfreter solution at 19-20°C. H^3-thymidin (10-30 nl:1mCi/ml) was injected into the yolk of the eggs of the donor embryos, just below the cells at the 2-16 cell stage (fig. 1b). At that stage the cells are still not separated from the yolk by a basal membrane. Transplantations were carried out 24-30 hours after fertilization between the 1st and the 16th somite stage (fig. 1c). At each stage the neural primordium at the place of the last formed 5-6 somites is mechanically excised together with the surrounding tissue, and isolated by incubation in 0.03% trypsin solution in a NRHC medium (Sigma, chem. comp. T8253) (fig. 1c, 1d, 1e). In most cases the notochord remains attached to the neural primordium after trypsinisation, permitting an exact orientation. The dorsal part of the neural primordium was excised mechanically to obtain small pieces of tissue that contain mainly neural crest cells among the cells of the dorsal part of the neural tube (fig. 1e). This cell material was injected into host embryos by micro-injection with glass capillaries, preferably dorsally between neural tube and somites, the place where normally the migration of the neural crest cells has its origin (figs. 1f, 1f'). The host embryos received grafts in the rhombencephalic and trunk region between the

1st and the 16th somite stage, just before the anus.

The migratory capacity of neural tube cells was checked by injecting cells of labeled ventral neural tube.

After transplantation the host embryos were kept for 1 to 5 days at 25°C. Embryos were fixed in Bouin solution, and cross-sectioned at 5 µm after embedding in Paraplast Plus (Sherwood). Sections were prepared for radioautography with Kodak NTB-2 emulsion. After 1 to 6 weeks exposure the slides were developed with Kodak D-19 developer and stained with haemalum and eosin.

RESULTS

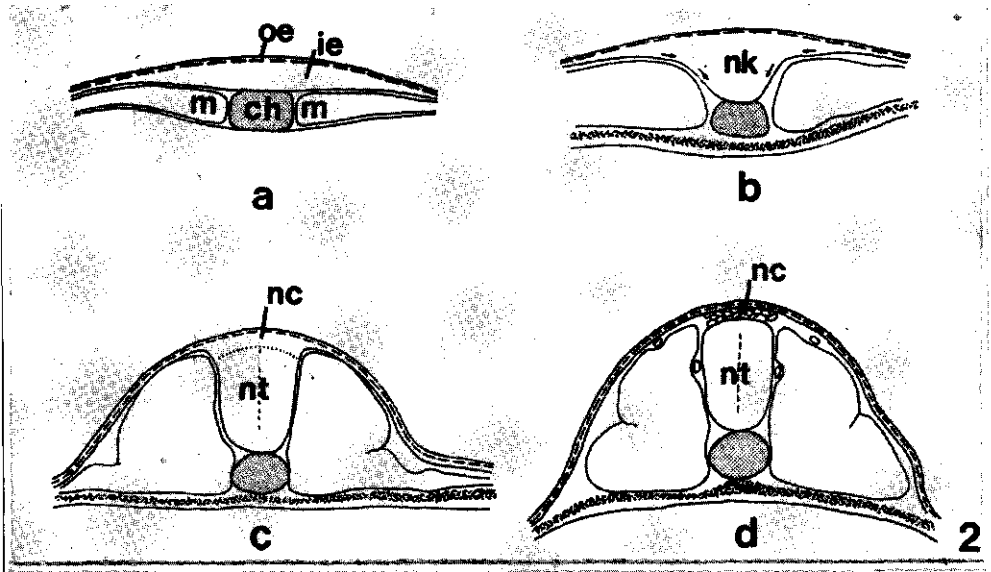
1. Marker

After injection of ^{3}H-thymidin, the embryos developed normally without any noticeable retardation in comparison to untreated control animals and no radiation damage has been observed. The cell nuclei proved to be heavily labele (fig. 4), at least until the larval stages (6 days). This method was suitable for our experiments, as after transplantation the cells can be easily recognize with radioautography.

Of the 410 host embryos that received grafts, 243 survived and in 120 of these labeled cells could be recognized. In most cases, clusters of probably neural tube cells remained at the place of injection; this served as a point of reference for the exact spot of injection. In 41 embryos this point of reference was located at the intended place, dorsally between neural tube and somites; these grafts can be considered as isotopic ones. Only this group has been used for studying migration. In another 40 embryos the grafts were traced

Fig. 2a-d. Scheme of the process of neurulation in the trunk region.

Fig. 3a-f. Cross-sections of embryos during several stages of neurulation (bar = 20 µm).
a) Notochord just formed and not yet separated from the endodermal layer. The inner layer of the ectoderm becoming thicker in the midline. Note the large periblast nuclei. b) The inner ectodermal layer becoming more wedge shaped in the midline, forming the neural keel. c) The process of neurulation more advanced than in b. d) Neurulation completed and the basement membrane is formed at the dorsal side. Neural crest becomes visible above the neural tube (arrow). e) Neural crest cells start to migrate (arrows) the notochord becomes vacuolized. f) Section in the region of the first somite (rhombencephalic). The neural anlage more pronounced than in the trunc region. The entodermal layer clearly visible in this region.
ch: notochord; e: endoderm; ie: inner ectodermal layer; m: mesoderm; n.c.: neural crest; n.k.: neural keel; n.t. neural tube; oe: outer ectodermal layer; p: periblast.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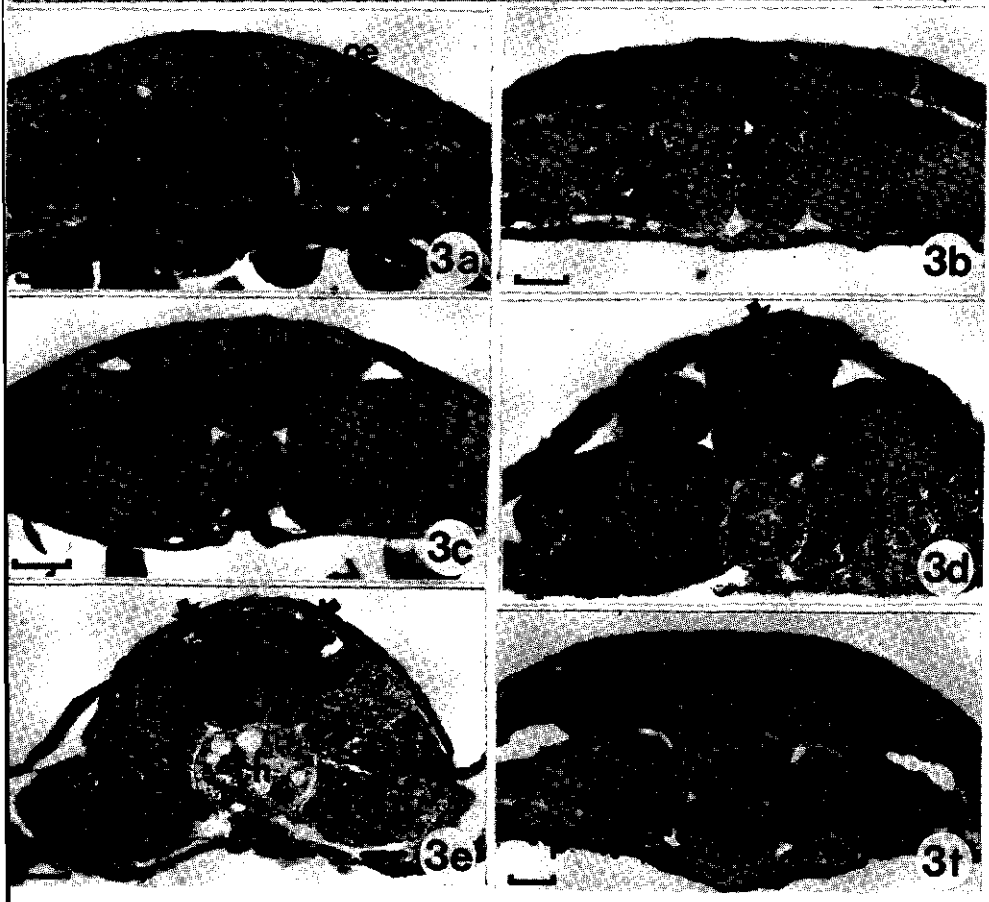
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in more ventral places, and can be considered as ectopic grafts; the periblast of 21 of these embryos contained labeled cells. The remaining group of embryos with labeled cells was not used for different reasons, e.g. no distinct point of reference, high background or inadequate fixation.

2. Early development and neurulation

Gastrulation takes place between 7 and 10 h (25°C) after fertilization. Within 1 h after closure of the blastopore the notochord becomes visible in the midline of the embryo (figs. 2a, 3a). Neurulation begins at the same time in the head region and continues in an anterior-posterior wave along the embryonic axis; cells of the inner layer of the dorsal ectoderm move towards the midline and form a wedge-shaped mass of cells, the neural keel (figs. 2b, 3b,c,f). Between 12 and 13 h the first somites are formed and the future optic vesicles appear as lateral enlargements of the forebrain. At 19-20 h, when all somites have developed (about 33), neurulation is completed in the caudal part of the embryo. The end of neurulation at a certain place is marked by the appearance of a basement membrane at the dorsal side of the neural tube; at that time neural crest cells can be recognized as a distinct group of cells between ectoderm and neural tube (figs. 2c, 3d). The cells within the neural cord become rearranged into right and left groups facing the midline. At the same time the neural crest cells become separated and begin to migrate (figs. 2d, 3e). The start of this cell migration is gradually transmitted in caudal direction and follows the formation of the somites. The duration of migration could not be determined; the beginning is noticed in the head region 14 h after fertilization, whereas some pigment cells still seem to be moving away from the neural tube 32 h after fertilization.

3. Migration of neural crest cells in isotopic grafts

After 2-6 h the injected cells were well incorporated in the host-tissue (fig. 5) and 6 h after transplantation a dispersion of these cells can be noti After 17 h the cells were found in different places, from neural tube to ventr region (fig. 6). At that time migration seems to be completed, and only growth and differentiation of the tissues take place in the following days of develo ment. No principal differences have been observed in the location of the migra cells at various developmental stages (1-6 days), and the results have been co bined. Only the cells that had distinctly moved away from the grafts are considered as migrated cells. The distribution of the migrated cells from isotopi

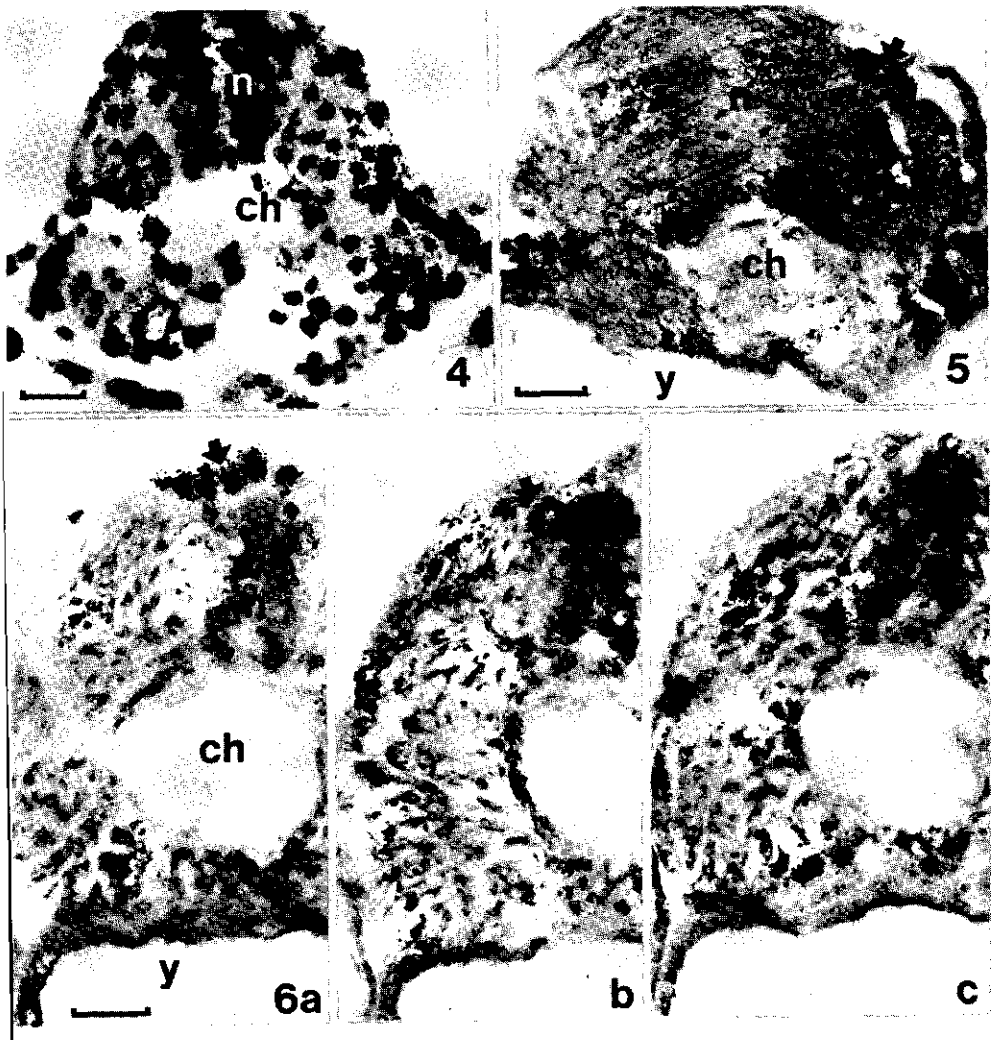


Fig. 4. Radioautograph 23 h. after ^{3}H-thymidin injection. All nuclei of the embryo heavily labeled (cross-section, bar = 20 µm).

Fig. 5. Radioautograph of a section at the level of the first somite 2h. after grafting. The graft (arrow) well incorporated in the host tissue (bar = 20 µm).

Fig. 6a,b,c. Radioautographs of sections at different levels of the same embryo 17 h. after grafting (bar = 20 µm).
a) a group of labeled cells located at the dorso-lateral side of the neural tube (arrow); these cells have not migrated, indicating the place of injection. b,c) sections at 35 µ and 90 µ caudally from a) respectively. A few cells are still located near the neural tube (arrow). Most cells have migrated from the graft in two directions, one in ventral and one in latero-ventral direction.
ch: notochord; n: neural tube; y: yolk.

grafts is presented in fig. 7. Neural crest cells migrate from their initial place along two ways (figs. 6, 7): one pathway in ventral direction passing the area between somite and neural tube/notochord, the other pathway in ventro-lateral direction between somites and ectoderm.

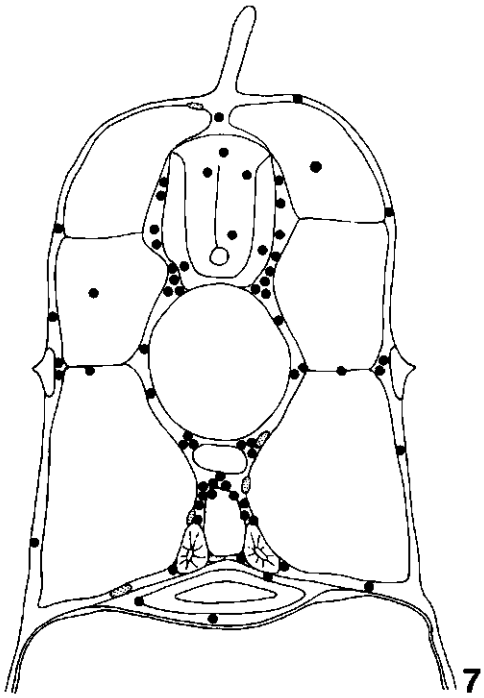


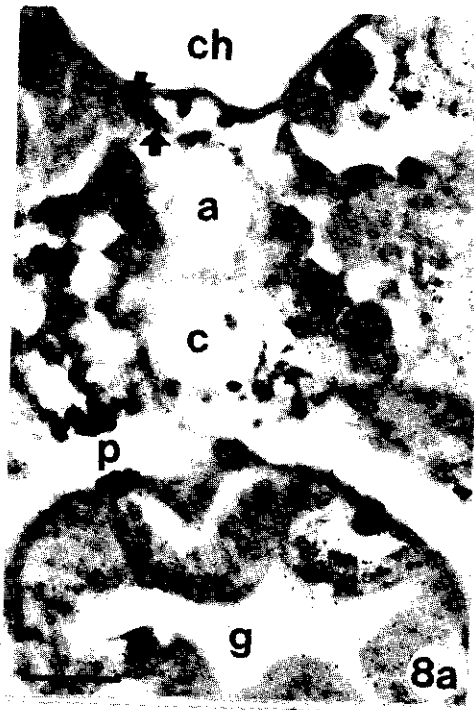
Fig. 7. Diagram of a cross-section through the trunk region of an embryo of 3-4 days showing the total number of migrated cells in different areas, summarized for 41 embryos. Each point represents 4 labeled cells.

Fig. 8. Radioautographs of a host embryo, 5 days after grafting (bar = 10 µm).
a) two labeled cells located near the aorta (arrows). In this region the symathetic ganglion will be formed.
b) one labeled cell between aorta and cardinal vein (arrow).

Fig. 9. Radioautograph of a host embryo 4 days after grafting; one labeled cell near the basement membrane of the gut epithelium, and two labeled cells between aorta and cardinal vein (arrows) (bar = 5 µm).

Fig. 10. Radioautograph of a host embryo, 3 days after grafting. The graft was injected near the developing primary urinary duct; four labeled cells in the epithelium of the primary urinary duct, and two cells near. Just below the basement membrane of the gut epithelium three labeled cells (bar = 5 µm).

Fig. 11. Radioautograph of a host embryo 6 days after grafting labeled cells into the yolk. Labeled cells with the characteristic large nuclei of the periblast. The section was made at a level where the yolk is completely absorbed and only the periblast is present underneath the gut (bar = 10 µm).
a: aorta; c: cardinal vein; ch: notochord; g: gut; p: pigment cell; u: urinary duct.



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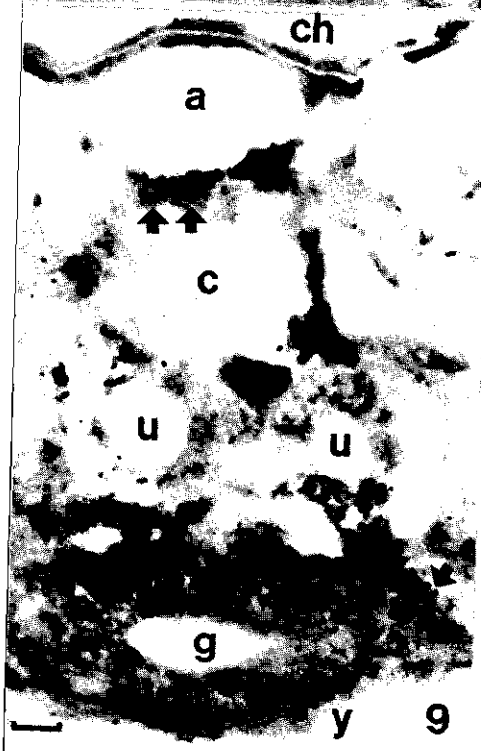

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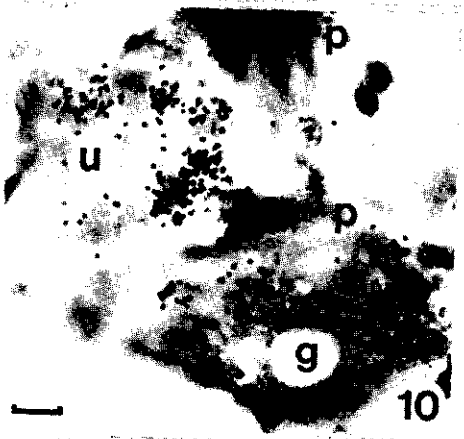

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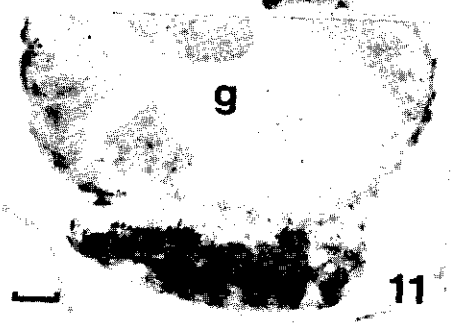

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Cells migrated via the first pathway have been found:

-- in the area of the developing spinal ganglia and within the ganglia.
-- at the periphery of the notochord; nucleï of these cells are oblong. Some labeled oblong nucleï are also found in the septa between the myotomes.
-- in the area between notochord and intestine where aorta, cardinal vein, and primary urinary ducts are located, and where sympathetic ganglia are formed (fig. 8); the cells are found ventro-laterally of the aorta, near the cardinal vein, in the walls of the cardinal vein and aorta, and near the primary urinary ducts.
-- in the area around the gut; labeled cells were not observed, however, in the intestinal epithelium (fig. 9).

Cells migrated via the second pathway have been found:

-- in the skin, where they are mainly differentiated as pigment cells; melanin formation reduces the possibility of recognizing the ^{3}H-thymidin label, and only some of the pigment cells could be traced.
-- near the lateral line organ and in the septa at the place of this organ.

In several embryos labeled cells appear to be located in the neural tube, and some were noticed in the myotomes. These are probably myoblasts or neural tube cells which had become intermingled with the graft.

4. Neural crest cells in ectopic grafts

In embryos in which the grafts had been injected at the ventral side of the embryos or even in the yolk, the following observations were made: When the graft had been injected near the future primary urinary ducts and intestine, distinctly labeled cells were noticed in the epithelium of the primary urinary duct but not in the intestinal epithelium, whereas several labeled cells are present in the gut wall (fig. 10). It is remarkable that the wall of the gut in ectopic grafts contains a much greater number of cells (n=34) than in the isotopic grafts (n=9). Participation of neural crest cells in the development of the primary urinary duct epithelium is not accompanied by abnormal shape or malformation.

When neural crest or neural tube cells accidentally had been injected int the yolk, some remained there but others appeared to have fused with the periblast. The labeled nucleï of these cells were just as large as other nucleï in the periblast (fig. 11).

5. Grafts of fragments from the ventral part of the neural tube

It is not yet clear whether the labeled cells in the neural tube are neural crest cells or cells of the dorsal part of the neural tube, which had been injected together with the neural crest cells. As a control on the migratory capacity of the neural tube cells, fragments of ventral neural tube were injected (± 40 grafts) to prevent any intermingling with neural crest cells. Migration of cells from the graft has practically not been noticed.

DISCUSSION

In the present study ^{3}H-thymidin was used as nuclear marker. This is the first reliable method for studying the migration behaviour of neural crest cells in fish, for no other cell marker is known in this class of vertebrates. Two migratory pathways of neural crest cells have been recognized, which lead to the destination of these cells in the ectoderm, lateral line organ, spinal ganglia, aorta, cardinal vein, urinary ducts and gut wall.
Although in our experiments the neural crest cells were added to those normally present in the embryo, abnormalities have not been observed in development, and migration pathways of the neural crest cells seemed to be normal. The preparation of the neural crest by trypsinisation in fish is more complicated than in birds because of the massive structures; in a number of cases the neural crest was not totally detached from the adjacent tissues (e.g. myoblasts and neural tube cells). Pollution of the graft with myoblasts may explain the presence of labeled cells in the myotomes. Neural tube cells do not migrate, as can be concluded from the experiments with ventral neural tube cells and from the clustering of dorsal neural tube cells at the place of injection. Moreover, ventral neural tube cells do not fuse with the host neural tube. However, it cannot be concluded that the labeled cells observed in the host neural tube are neural crest cells, because the experiments are not conclusive as to the (migratory) capacities of dorsal neural tube cells.

With the applied transplantation method it is possible to study the migration and fate of the neural crest cells of fish; till now only suggestions were available from experiments in birds and amphibia and from scarce extirpation and explantation experiments in fish. Our results confirm the findings of Newth (1951, 1956) and Lopashov (1944) who found in the trunc region a contribution of neural crest cells to the spinal ganglia, as already generally known for amphibia and birds (c.f. Weston, 1970). The results obtained for pigment cells are in agreement

with theories on their origin in fish by Orton (1953), Newth (1956) and Shepard (1961) and with the generally accepted theories on their origin in the neural crest (c.f. Weston, 1970). Newth (1951, 1956), Lopashov (1944) and Damas (1951) suggested that the neural crest of the head region participates in the formation of the head ganglia, the arteries and the skeleton of the head. As our experiments were restricted to the trunc region their view could not be confirmed.

It has first been proven in chick embryos that the migration of the neural crest cells take place in two well-defined pathways (Weston, 1963): a dorso-lateral stream of cells towards and in some places below the ectoderm and a ventral stream towards the mesenchyme between the neural tube and the myotome and penetrating the somitic mesenchyme. The present study indicates a similar process in fish as observed in birds, but there is one difference i.e., the ventral stream does not penetrate the mesoderm but migrates within the space between the neural tube/notochord and the somite; as a result the spinal ganglia develop in the space between the somites and the neural tube, and not in the somites as they do in birds and amphibians (Weston, 1970). The explanation may be that contrary to the developmental rate of the other organs, in fish the somites have an explosive growth and differentiation in the early developmental stages and become functional within a few hours after their formation.

The present study clearly indicate a contribution of the neural crest to the spinal ganglia and to the region of aorta and postcardinal vein. It is known that the sympathetic ganglia appear near the aorta and that the nephritic tissue is formed around the postcardinal vein. Chromaffin elements are scattered in the nephritic mesenchyme, always near or just below the endothelium of the postcardinal vein. This tissue is analogous to the adrenal medulla and paraganglia of higher vertebrates (Grassé, 1958; Bolk et al., 1934). This indicates participation of neural crest cells in the formation of the sympathetic ganglia and chromaffin tissue. Confirmation requires information on the differentiation of these cells during later developmental stages.

A distinct number of cells with an oblong nucleus have been observed near the notochord and in the septa between the myotomes. These cells probably correspond to Schwann sheat cells; a relation of these cells to the neural crest has been established in birds and amphibia (c.f. Weston, 1970).

Regularly cells were found near the developing lateral line organ. Grafting experiments in amphibia indicated that neuromasts of the lateral line organ are derived from the auditory placode by backward migration of cells out of this

region (Harrison, 1903). Several authors assumed that neuromasts in fish develop from epithelial cells, possibly induced by the lateralis nerve (c.f. Grassé, 1958). In the present study, neural crest cells were found near the future lateral line organ before nerves can be detected; therefore, these crest cells might be involved in the differentiation of ectoderm cells into neuromasts.

The neural crest cells were found to penetrate the urinary duct epithelium only when the graft is placed in the vicinity of this future organ. In birds, neural crest cells are contributing to the nephritic mesenchym (Fontaine, 1974), but a contribution to the primary urinary duct or future urethra has never been reported. The affinity of the neural crest cells to the urinary duct epithelium may be due to the ectopic location of the neural crest.

Several neural crest cells are found in the wall of the gut, and their number increases strongly when neural crest was grafted near the future gut; in the investigated stages (up to 6 days), however, none of these cells penetrated into the future intestinal epithelium. This is in agreement with the results obtained in birds of Le Douarin & Teillet (1973) by transplanting neural tubes including neural crest from quail to chick, and of Le Douarin & Teillet (1974), Smith et al. (1977) and Teillet et al. (1978), who cultured quail neural primordia together with chick aneural hindgut fragments. The latter experiments are comparable to our ectopic neural crest grafts and indicate that neural crest cells grafted in this way maintain their migratory capacity and their ability to differentiate in a normal way. It may be concluded that neural crest cells of *B. conchonius*, just as in birds, do not have any affinity to the future intestinal epithelium (endoderm); such a contribution was clearly shown to be possible for the epithelium of the primary urinary duct (mesoderm). The neural crest cells in the gut wall probably contribute to enteric ganglia, as was described by Le Douarin and her group. In birds these structures originate in distinct levels of the neural axis (the region between somite 1-7, and caudally of 28 S; Le Douarin & Teillet, 1973). Our method does not yet permit to establish the exact origin.

The present study indicated a trunc-or rhombencephalic-neural crest origin as unlikely for enteroendocrine cells of *B. conchonius*, contrary to Pearse (1969, 1973) and Pearse & Polak (1971). This confirms the results obtained in birds by Andrew (1963, 1974, 1976) and Le Douarin & Teillet (1973), but the conclusion of an endodermal origin of the enteroendocrine cells, presented in the unitarian hypothesis of Cheng & Leblond (1974) and suggested by Rawdon et al. (1980), seems to be premature. A neural origin of the enteroendocrine cells cannot be

rejected because of the following arguments:

- the permanent or short time presence of the APUD characteristics and, consequently, the belonging to the APUD series (c.f. Pearse & Takor Takor, 1979; Rombout et al., 1978).
- the presence of several peptides common to gut endocrine cells and nervous system (Pearse, 1977, Pearse & Takor Takor, 1979).
- the presence of a neurone-specific enolase in cells of the APUD series, and in some GEP endocrine cells (Schmechel et al., 1978).
- the neuron-like processes in some gut endocrine cells (Larsson et al., 1979).
- the penetration of endocrine cells from the lamina propria into the gut epithelium of 4-5 months old human fetuses (Osaka & Kobayashi, 1976).

When the neural crest, and even the whole neurectoderm (Fontaine & Le Douarin, 1977) had been excluded as a possible origin of the gut endocrine cells in birds, Pearse (1977, 1979) modified his hypothesis on the origin of the GEP endocrine cells, and now considers them to be derived from neuroendocrine-programmed ectoblasts. This hypothesis is supported by recent views on the gastrulation in birds and mammals (Levak-Svajger, 1974; Pearse & Takor Takor, 1979), that formation of the definite endoderm (the secondary hypoblast) begins at the primitive streak stage. Possibly cells "retaining a neuroendocrine program" may accompany the endodermal cells during migration from the ectoblast through the primitive streak. The classical view on the gastrulation process in fish has recently also been disputed by Ballard (1973). He concluded from his experiments that future endoderm cells are located at the most ventral side of the blastodisc and in contact with the periblast, which eliminates the need of dorso-ventral migration of future endodermal cells. Therefore, the endodermal layer must be formed by a rearrangement of the deep-lying cells of the blastodisc. As the future endoderm and ectoderm are located close to each other in the blastodisc, "neuroendocrine-programmed ectoblasts" might be intermingled with future endoderm. However, direct experimental evidence is not yet available to support or reject a neural origin of the GEP endocrine cells, and the concept of GEP endocrine cells being members of the Diffuse Neuro Endocrine System (DNES; Pearse, 1977) or paraneurons (Fujita, 1976) should be considered with caution; it cannot be applied to all types of GEP endocrine cells. The question whether all enteroendocrine cells are of neural origin, can only be anwered by additional embryological experiments.

The phenomenon that neuroectodermal cells may loose their neural properties and (de)differentiate into cells of another germ lager, the periblast,

might be attributed to a strong inducing influence of the yolk or periblast. To our knowledge, this phenomenon has never been mentioned.

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Chapter VI

Proliferation and differentiation of intestinal epithelial cells during development of *Barbus conchonius* (Teleostei, Cyprinidae).

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SUMMARY

A discussion is presented on proliferation, cell division and differentiation of intestinal epithelial cells during development. After 3 days of development, all cells of the presumptive gut proliferate and have a cell cycle of about 22 hours. The intestinal epithelium continues to differentiate and this results in different cell types, in a decreasing percentage of proliferative cells, and concomitantly in an increasing duration of the cell cycle.
From the 5th day when intestinal folds start to develop, the proliferative cells move towards the basal location of the future folds.

Ultrastructural examination of DNA-synthesizing cells and mitotic cells of 6 days old larvae shows that functional enterocytes are proliferative in all the three segments of the digestive tract; proliferating goblet cells, however, were not observed. The undifferentiated "dark" cells (many free ribosomes and only a few organelles) will be considered as stem cell for the goblet cell. Proliferating enteroendocrine cells have not been found, probably owing to their scarcity and long turn-over time. As the granule-containing cells of endocrine corpuscle of Stannius appear to be proliferative, the same may be suggested for the enteroendocrine cells.

A common feature of all dividing cells is the presence of isolated spherical to cylindrical cisternae, which are supposed to have lost the contact with the cell membrane during the prophase; they probably regain this contact by fusion with the cell membrane at the end of mitosis.

The lymphoid cells may proliferate within the intestinal epithelium.

INTRODUCTION

The renewal of the intestinal epithelium of cyprinids occurs in the basal parts of the mucosal folds (Hyodo-Taguchi, 1970; Gas and Noaillac-Depeyre, 1974; Stroband and Debets, 1978). In contradistinction to mammals (Cheng and Leblond, 1974a; Van Dongen et al., 1976) the juvenile grasscarp does not possess non-functional stem cells in the proliferative part of the gut epithelium. Therefore Stroband and Debets (1978) suggested that functional absorptive cells in fish are proliferative. Experimental data are not yet available to support this hypothesis.

In mammals, the absorptive cells, goblet cells and enteroendocrine cells were assumed to develop from the same cell, the crypt-base columnar cell (Cheng and Leblond, 1974b). If the endocrine cells of the digestive tract have a neural origin, as suggested by Pearse (1973, 1977, 1979), there must be more than one cell, but attempts to prove a neural origin have failed (for birds: Fontaine and Le Douarin, 1977; Andrew, 1974). In order to ascertain whether the intestinal epithelium of *B. conchonius* contains one or more functional or non-functional stem cells, proliferation and differentiation have been studied with histological, autoradiographical and electron-microscopical techniques. Embryos and larvae were used, as proliferating cells are commonly present in these developmental stages. The proliferation of the endocrine cells of the pancreas, corpuscle of Stannius, and the exocrine pancreas cells have been included in the discussion. The urinary duct cells will be also involved in our considerations because of the ultrastructural similarity with some intestinal absorptive cells.

MATERIALS AND METHODS

A. Autoradiography

Embryos (2, 3, 4, 5 days old) and larvae (6, 7 and 19 days old) were incubated at 25°C in 200 µ Ci/ml methyl-^{3}H-thymidin (spec. act.: 20 Ci/mmol, Radiochemical Centre, Amersham, England) for 4 h. For a long incubation time (18 h) embryos and larvae were injected in the anterior yolk with 20-30 nCi methyl-^{3}H-thymidin (spec. act., 24 Ci/mmol) by means of a fine glass-capillary. Larvae were fed with *Artemia* nauplii just before being exposed to ^{3}H-thymidin. Specimens were fixed in Bouin's fixative (24 h) and vacuum embedded in Paraplast Plus (Sherwood). Cross and sagittal sections of 4 µm were prepared for autoradiography with a

Kodak NTB-2 emulsion. After an exposure time of 2 weeks the slides were developed with Kodak D-16 developer and stained with haemalum. These autoradiographs were used for studying proliferation during development; for each age 3 specimen are used.

B. Electron microscopy

Embryos of 2, 3 and 5 days old and larvae of 6 days old (after first feeding) were fixed for 15 min at $0^{o}C$ in a mixture of 1% OsO_4, 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2), and postfixed 1 h in the same mixture to which 1% potassium dichromate was added. After embedding in Epon 812, ultra-thin sections were made on a Reichert OMU-IV microtome. Sections were collected on copper grids and stained with saturated uranyl acetate followed by lead citrate.

C. Comparison of light microscopial autoradiographs and electron micrographs

Larvae if 6 days old were incubated shortly after their first feeding in ^{3}H - thymidin, then prepared for electron microscopy. Immediately before and behind the series of ultra-thin sections, sections of 1 micron were made. These adjacent semi-thin sections were prepared for autoradiography and developed after 6 weeks. Autoradiographs were stained with p-phenylenediamine or toluidin blue and compared with adjacent ultra-thin sections. Ultrastructurally no radiation damage was observed.

RESULTS

A. Morphology

After the first food-uptake on the 6th day, the digestive tract becomes functionally active and the following zones can be distinguished.

Esophagus

The short esophagus consists of two distinct parts. The epithelium of the first part contains many secretory cells, resembling the goblet cells of the intestinal epithelium (fig. 1). Small slender undifferentiated cells (characterized by many free ribosomes and only a few scarcely developed organelles) are found between the secretory cells. In the second part the epithelial cells contain many small granules at the apex (fig. 2b). Light-microscopical staining shows that the apical area is PAS-positive and metachromatic (fig. 2a) like the secretory cells of the first part; this indicates mucous-like contents of these granules. The lateral cell membranes show interdigitations (fig. 3b).

The transition of esophagal to intestinal epithelium is rather abrupt. The cells of the short transitional zone have characteristics of both parts (fig. 3).

Intestine

On the basis of the morphology of the absorptive cells 3 distinct segments can be distinguished in the intestine of *B. conchonius* larvae.

1. *First or proximal segment* (70-75% of the total length of the intestine) The absorptive cells of this segment contain many chylomicrons in their endoplasmic reticulum and Golgi apparatus and many lipid-containing vacuoles are frequently present (fig. 4). With the light microscope the lipid vacuoles can be seen as clear vesicles or (after OsO_4 fixation followed by p-phenylene-diamine staining) as dark droplets (fig. 4a). The ultrastructural characteristics have not been observed in starved larvae of the same age. This segment contains long lamellar structures, (fig. 4d), in contact with the lateral and basal plasma membrane as described for juvenile grasscarp by Stroband and Debets (1978). These structures, which are characterized by an apperantly thicker membrane, are probably cisternae in three-dimensional view. The transition of the first segment to the second segment is abrupt and no cells have been found with characteristics common to both segments.
2. *Second or middle segment* (20-25% of the total length of the intestine) Epithelial cells of the second segment are characterized by the presence

Fig. 1 Sagittal section of the esophagus of a 19 days old larva. Numerous goblet-like cells in the first part but not in the second part of the esophagus. x 300
PH = pharynx; E_1, E_2 = first and second part of the esophagus; B = intestinal bulb.

Fig. 2a,b Esophagus of a 6 days old larva;
a. autoradiograph of a semi-thin section of the second part of the esophagus, stained with toluidin blue, which reacts metachromatic to the apical part of the epithelial cells (small arrows), x 1,25(
b. Electron micrograph of an adjacent ultra-thin section showing apic side of a DNA-synthesizing cell (thick arrow in fig. 2a). A dark staining cell (asterik) near the labeled cell is indicated in both figures as a reference. Note the many small granules in th apex of this cell. Section made near the transition to the intest where microvilli are common. x 7,500 N: nucleus

Fig 3a,b Transition from esophagus to intestinal bulb of a 6 days old larva;
a. Electron micrograph of an epithelial cell with characteristics of an esophageal cell (interdigitations, granules) and of a flat absorptive cell in the first segment (lipid-containing vacuoles, chylomicrons) x 11,000
b. Magnification of the outlined part x 22,000 thin arrows: granules; thick arrows: interdigitations L = lipid-containing vacuole; c = vesicles containing chylomicron


B
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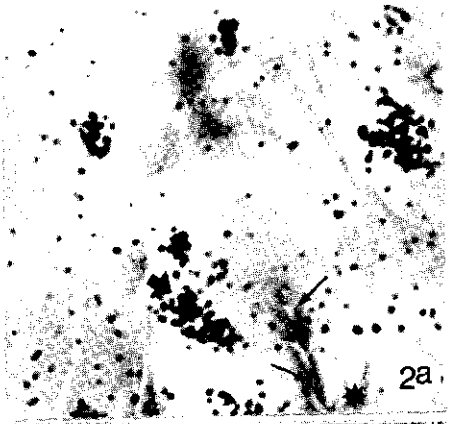
E
1
PH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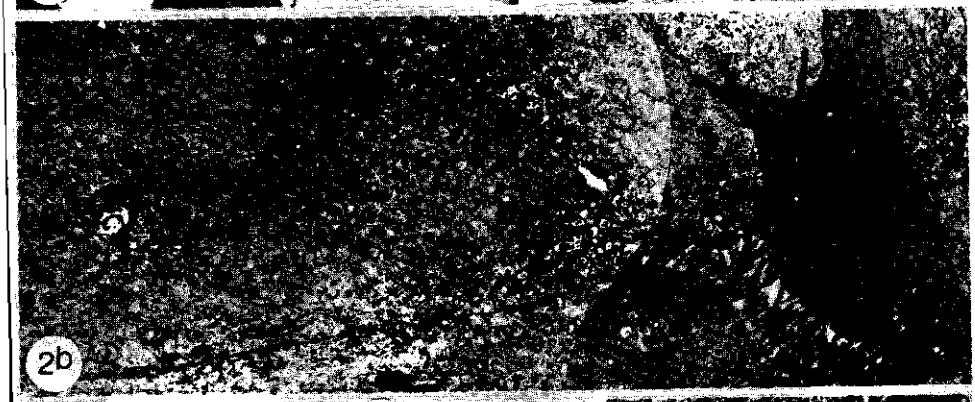
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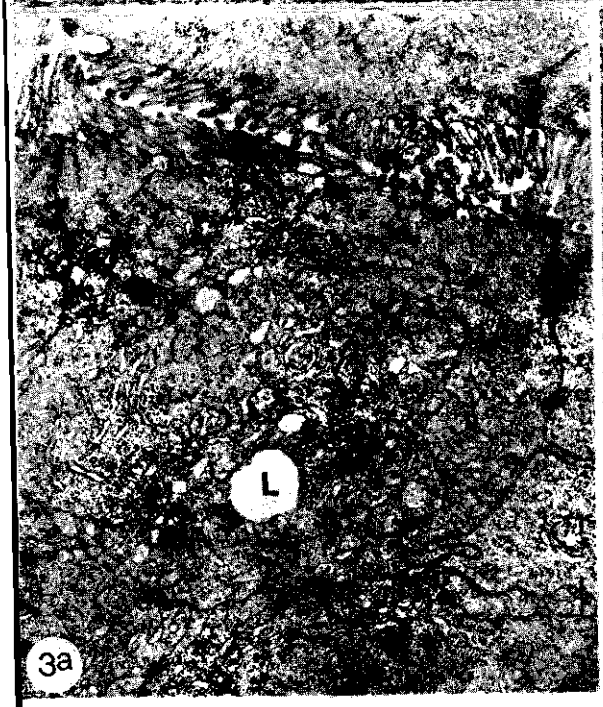
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of supranuclear vacuoles and an abundant pinocytotic activity (fig. 5). Just before first food-uptake the pinocytotic activity is slight and small vacuoles are scarce. The basal part of these cells contains a network of rough endoplasmic reticulum (fig. 5b).

Large lysosomes are often present, and they may fuse with the supranuclear vacuoles. The cisternae as described for the first segment are less common and poriferated in this segment.

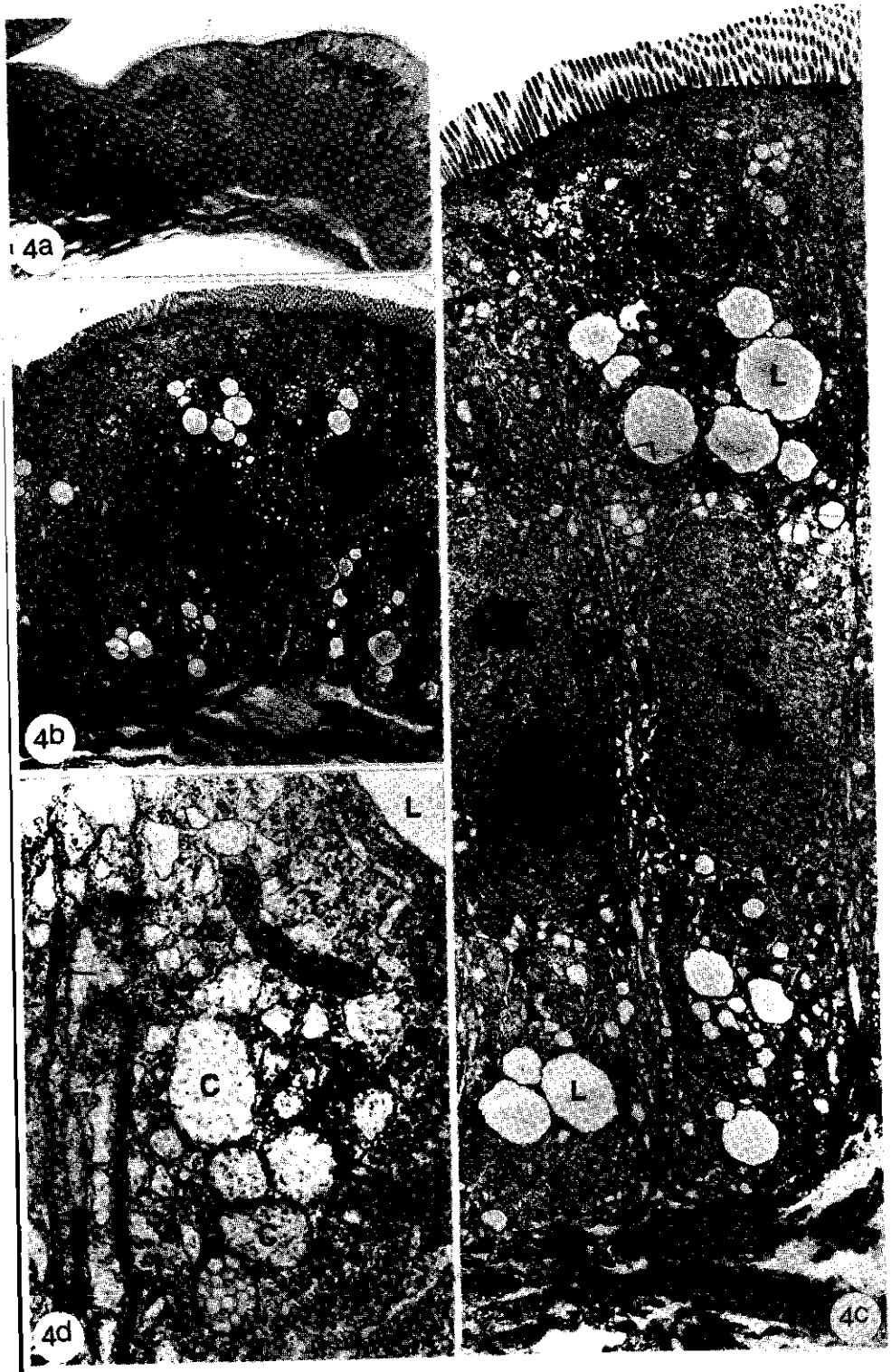
3. *Third or distal segment* (5-10% of the total length of the intestine)
Epithelial cells in the rostral part of this segment carry short microvilli covered with a fuzzy coating (fig. 7). The lateral and basal plasma membra show more lamellar structures than the cells in the first segment. Stacks these structures, as described for juvenile grasscarp by Stroband and Debe (1978), have not yet fully developed at this stage in the enterocytes of *B. conchonius*.

The relatively short caudal part of the third segment, near the anus, is characterized by the absence of microvilli and the presence of vesicles in the apical part of the cells (fig. 8). These vesicles resemble the granules of the cells in the second part of the esophagus. Interdigitations of the lateral cell membranes, as described for the esophagus, are present also in this part of the gut.

Next to absorptive cells, the intestinal epithelium contains goblet cells, enteroendocrine cells and "dark" cells. Most of the goblet cells are found in the middle and third segments. Of the enteroendocrine cells only the types I and II (Rombout, 1977) are present at this stage (6 days old). In the middle and third segments, mainly type I is present, but the first segment contains both types.

"Dark" cells are undifferentiated cells, as indicated by the large number of free ribosomes, the scarce mitochondria, and the absence of a

Fig. 4a-d First segment of the gut of a 6 days old larva;
a. Autoradiograph of a semi-thin section stained with p - phenylene-diamine. Note the black lipid containing vacuoles and two DNA-synthesizing cells (arrows) x 1,000
b. Electron micrograph of the same cells in an adjacent ultra-thin section (arrows) x 2,500
c. Magnification of fig. b. Note the lipid containing vacuoles. x 7,0
d. Detail of the outlined part of fig. c. x 33,000
Many chylomicrons in the endoplasmic reticulum. Note the relatively thick membrane of the long lamellar structures (arrows)
c ; chylomicrons; L = lipid-containing vacuoles; N = nucleus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well-developed endoplasmic reticulum and Golgi-apparatus (fig. 10). They are always in contact with the basement membrane, but usually without an extension towards the lumen of the gut.

At the age of 5 days, the different heights of the epithelial cells may indicate future locations of the intestinal folds. Mucosal folds are developed from the 7th day.

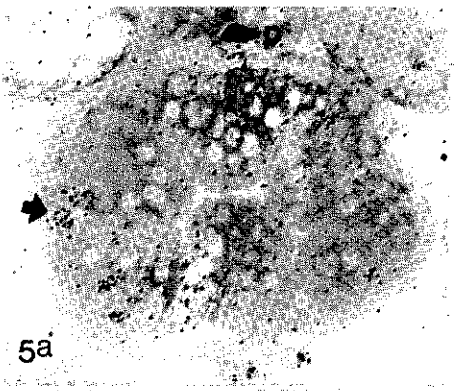
B. Proliferation

Three methods have been used for studying the proliferation of intestinal epithelial cells during embryonic and larval stages:

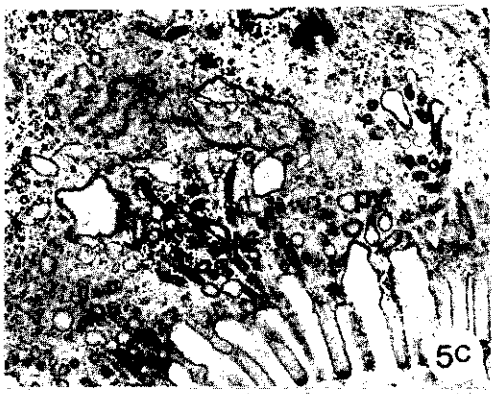
1. Expressing the numbers of mitotic figures in the intestinal epithelium in percentages (table 1). Two locations of mitotic cells can be distinguished in the epithelium: one near the basement membrane (fig. 17), the other near the apices of the absorptive cells (fig. 12). Lymphoid cells are present in the intestinal epithelium from the 4th day, but they are scarce in early developmental stages and have not been counted separately. Only at the age of 19 days, when the number of lymphoid cells is increased considerably, apical and basal mitotic cells were counted separately.
2. Calculating the average percentage of DNA synthesizing cells in the intestinal segments after incubation for 4 or 18 h in tritiated thymidin (figure I; table 1).
   In order to establish the effect of cell division on the percentage of DNA-synthesizing cells after 4 h incubation, the percentage of labeled mitotic stages is computed at different ages. At each of these ages about 60% of pro- and metaphases and 30% of ana- and telophases are labeled.

Fig. 5a-c Middle segment of the gut of a 6 days old larva;
- a. Autoradiograph of a semi-thin section stained with p-phenylene-diamine. x 500
- b. Electron micrograph of an adjacent ultra-thin section of a DNA-synthesizing cell as indicated in fig. a (arrow). Note the abundant basally located rough endoplasmic reticulum and the supranuclear vacuole. x 8,000
- c. Magnification of the apical part of the cell as outlined in fig. b. Note the pinocytotic activity. x 18,000

Fig. 6 Electron micrograph of a non-ciliated cell in the proximal part of the urinary duct of a 6 days old larva, showing pinocytotic activity and supranuclear vacuoles. x 7,000
bm = basement membrane; N = nucleus; p = pigment cell; pv = pinocytotic vesicles, sv = supranuclear vesicle.



5a



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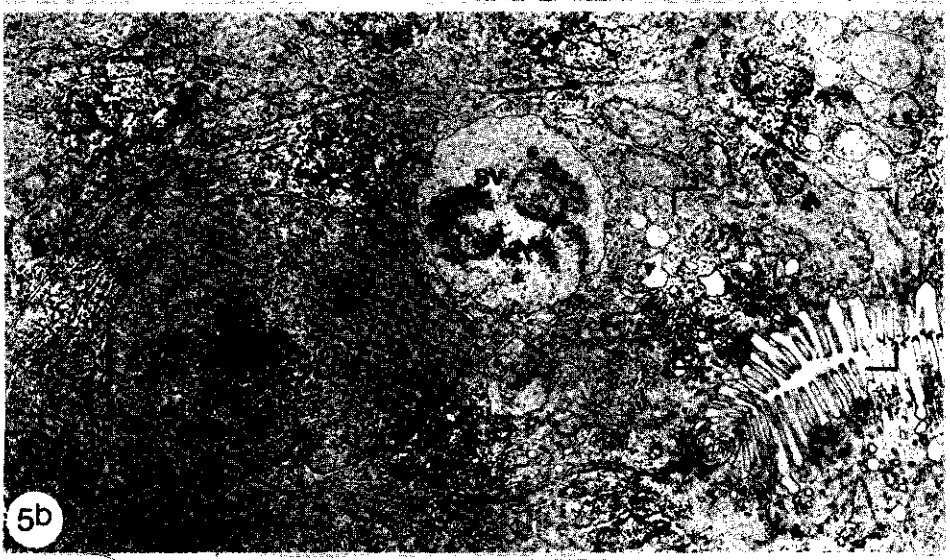

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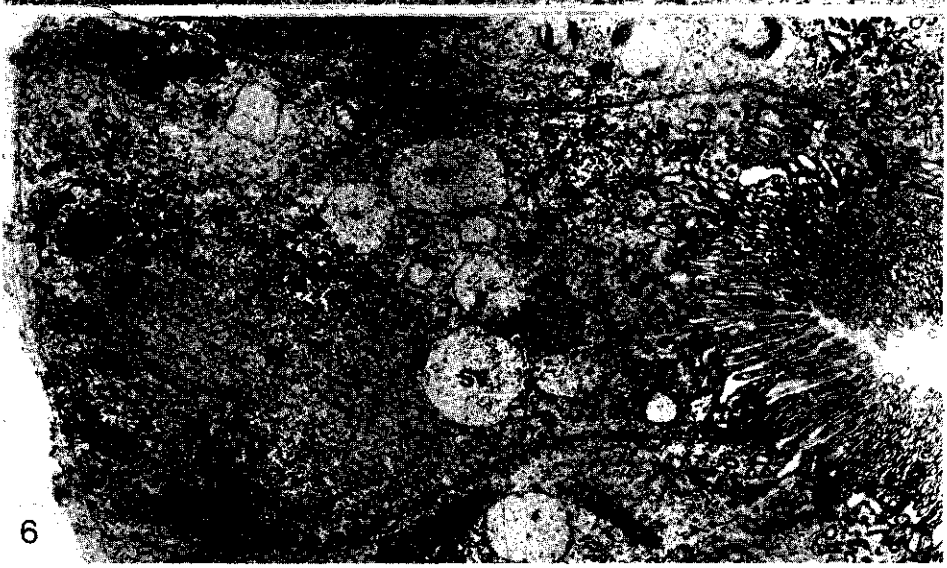

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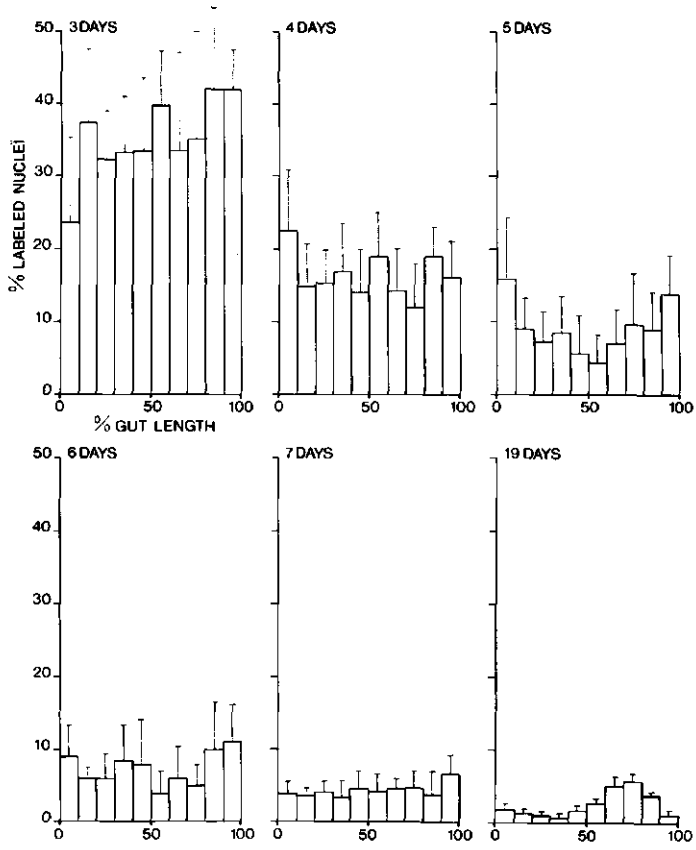


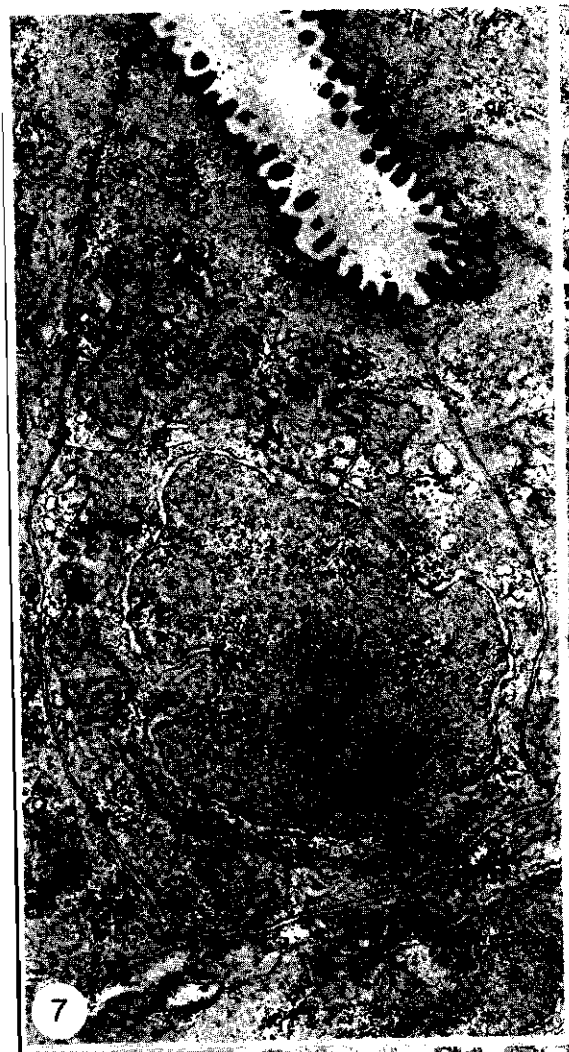
Figure I: Percentages of labeled nuclei in zones of the intestine at different ages after 4 h incubation in ^{3}H-thymidin (bar:SD). Each bar represents the average results of 3 specimens; 6 sections per specimen counted for each part of the intestine.

Fig. 7 Electron micrograph of the rostral part of the third segment of the gut of a 6 days old larva, showing short microvilli with a fuzzy coating and developing stacks of lamellar structures (arrows). x 11,000

Fig. 8 Electron micrograph of the apical part of an epithelial cell in the caudal part of the third segment of a 6 days old larva, showing apically located granules (small arrows) and interdigitations (thick arrow). x 13,000

Fig. 9 Electron micrograph of the basal part of an urinary duct cell (distal part) of a 6 days old larva, showing membranous stacks ("basal labyrinth") between mitochondria. x 21,000

Fig. 10a,b a. Autoradiograph of a semi-thin section with a labeled "dark" cell (arrow). x 1,000
b. Electron micrograph of the adjacent section of a., with a DNA-synthesizing "dark" cell in the first segment of the gut of a 6 days old larva. x 12,000
bm = basement membrane; m = mitochondrium; N = nucleus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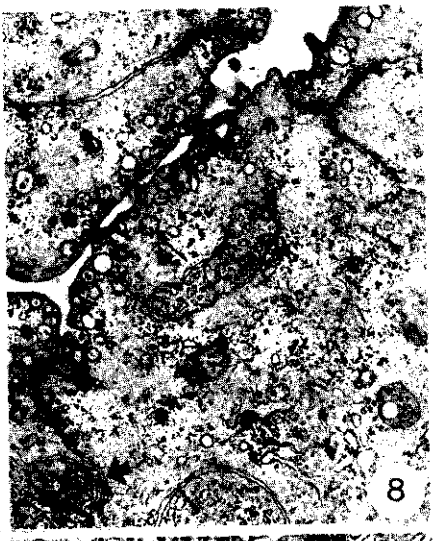
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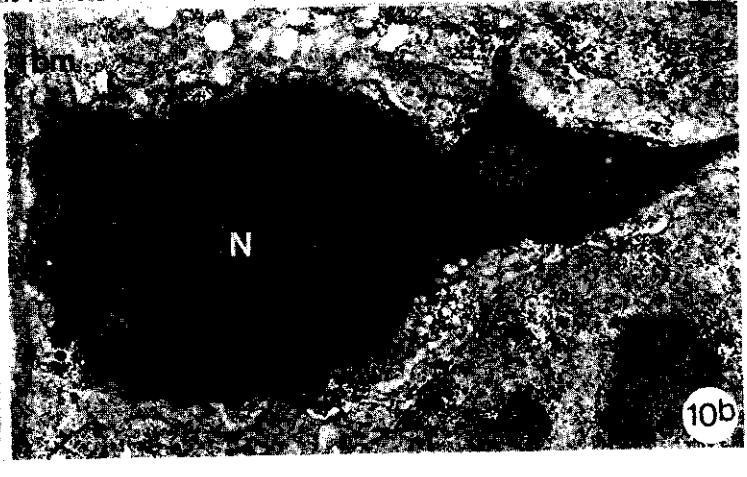
9


10a


bm
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Therefore, the period between S phase and the end of the M phase will be approximately 4 h at each age. Only a few tritiated cells appear to divide during the 4 h incubation time; consequently the percentage of DNA-synthesizing cells is only marginally affected by the division of labeled cells.

3. Measuring the increase in width of the intestine at different ages by determining the average number of cells per intestinal cross section (fig. II). As the length of the digestive tract hardly increases between

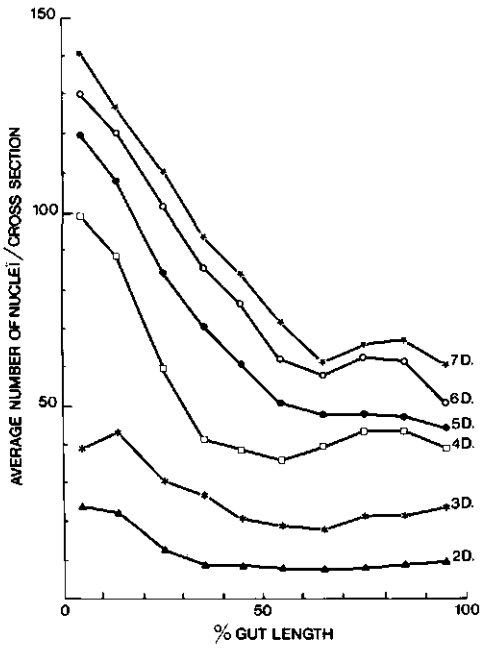


Figure II:
Increase in the number of epitheli cells in zones of the intestine at different ages. Each point represents the mean value of 3 specimen 6 sections per specimen counted fo each point.

Fig. 11 Electron micrograph of an early prophase of an absorptive cell in the first segment of the gut of a 6 days old larva. Cell without contact with basement membrane and retracted to the apical side of epithelium. Note the lipid-containing vacuoles and the disappearing nuclear envelope. x 7,500

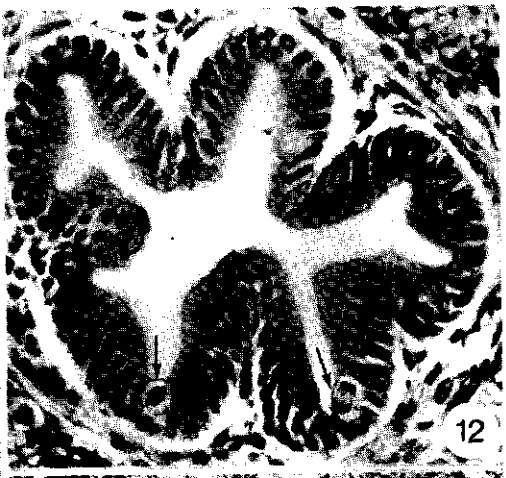
Fig. 12 Cross section of the first segment of the gut (near the esophagus) of a 5 days old embryo. Two apically located mitoses in the epitheliu (arrows). x 700

Fig. 13 Electron micrograph of a dividing cell (prophase) in the first segmen of the gut of a 6 days old larva. Only a few small vesicular structures represent the remnant of the nuclear envelope (arrows). Note th lipid-containing vacuoles and chylomicrons. x 40,000

Fig. 14 Electron micrograph of a late prophase of an absorptive cell in the first segment of a 6 days old larva. The nuclear envelope has disappeared. Note the chylomicrons and rounded cisternae (arrows). x 18,5

bm: basement membrane; c: chylomicrons; ch: chromatin; L: lipid-conta ing vacuoles; m: mitochondrium


L
L
11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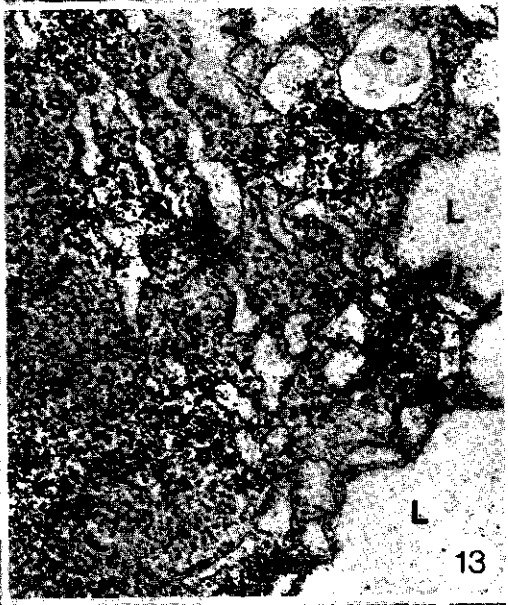
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L
L
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14

2 and 7 days, the average increase in the number of epithelial cells in this period can be calculated for the whole gut (table 1).

Table 1: Proliferation of intestinal epithelial cells at different ages as measured by the three different methods.

	2d	3d	4d	5d	6d	7d	19d
1. % mitosis (SD)	1,9 (0,4)	2,2 (1,2)	1,35 (0,75)	0,95 (0,25)	0,1 (0,01)		*A:*0,09 (0,06) *B:*0,06 (0,04)
2. % DNA-synthesizing cells[a] after 4 h incubation (SD)		35,5 (11,3)	16 (6,7)	8,9 (6)	7 (4,7)	4,2 (2,4)	*1:* 2,3 (1,7) *2:* 5,0 (1,3) *3:* 1,3 (0,6)
% DNA-synthesizing cells[b] after 18 h incubation (SD)	100	93,6 (5,7)	87,3 (2,3)	52 (6,7)	19,8 (1,8)		
3. % increase in number of cells in 24 h[a]		113	104	30	18	10	13/day[c]

a) These percentages are deduced from figure I and II respectively.

b) Incubation time: 1d 6h - 2d; 2d 6h - 3d; and so on.

c) Corrected to a 15% increase in gut length, the increase in number of cells is 154% between 7 and 19 days.

1, 2, 3: first, second and third segment respectively. *A:* Apically located mitosis; *B:* Basally located mitos

All three methods indicate that the rate of proliferation decreases strongly between the 3rd and 7th day. From the 7th day, the rate of proliferation is stabilized in the second segment, as the proportional increase of epithelial cells remains the same. Only the number of DNA-synthesizing cells in the first and third segments continues to decrease.

Until the 6th day tritiated cells and mitotic cells are randomly distributed in the epithelium. After the 6th day these cells are increasingly located in the basal parts of the developing folds.

Fig. 15a-d Electron micrograph of a late teleophase in the first segment of the gut of a 6 days old larva.

a. Note the lipid-containing vacuoles (L), the chylomicrons (c), the rounded cisternae (arrows), and the reappearing nuclear envelope in one of these cells. x 11,000

b. Magnification of the outlined apical part, with the midbody (M) and many small vesicles (arrows). x 33,000

c. Magnification of the outlined central part of this cell. Rounded cisternae and re-shaped cisternae (arrows) are present. x 22,000

d. Magnification of the outlined basal part of this cell, showing a fused cisternae (arrow). x 22,000


L
L
G
L
15a
M
15b
15c
L
15d

C. Differentiation

In order to study the degree of differentiation of intestinal absorptive cells, amount was made of the number of free ribosomes per unit area. Furthermore, attention was paid to the presence of structures indicating functionality e.g. chylomicrons, lipid-containing vacuoles, pinocytotic vesicles, supranuclea vacuoles, and lamellar structures. The number of ribosomes as counted in electr micrographs is decreasing from the 2nd day to the 6th day (fig. III). At that age the number of ribosomes in the cells is about half the original number. "Dark" cells, however, contain about the same number of ribosomes as the undiff entiated entodermal cells at the age of 2 days. The cell organelles are scarcel developed in the "dark" cells; they may represent stem cells, and are preferentially located near the basement membrane and only a few have an extension towards the lumen; in that case they have characteristics of immature goblet cell

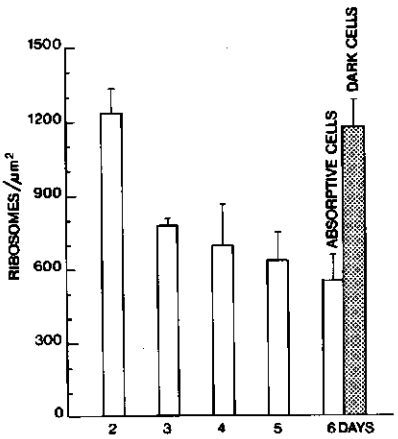
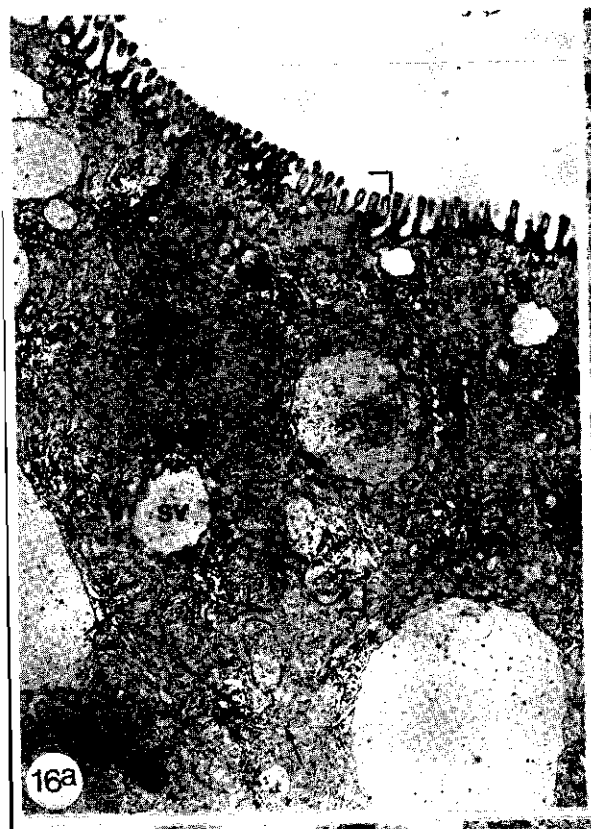


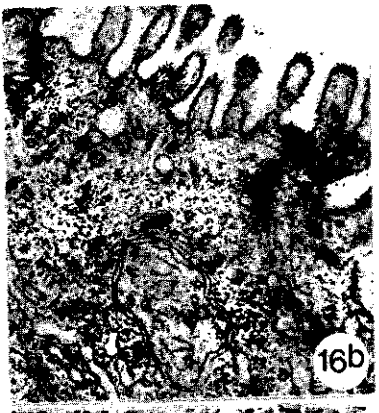
Figure III:
Relative number of free ribosomes in absorptive intestinal cells. Ribosomes counted in electron micrographs, in areas without organelles. Each bar represents the arithmetic mean of at least 3 cells (± SD)

Fig. 16a-c a. Electron micrograph of a late telophase in the second segment of the gut of a 6 days old larva: the outline indicated with arrow During mitosis the "supranuclear vacuoles" (sv) can also be locate below the nucleus (N). x 7,000
b. Magnification of the outlined apical part. Note the low pinocytoti activity. x 22,000
c. Magnification of the outlined middle part with poriferated rounded cisternae (arrows). x 22,000

Fig. 17 Sagittal section of the gut of a 7 days old larva, with basally locat mitosis (arrow). x 1,400

Fig. 18 Electron micrograph of a lymphoid cell in the intestinal epithelium of the third segment of a 6 days old larva, labeled in the autoradiograph of an adjacent section. Note the round shape and the small rim of cytoplasm. x 15,000 bm = basement membrane N = nucleus


SM
16a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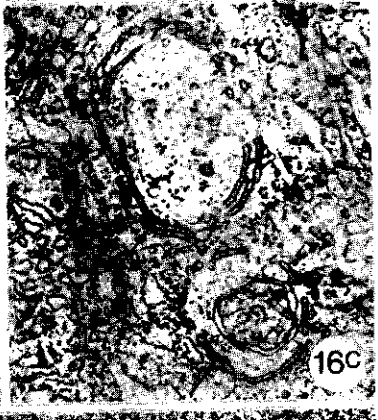
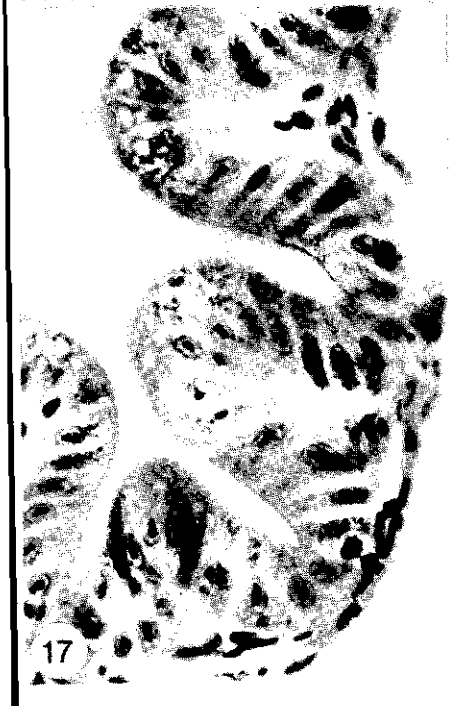

16c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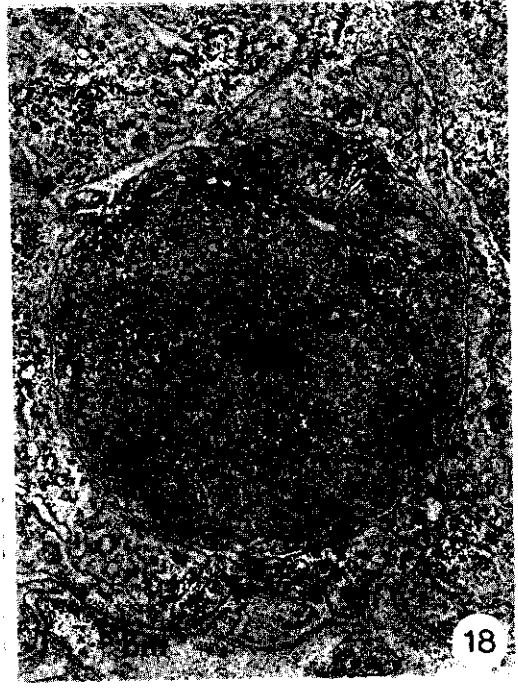
18

D. Ultrastructure of DNA-synthesizing cells

Comparison of light microscopic autoradiographs and electron micrographs of adjacent sections show the ultrastructure of DNA-synthesizing cells in all parts of the digestive tract . As shown in table 2 and illustrated in figs. 4 and 5, most of the tritiated intestinal epithelial cells are functionally activ with the exception of the labeled cells in the first part of the esophagus. The epithelium of the third segment is only poorly developed at this stage (fig. 7) It contains tritiated functionally active absorptive cells, and also tritiated "dark" cells (fig. 10) and lymphoid cells (fig. 18). Labeled goblet cells and enteroendocrine cells have not been observed. Tritiated pancreatic endocrine cells are found in the autoradiographs, but only after a relatively long incubation in ^{3}H-thymidin (18 h).

E. Ultrastructure of dividing cells

Mitotic cells have the same general features as DNA-synthesizing cells, viz. they are to some degree functionally active (figs 11, 13, 14, 15), apart from a decreasing pinocytotic activity in the enterocytes of the middle segment (fig. 16). Dividing absorptive cells can be recognized because they become detached from the basement membrane and rounded at the apical side of the epithelium (figs. 11, 12). During this process the long lamellar structures (cisternae) loose the contact with the lateral plasma membrane and become round ed in the cytoplasm (fig. 14, 15, 16); adjacent sections show these structures, which are often associated with mitochondria, to be spherical to cylindrical. Simultaneously the nuclear envelope becomes vesicular and disappears (fig. 13, 14). During the telophase the nuclear envelope appears again and the rounded cisternae regain their original shape and re-establish the contact with the lateral plasma membrane (fig. 15c, d). The cleavage furrow starts from the basal side of the dividing cell. Consequently, in the late telophase the mid-body (remnant of the spindle) is located in the apical part of the cell near the future junctional complex (fig. 15 b).

F. Proliferating cells in other organs

Two distinct parts can be distinguished in the urinary duct: a proximal part, of which the unciliated cells ultrastructurally resemble the intestinal epithelial cells of the second segment (fig. 6), and a distal part, where the cells have the same characteristics as the intestinal epithelial cells of the first part of the third segment. In contrast with these cells of the third

segment the cells of the distal part of the urinary duct already show a "basal labyrinth" at the mentioned stages (fig. 9). As shown in table 2 functional cells of the urinary duct are able to proliferate and so the same is suggested for fully differentiated intestinal epithelial cells of the third segment. In addition granule-containing cells i.e. pancreatic exocrine cells and endocrine cells of the corpuscles of Stannius are able to proliferate (table 2).

Table 2: Number and ultrastructural characteristics of DNA-synthesizing cells in 6 days old larvae.

	functional cells		undifferentiated cells[a]	lymphoid cells[b]
GUT: oesophagus, part 1	goblet-like cells	-	19	
part 2	mucous cells	33		
segment I	only chylomicrons	32	11 ("dark cells")	6
	chylomicrons and fat vacuoles	49		
segment II	pinocytosis and small supranuclear vacuoles	17	8 ("dark cells)	3
	pinocytosis and large supranuclear vacuoles	21		
segment III, part 1	stacks (basal labyrinth)	14	1 ("dark cell")	3
part 2	granules	9		1
URINARY DUCT, part 1	pinocytosis and vacuoles	1		
part 2	basal labyrinth	2		
PANCREAS (exocrine)	RER, without zymogen granules	3		
	with zymogen granules	16		
CORPUSCLE OF STANNIUS	with endocrine granules	3		

a) many free ribosomes, few organelles and contact with the basement membrane.

b) no contact with the basement membrane

The arabics represent the number of labeled cells examined in 3 specimens.

Discussion

The ultrastructure of the intestinal absorptive cells of larval *B. conchonius* resembles that of other larval, juvenile and adult cyprinids (Yamamota, 1966;

Iwai, 1969; Gauthier and Landis, 1972; Noaillac-Depeyre and Gas, 1976; Stroband and Debets, 1978). The first segment shows the characteristics of lipid absorption. The second segment has a vigorous pinocytotic activity; Stroband et al. (1979) proved an uptake of macromolecules in this segment of cyprinid larvae. This might explain the resemblance of these cells to epithelial cells of the proximal urinary duct, and permits resorption of large molecules from the pre-urine.

The first part of the third segment might be involved in water and ion transport (Noaillac-Depeyre and Gas, 1976). This assumption is based on the presence of a "basal labyrinth" in enterocytes of this segment, a common featu in urinary duct cells.

At the third day of development, when lumen formation and cell differentiation starts (Rombout et al., 1978) all cells seem to be able to proliferate as nearly all of them appear to be tritiated after 18 h incubation in ^{3}H-thymidin. The rapid increase in the number of cells (about 110% in 24 h) at the third day indicates a cell cycle of about 22 h for these relatively undifferentiated cel The strong decrease of proliferation between 3 and 7 days may be attributed to (1) a strongly increasing duration of the cell cycle and (2) a decrease in the percentage of proliferative cells. The latter feature is related to the transition from the randomly distribution of many proliferative cells in embryos to normal accumulation of proliferative cells in the basal part of the folds in the larval stages. A comparable change was observed in the intestinal villi of rats around birth (Hermos et al., 1971) and in the gut of *Xenopus laevis* aroun metamorphosis (Marshall and Dixon, 1978).

Proliferative enterocytes are functionally active in larval *B. conchonius*. The same was described for the intestinal epithelium of *Xenopus laevis* tadpole (Marshall and Dixon, 1978); after metamorphosis, proliferation is restricted to relatively undifferentiated cells, localized in the basal part of the folds (McAvoy and Dixon, 1977). In fish the proliferative cells become also concentrated in the basal part of the folds, but, as already suggested by Stroband and Debets (1978) for grasscarp juveniles, these cells remain functionally active in later stages. So, in contrast with mammals, enterocytes of fish loose their proliferative activity in a relatively late stage of differentiation. In mammals (Lipkin, 1973), apparently more than in fish, intracellular changes seem to be very important in inhibiting proliferation during differentiation of gastrointestinal cells.

In larval *B. conchonius* most proliferative cells of the first segment

contain chylomicrons and lipid-containing vacuoles, whereas in older fish these vacuoles are present only in epithelial cells at the tip of the mucosal folds; Stroband and Debets (1978) suggested fat accumulation caused by ageing of these cells. In proliferative cells of larvae these vacuoles might be attributed to an incomplete fat processing mechanism in the not fully differentiated absorptive cell.

In the first 7 days no significances are found in the rate of proliferation in the different parts of the gut. From that time, the percentage of proliferative cells is stabilized in the middle segment, but the decrease continues in the first and third segments. This confirms the data of Stroband and Debets (1978) who also found 2-3 times more proliferative cells in the middle segment of juvenile grasscarp than in other segments.

Contrary to the results of Marshall and Dixon (1978) proliferating mature goblet cells have not been found in the gut of larval *B. conchonius*, not even in the parts of the digestive tract with large numbers of goblet cells. As several transitional stages are found between "dark" cells (strongly resembling the undifferentiated endodermal cell) and the young goblet cells, a stem cell function is suggested for these cells. Whether these stem cells are also involved in the renewal of other epithelial cell types is not known. As their presence is low compared of functional absorptive cells, they might play a secondary role in the renewal of absorptive cells.

Proliferating enteroendocrine cells have not been observed. This may be attributed to the scarcity and the long turn-over time, just as for the related pancreatic endocrine cells. Contrary to Cheng and Leblond (1974b), who suggested the same turn-over time (2-4 days) for all types of epithelial cells in the small intestine of the mouse, several authors reported a slower rate of renewal of the enteroendocrine cells (Fujita and Kobayashi, 1977; Tsubouchi and Leblond, 1979). These authors supposed a stronger adhesion of these cells to the basement membrane, and this may cause a slower moving upwards into the folds. The same was suggested by Rombout et al., (1978) for enteroendocrine cells of *B. conchonius*. The proliferation of granule-containing endocrine cells of the corpuscle of Stannius in this species corresponds to the results obtained in several mature-type endocrine cells of mammals, such as thyroid parafollicular cells, pancreatic islet cells and even enteroendocrine cells (Fujita and Kobayashi, 1977). The same may be suggested for the enteroendocrine cells of *B. conchonius*. In view of the supposedly neurectodermal origin of the enteroendocrine cells (Pearse

and Takor Takor, 1979) another possibility might be suggested, namely differentiation from a more or less differentiated extra-epithelial stem cell resting below the basement membrane (Dias-Amado, 1925; Osaka and Kobayashi, 1976; Rombout et al., 1978). If enteroendocrine cells are not of neurectodermal origin as concluded by Fontaine and LeDouarin (1977) and Andrew (1974) from experiments in birds, the presence of the APUD (Amine Precursor Uptake and Decarboxylation) characteristics and of many peptides common to the gut endocrine cells and the nervous system (Pearse, 1977; Pearse and Takor Takor, 1979) remain to be explained. Therefore, Pearse (1977) adjusted his hypothesis on the origin of the gastro-entero-pancreatic (GEP) endocrine cells to a possible origin in neuroendocrine programmed epiblasts. Enteroendocrine cells of *B. conchonius* possess the APUD characteristic only a few days during development (Rombout et al., 1978). Possibly this short time APUD facility is a necessary step in the process of differentiation from stem cell to endocrine receptor cell. Consequently the renewal of this cell type in older stages has to be brought about by mature-type enteroendocrine cells. Ultrastructural examination shows that epithelial cells of the first segment retain their functional characteristics during mitosis. In the middle segment of the gut, however, pinocytotic activity is nearly nil during mitosis, and this indicates a brief inactivation during this phase.
A common feature of all dividing absorptive cells is the presence of isolated spherical to cylindrical cisternae without any contact with the cell membrane. These structures, which have not been previously described in mitotic cells, develop from the long lamellar structures (cisternae) at the start of mitosis; they can be recognized by the thick membranes and the regular distance between them, as described by Noaillac-Depeyre and Gas (1973) and Stroband and Debets (1978). The loss of contact between these structures and the cell membrane might be facilitate retraction of the mitotic cell as their membranes seem to be more "solid" than the cell membrane in electron micrographs. At the end of mitosis these structures fuse again with the cell membrane and form similar lamellar structures. Possibly they participate in the enlargement of the cell.

Lymphoid cells, present in the intestinal epithelium from the 4th day, appear to be labeled after a short incubation period (4 h), it cannot be excluded that these cells invade the gut epithelium and that DNA-synthesis takes place elsewhere. The presence of small basal mitotic cells in the intestinal epithelium and the results of Marshall and Dixon (1978), however, suggest a proliferation of these cells within the intestinal epithelium.

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General summary

Information on the endocrine regulation of digestion in fish is scarce especially on stomachless cyprinids. In the present study (chapters I, III) 3 distinct enteroendocrine cell types will be described for the intestinal epithelium of *Barbus conchonius*. With the light microscope, enteroendocrine cells only stained moderately after some argyrophil reactions; therefore, the distinction is mainly based on the size of the basally located secretory granules. Cell type I (small granules) is distributed throughout the intestine, but with the highest frequency in the third segment; cell type II (intermediate granules) is mainly present in the first segment, whereas most of the cells of type III (large granules) are found in the intestinal bulb.

Most if not all enteroendocrine cells are of the "open" type, which has a dendrite-like process to the intestinal lumen. The ultrastructure of the apical part (pinocytotic vesicles, cilium, microtubules) indicates that these cells probably have a chemo-receptory function. In contrast to the strongly innervated pancreatic endocrine cells, nerve endings are not found in the vicinity of the enteroendocrine cells. Consequently, the gut endocrine cells seem to be self-sufficing in their functioning; they receive adequate stimuli at their apical end, that activate or inhibit the basal granule release. However, a paracrine impact from one cell to another cannot be excluded.

Comparison of enteroendocrine cells with pancreatic endocrine cells (chapter II) revealed only one common cell type for gut and pancreas, viz. cell type III resembles the pancreatic A_{2r} cell. Therefore, cell type III is probably involved in the secretion of a pancreatic hormone, possibly a glucagon-like immunoreactive peptide (GLI, formerly enteroglucagon) or, as discussed in chapter III, pancreatic polypeptide (PP). In contrast with mammals, the D (= A_1) cells of fish, which are assumed to produce somatostatin, are abundantly present in the pancreatic islets but not in the intestinal epithelium.

The possible functions of the two other cell types is discussed in

chapter III. Cell type II is probably involved in CCK-PZ secretion. As this type contains intermediate granules the indication "I" cell, used for mammals, may be maintained for this cell type. The location throughout the intestine suggests that cell type I produces a hormone that controls gut motility. As serotonin cannot be demonstrated in the intestinal epithelium of fish, the hormones motilin, vasoactive intestinal peptide (VIP), and neurotensin may be considered for this function.

As a consequence of the absence of peptic digestion and multicellular intestinal glands, the digestive tract of cyprinids contains a relatively simple endocrine regulatory system, in which only 3 distinct enteroendocrine cell types can be recognized. Therefore, hormones directly or indirectly related to the presence of a stomach cannot be expected, such as gastrin, histamin, gastric inhibitory peptide (GIP) and secretin. Moreover, the evolution of the endocrine system in fish may be less developed; consequently some "primitive hormones" resembling two or more mammalian hormones might be expected.

Neither serotonin nor catecholamines can be demonstrated in the enteroendocrine cells of adult fish, even not with amine precursors (5-HTP or L-DOPA) consequently, the APUD characteristic (*A*mine *P*recursor *U*ptake and *D*ecarboxylation) is absent in these cells of adult specimen (chapter I). On the other hand, APUD cells appear to be present in the intestinal epithelium from day 3 untill day 6 of development (chapter IV). This short-time APUD facility has probably to be considered as a rudiment of a neural origin of the enteroendocrine cells. The present study (chapter V) shows that a neural crest origin is hardly possible, and presumptive enteroendocrine cells are supposed to migrate in the early developmental stages from the neurectoderm. It is not certain whether a similar origin can be suggested for all enteroendocrine cell types. Particularly for type III (= A_{2r}) cells, a neurectodermal origin is hardly conceivable, as their granules are found in intermediate cells of the pancreas, which contain both exocrine and endocrine granules (chapter II).

The absence of the APUD characteristics as from the larval stage onwards is in contrast with the presence of this facility in gastro-enteric endocrine cells of birds and mammals. This may be explained by assuming that granule-containing enteroendocrine cells are able to proliferate (chapter VI). Hence differentiation from stem cell to enteroendocrine cell may occur only during embryonic development, and the APUD facility must possibly be considered as a differentiation characteristic. Whether such mature-type enteroendocrine cells

proliferate in or outside the epithelium is not yet known.

In chapter VI it is shown that the turn-over time of the enteroendocrine cells is considerably longer than that of absorptive cells. Thus, the moving upward into the folds must be much slower than of other enterocytes. This might be attributed to a stronger adhesion of the enteroendocrine cells to the basement membrane.

The present study can only give assumptions with respect to function, origin and renewal of the enteroendocrine cells of a cyprinid species. Additional experiments remain to be done to provide further information.

Samenvatting

Bij de start van dit onderzoek was weinig bekend over de endocriene regulatie van de spijsvertering van vissen, met name de maagloze karperachtigen zijn niet eerder onderzocht. Lichtmicroscopisch bleken enteroendocriene cellen moeizaam aantoonbaar te zijn. Dientengevolge is overgegaan op een electronenmicroscopische identificatie van deze hormoon producerende cellen.

Met zekerheid konden slechts 3 enteroendocriene celtypes onderscheiden worden, voornamelijk gebaseerd op de grootte van de secretiegranula. Celtype I heeft de kleinste granula en komt in alle segmenten van de darm voor. Daarentegen zijn de celtypes II (intermediaire granula) en III (grootste granula) voornamelijk gelocaliseerd in het eerste segment van de darm.

De meeste enteroendocriene cellen staan met een smalle dendrietachtige uitloper in open verbinding met het darmlumen; daarom wordt dit celtype "open" genoemd, dit in tegenstelling tot de meeste endocriene cellen in de maag van hogere vertebraten die deze uitloper naar het lumen missen ("closed"). De enteroendocriene cellen worden basaal niet geinnerveerd. Stimulatie van deze cellen moet zodoende vanuit het darmlumen plaats vinden. Adequate stimuli, mogelijk specifiek voor ieder celtype, inhiberen of stimuleren de hormoonafgifte aan de basis van deze cellen. Dientengevolge dienen de enteroendocriene cellen als zelfstandig opererende units beschouwd te worden; hoewel een onderlinge paracriene beïnvloeding niet uitgesloten mag worden.

Bij vergelijking van de endocriene cellen van pancreas en darm blijkt slechts één celtype in beide organen voor te komen. Celtype III komt namelijk ultrastructureel overeen met een van de twee A_2 cellen (de A_2 cel met de ronde granula) van de eilandjes van Langerhans. Vandaar dat voor celtype III de produktie van een pancreashormoon verondersteld wordt; "pancreatic polypeptide" (PP) of een glucagonachtig hormoon. In tegenstelling tot zoogdieren is de somatostatin-producerende D ($=A_1$) cel bij de vis in grote aantallen in de eilandjes van Langerhans aanwezig en niet in de darm. Celtype II secreteert waarschijnlijk het hormoon cholecystokinine-pancreozymine (CCK-PZ), dat de afgifte van pancreassappen en gal reguleert. Vanwege het voorkomen in alle segmenten van de darm wordt voor celtype I de produktie van een darmperistaltiek beïnvloedend hormoon verondersteld. Daar serotonine bij deze vis niet aantoonbaar is komen de hormonen motiline, "vasoactive intestinal peptide" (VIP) en neurotensine voor deze functie in aanmerking.

Waarschijnlijk ten gevolge van de afwezigheid van een maag en darmklieren bezitten karperachtigen een relatief eenvoudiger endocrien regulatiesysteem in het spijsverteringskanaal. Hormonen die direct of indirect gerelateerd zijn aan het functioneren van een maag, zoals gastrine, "gastric inhibitory peptide" (GIP) en secretine, zijn bij karperachtige vissen zeer waarschijnlijk afwezig. Tevens is het niet onmogelijk dat in de vis minder geëvolueerde (primitieve) hormonen voorkomen met de werking van twee of meer zoogdierhormonen.

Naast serotonine zijn ook catecholamines niet aantoonbaar in het darmepitheel van de vis, zelfs niet na toediening van 5-hydroxytryptofaan of L-DOPA als amine-precursor. De enteroendocriene cellen van de vis bezitten blijkbaar niet de APUD-eigenschap (Amine Precursor Uptake and Decarboxylation), in tegenstelling tot de endocriene cellen in het maagdarmkanaal van zoogdieren. Echter tijdens de embryonale ontwikkeling bleek deze eigenschap wel, zij het kortstondig (tussen 3 en 6 dagen), aanwezig te zijn in de differentierende enteroendocriene cellen van de vis. Omdat de enteroendocriene cellen de APUD-eigenschap gemeen hebben met cellen die afkomstig zijn van de neurale lijst, zoals pigment cellen, sympathische ganglioncellen, bijniermergcellen, paraganglioncellen en calcitonine-producerende cellen, en omdat in de endocriene cellen van het spijsverteringskanaal van zoogdieren diverse neuropeptides zijn aangetoond wordt een neurale herkomst ook voor de enteroendocriene cellen verondersteld. De tijdelijke aanwezigheid van de APUD-eigenschap in de enteroendocriene cellen van de embryonale vis moet dan misschien beschouwd worden als rudiment van de neurale herkomst. De in dit onderzoek uitgevoerde neurale lijst transplantaties toonden echter aan dat een neurale lijst herkomst van de enteroendocriene cellen van deze vis onwaarschijnlijk is. Indien deze cellen toch een relatie met het neurale systeem hebben moet migratie van neuroendocrien-geprogrammeerde cellen naar het entoderm in een vroeger stadium van de ontwikkeling plaatsvinden, mogelijk reeds tijdens de gastrulatie. Het is onzeker of een dergelijke herkomst voor alle enteroendocriene celtypen geldt, vooral omdat in de pancreas intermediaire cellen gevonden zijn die zowel exocriene als endocriene granula (type III) bevatten.

Daar catecholamines in de enteroendocriene cellen van de vis blijkbaar niet functioneel zijn, moet de APUD-eigenschap in deze cellen van embryonen mogelijk als een differentiatiekenmerk beschouwd worden. Hierop sluit de waarneming aan dat celvernieuwing waarschijnlijk plaats vindt via prolife-

ratie van granula-bevattende endocriene cellen, al dan niet gelegen buiten het epitheel. Derhalve behoeft differentiatie van stamcel tot endocriene receptorcel alleen tijdens de embryonale ontwikkeling plaats te vinden.

De celvernieuwingstijd van de enteroendocriene cellen is aanzienlijk langer als van de absorberende cellen. Het opschuiven van deze cellen naar de toppen van de plooi moet dientengevolge veel langzamer plaatsvinden; waarschijnlijk door een steviger binding van de enteroendocriene cellen met de basaalmembraan.

In dit proefschrift zijn een aantal veronderstellingen gedaan met betrekking tot functie, herkomst en celvernieuwing van de enteroendocriene cellen van een karperachtige vis. Om echter de vele gerezen vragen in de toekomst op te kunnen lossen zullen nog vele aanvullende experimentele studies aan dit endocriene regulatie systeem noodzakelijk zijn.

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Curriculum vitae

Op 2 oktober 1948 ben ik in Tilburg geboren. Na het behalen van het HBS-diploma aan het St. Paulus Lyceum te Tilburg werd in 1966 begonnen met de studie Biologie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen werd afgelegd in december 1969. Vanaf 1969 tot 1973 ben ik als student-assistent verbonden geweest aan de sectie Histologie en Celbiologie van de vakgroep Algemene Dierkunde. In 1971 ben ik tijdelijk leraar geweest aan het Stedelijk Gymnasium te 's-Hertogenbosch. In oktober 1972 werd het doctoraalexamen, met hoofdvak histologie, bijvakken biochemie en plantenfysiologie, en aantekening didactiek, afgelegd. Vanaf november 1972 ben ik werkzaam binnen de sectie Histologie en Ontwikkelingsbiologie van de vakgroep Experimentele Diermorfologie en Celbiologie (voormalige vakgroep Dierkunde) van de Landbouwhogeschool. In de beginfase ben ik uitsluitend betrokken geweest bij de "opbouw" van het zoologieonderwijs van de in 1971 gestartte studierichting Biologie. In 1975 is naast een taak bij het onderwijs in de histologie en ontwikkelingsbiologie een onderzoeksproject gestart wat geresulteerd heeft in dit proefschrift.