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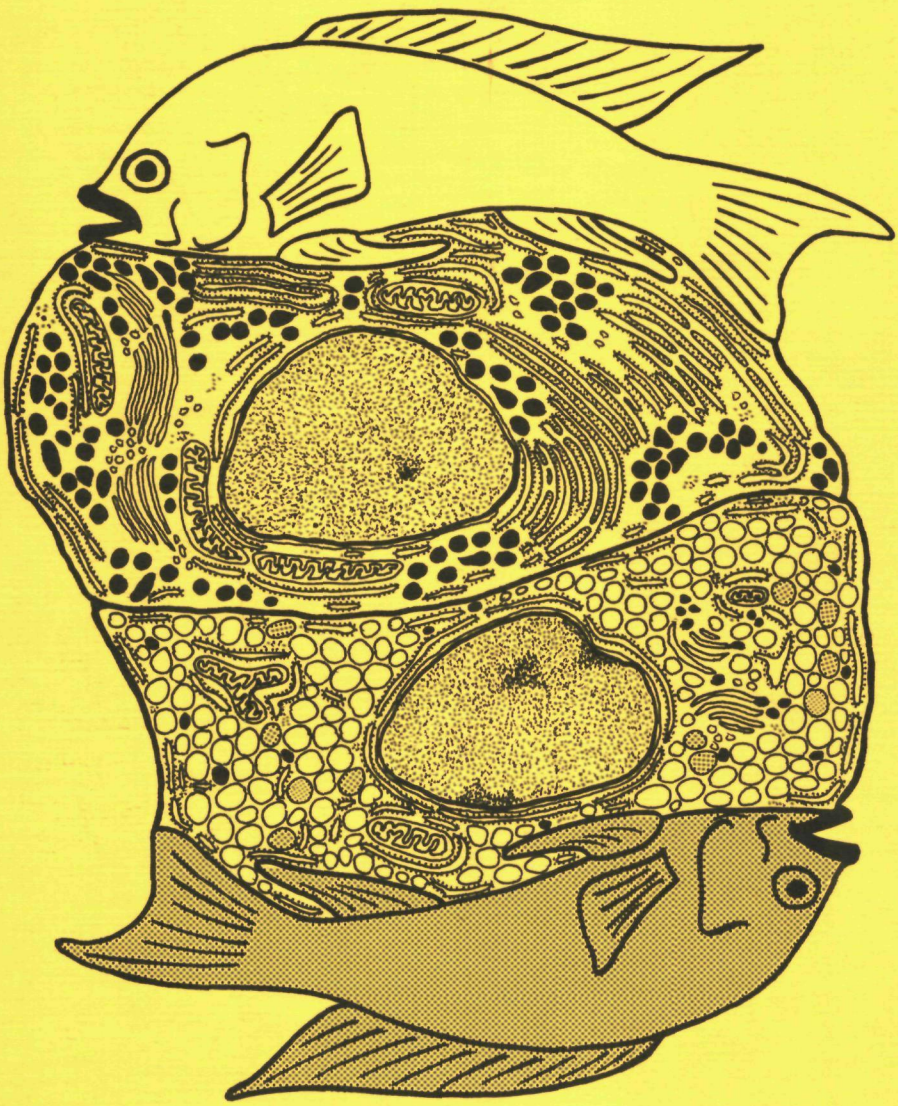

GJJM van Eys

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF THE TELEOSTEAN PARS INTERMEDIA

Promotores:

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PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF THE TELEOSTEAN PARS INTERMEDIA

P R O E F S C H R I F T

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF. DR. P.G.A.B. WIJDEVELD,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP MAANDAG 15 JUNI 1981
DES NAMIDDAGS TE 2 UUR PRECIES

door

GUILLAUME JOHANNES JOZEF MARIA VAN EYS
geboren te Schin op Geul

Nijmegen, 1981

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CONTENTS page

GENERAL INTRODUCTION

Structure of the pars intermedia in teleosts. In the pituitary gland of teleosts three regions can be distinguished: the pars distalis, the pars intermedia and the neurohypophysis (Fig. 1). The rostral pars distalis contains prolactin cells and ACTH cells, whereas gonadotropes, somatotropes and thyrotropes are located in the proximal part of the pars distalis. The posterior region of the pituitary gland represents the pars intermedia. The pars intermedia, and to a smaller extent, the pars distalis are invaded by the neurohypophysis. In addition to being penetrated by the neurohypophysis, the teleost pars intermedia differs from its equivalent in other vertebrate groups by the presence of two distinct glandular cell types, which can be differentially stained for light microscopy by the periodic acid-Schiff (PAS) technique, followed by staining with lead haematoxyline (PbH) (Stahl, 1958; Olivereau, 1970; Baker, 1972). At the ultrastructural level the two cell types can easily be distinguished on the basis of cellular organisation and electron density and shape of the secretory granules (Thornton and Howe, 1974; Leatherland, 1970).

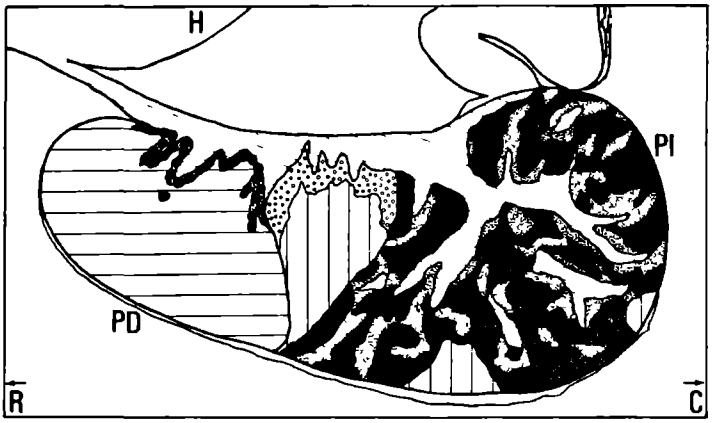

Fig. 1: ▭: Prolactin cells; ▬: ACTH cells; ▭: Gonadotropic and thyrotropic cells; ▭: Somatotropic cells; ▬: MSH (PbH positive) cells; ▭: PAS positive cells; ▭: Neurohypophysis; PD: pars distalis; PI: pars intermedia; H: hypothalamus; R: rostral; C: caudal.

The melanocyte stimulating hormone (MSH) producing, PbH positive cells. As in other vertebrates, most studies on the teleosts pars intermedia dealt with the role of this part of the pituitary gland in pigmentary control. Many teleosts are able to adapt their skin colour to background, generally having a dark appearance on a black background and a pale one on a white background (Waring, 1963; Fingerman, 1970; Abboth, 1973). In contrast to amphibians, in which pigmentation is hormonally controlled, background adaptation in teleosts is regulated by a dual control mechanism. In many teleosts the physiological background response (aggregation and dispersion of pigment in the melanophores) is often partly or totally under nervous control (Fujii, 1969; Fujii and Novales, 1969; Abboth, 1973; Novales, 1973). The role of the pars intermedia in such teleosts is limited to the morphological background response: the increase, maintenance and decrease of the number of melanophores and of the amount of pigment in these melanophores (Odiorne, 1957; Pickford and Kosto, 1957; Waring, 1963; Chavin, 1969; Shan-te Chen and Tchen, 1970; Jain and Bhargave, 1978).

Many reports have described a relationship between background colour and the activity of the PbH positive cells (Waring, 1963; Olivereau, 1971, 1972; Malo-Michele, 1977; Baker, 1972; Baker and Ball, 1970; Thornton and Howe, 1974). Placing fish on a black background led to an increased activity of the PbH positive cells, whereas transfering black adapted fish to a white background is followed by a reduction of the activity of these cells. Thus it was concluded that substances produced by the PbH positive cells of the pars intermedia play a role in the regulation of background adaptation processes in the teleostean skin. In some teleosts the PbH positive cells have been reported as reacting to changes in the electrolyte concentration of the ambient water (Olivereau, 1969; Malo-Michele, 1975). However, other investigators could not find such an effect (Mattheij et al., 1971; McKeown and Leatherland, 1973).

On the basis of physiological response as well as immunoreactivity it may be concluded that the PbH positive cells are comparable with the pars intermedia cells of other vertebrate groups. From studies in a number of vertebrate species, it has become clear that the pars intermedia cells produce several biologically active hormone-like peptides. Most of these investigations demonstrated the existence of a common precursor for these peptides (Mains and Eipper, 1976, 1978, 1979; Mains

et al., 1977; Loh and Gainer, 1977; Roberts and Herbert, 1977a,b; Roberts et al., 1979; Crine et al., 1978; Nakanishi et al., 1979; Jenks et al., 1979). This precursor is a glycoprotein with a molecular weight of about 30.000 daltons. Cleavage of this precursor results in a number of intermediates, of which β-LPH and ACTH are best characterized. The final products of this biochemical processing are endorphins and melanotropins (Fig. 2; Mains and Eipper, 1979; Loh and Gainer, 1977; Gianoulakis et al., 1979; Rubinstein et al., 1977; Jenks et al., 1979).

Investigations on the biosynthesis of the teleost pars intermedia are rather scarce. The presence of substances with melanotropic, corticotropic and opiate activity in the teleost pars intermedia was demonstrated by means of bioassays (Baker, 1972; Baker and Ball, 1975; Scott and Baker, 1975; Carter and Baker, 1980; Hunter and Baker, 1979). Kawauchi and coworkers (1980a,b,c,d) were able to isolate several MSH and β-endorphin peptides from the pituitary of Oncorhynchus keta. These findings have been confirmed by the results of immunohistochemical studies, which indicated the presence of MSH, ACTH and endorphins in the teleostean MSH cells (Follenius and Dubois, 1974, 1976, 1980; Malo-Michele et al., 1976; Olivereau et al., 1976a,b; Romain et al., 1974; Dubois et al., 1979).

Investigations on the regulation of biosynthesis and release in the MSH cells have shown that in vertebrates in general control of these processes is mainly of an inhibitory nature. Transection of the hypophyseal stalk leads to continued release of melanotropic peptides (Etkin, 1962; Ito, 1968; Kastin and Ross, 1964, 1965; Meurling and Fremberg,

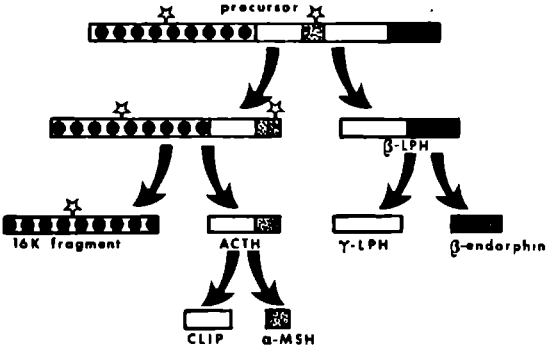

Fig. 2: Model of processing of precursor for α-MSH and β-endorphin in the pars-intermedia cells. (After: Mains and Eipper, 1979).

1974; Fremberg et al., 1977; Taleisnik et al., 1967). Two possible explanations for this result may be given. First, the continued release is the consequence of a disruption of a portal system connecting the hypothalamus and the pituitary gland. This would be in line with the hypothesis that the control of the pars intermedia is mediated by blood-borne factors (Kastin and Schally, 1966; Taleiskin and Tomatis, 1967a,b, Bower and Hadley, 1971). Secondly, the transection causes a disruption of aminergic fibers originating in the hypothalamus, which would be in agreement with the idea of a direct innervation of the pars intermedia (Iturriza, 1966, 1967; Zambrano et al., 1972; Penny et al., 1979; Fremberg et al., 1977; Fremberg and Meurling, 1975; Bern et al., 1971). Pharmacological studies suggest that the inhibitory control is mediated through dopaminergic fibers, that make synactic contacts with the MSH cells (Olivereau, 1972, 1978; Fremberg and Olivereau, 1973, Zambrano et al., 1972). In teleosts this inhibitory control system has been reported to be activated by light received by the eyes. The influence of another photoreceptor, the pineal organ, has received little or no attention in this context. In mammals pinealectomy has been shown to increase the pituitary content of MSH (Kastin et al., 1967) while administration of melatonin, a substance present in the pineal organ, lowers pituitary MSH level (Kastin and Schally, 1967). There are indications that in teleosts too the pineal organ influences the activity of the MSH cells, and that the effect of the pineal organ on skin melanophores is mediated by the MSH cells (Kavaliers and Abboth, 1977; Kavaliers et al., 1980). In addition, there is little experimental evidence in teleosts that melatonin or the pineal organ exercises an effect on whole body chromatic responses, like background adaptation (Abboth, 1973; Kavaliers and Abboth, 1977; Kavaliers et al., 1980; Owens et al., 1978). However, most investigators interpret pallor of the skin after administration of melatonin or a stay in darkness, and the disappearance of this pallor after pinealectomy, as a result of direct action of the pineal organ on the melanophores (Ruffin et al., 1969; Reed et al., 1969; Joss, 1973; Hafeez, 1970; Hafeez and Quay, 1970).

The PAS positive cells. The PAS positive cells of the teleosts pars intermedia represents a cell type that is likely unique for the vertebrate adenohypophysis. The substances produced by these cells are probably glycoproteins, as may be concluded from the histochemical characteristics, of which a high affinity for the Schiff reagent in the

PAS reaction is the most prominent. The products of the PAS positive cells may have some structural resemblance with prolactin, since Rawdon (1979), working with Sarotherodon mossambicus, found these cells to bind anti-ovine prolactin, even after pretreatment of the antiserum with fish prolactin.

Not only is there an almost complete lack of knowledge about the products of the PAS positive cells; also the data concerning the physiological significance of these cells are scarce and unrelated. Investigations of the PAS positive cells are limited to analyses of changes in cellular and nuclear volumes in response to different environmental conditions. Thus PAS positive cells have been reported to react to changes in salinity (Baker, 1972; Olivereau, 1969; Olivereau and Ball, 1970; Malo-Michele, 1975; Leatherland, 1970) or to changes in calcium levels of the ambient water (Olivereau, 1969; Pang et al., 1973; Olivereau et al., 1980, 1981). In addition, a few investigators have reported that the background colour affects these cells: PAS positive cells were active in fish on a black background and inactive in fish on a white background (Ball and Baker, 1969; Sage and Bern, 1971; Baker and Ball, 1970; Malo-Michele, 1977).

The aim of the present investigation. The present study was directed towards elucidating the physiological significance of both endocrine cell types of the teleost pars intermedia. The species studied was the African cichlid fish Sarotherodon mossambicus (Tilapia mossambica), an euryhaline freshwater species.

The study was started with an analysis of the effects of illumination and background reflectivity on the activity of MSH cells and PAS positive cells. Changes in activity of both cell types were investigated by morphometrical analysis (Chapter 1 and 2). From the results a new hypothesis was developed concerning the mode of control of the activity of the MSH cells, in which both the eyes and the pineal organ play a role. In chapter 3 experiments are described that were performed to test this hypothesis. In chapter 4 the relation between activity of the MSH cells and PAS positive cells and the chromatophore behavior in the skin is reported. In addition, the effects of α-MSH on the chromatophores as well as on the two endocrine cell types of the pars intermedia were investigated.

Several different techniques were applied to investigate the nature

of the products synthetized by the MSH cells and the PAS positive cells. By means of immunocytochemical techniques at light and electron microscopic level, the presence and localization of substances reactive to anti-α-MSH, anti-$ACTH_{1-24}$ and anti-β-endorphin was investigated (Chapter 5). Chapter 6 reports on the biosynthesis of the pars intermedia in vitro. Several techniques were used in an attempt to differentiate between the products of MSH cells and PAS positive cells. Cell separation by Percoll density gradient appeared to be unsuccesful. Therefore, we used the selective and extreme suppression of the activity of the PAS positive cells, as was observed during morphometrical studies in fish kept on a white background. Biosynthesis of the pars intermedia of such fish was analysed by SDS polyacrylamide gelelectrophoresis and high pressure liquid chromatography, and compared with the biosynthesis in fish kept under different environmental conditions.

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CHAPTER 1

Structural changes in the pars intermedia of the cichlid teleost Sarotherodon mossambicus as a result of background adaptation and illumination.

I. The MSH-producing cells.

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Structural Changes in the Pars Intermedia of the Cichlid Teleost *Sarotherodon mossambicus* as a Result of Background Adaptation and Illumination

I. The MSH-producing Cells

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Summary. The pars intermedia of *Sarotherodon mossambicus* (*Tilapia mossambica*) contains two cell types which can be differentiated at both the light and electron microscopic level The predominant cell type is lead haematoxyline positive, and has been shown to be the MSH producing cell type by means of immunocytochemical staining at the ultrastructural level The changes in cellular and nuclear volume, as well as the results of stereological measurements on the cytoplasmic organelles, show that the activity of MSH cells is high on a black background and low on a white background or in total darkness In blinded fish under a normal day-night regime the activity of the MSH cell is as high as that in black adapted fish, whereas the activity is low when the blinded fish are kept in total darkness From the observed differences in activity of the MSH cells between the experimental groups, it is concluded that the MSH cells are not activated by the absence of reflected light, but by a high ratio between direct and reflected light A second light-sensitive organ, supposedly the pineal gland, is also involved in the background response of the MSH producing cells

Key words: Teleost – Pars intermedia – MSH – Morphometric analysis – Light perception

In many teleosts the pars intermedia has been shown to contain two distinct endocrine cell types For light microscopy the periodic acid Schiff (PAS) technique, followed by lead haematoxyline (PbH) staining, is the procedure of choice for discrimination between the two cell types (Baker 1972, Olivereau 1970, Stahl 1958) With the electron microscope the two cell types can be distinguished by electron

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density and shape of the secretory granules. The granules are electron dense and often oblong in the PAS positive cells, whereas they are round in shape and vary from electron dense to electron translucent in the PbH positive cells (Leatherland 1970; Thornton and Howe 1974). By means of immunocytochemical techniques at light microcopic level, the latter cell type has been shown to produce MSH (Follénius and Dubois 1974, 1976; Malo-Michele et al. 1976; Olivereau et al. 1976a, 1976b).

The correlation between background and dispersion of pigment in melanophores has been investigated in fishes (for review: Waring 1963) and amphibians (Hogben and Slome 1936), and the influence of total darkness and of blindness on these processes has also been studied. The pars intermedia, and especially the MSH producing cells, appear to play a crucial role in the control of background adaptation (Hogben and Slome 1936; Olivereau 1971, 1972; Baker 1972; Thornton and Howe 1974; Malo-Michele 1977), and it has been demonstrated that a black background results in a high activity of the MSH cells, whereas low activity is associated with a white background. For amphibians two different hypotheses have been proposed for the control of melanophore activity. The first, suggested by Hogben and Slome (1936), proposes a differentiation of the retina in two functionally different areas and the second, proposed by Charlton (1966), proposes a role for the pineal gland.

Similar investigations in teleosts have been hampered by the complexity of the control of the melanophore response, which varies from entirely nervous to completely hormonal (Fingerman 1963; Novales and Novales 1966; Abboth 1973), and data on the effects of darkness and blinding on pigment dispersion and aggregation are sparse. The present study investigates the distribution of anti-α-MSH immunoreactive material in the pars intermedia of *Sarotherodon mossambicus* at electron microscopic level. The influence of background on the activity of the MSH cells has been studied by measurement of cellular and nuclear volume as well as morphometrical analysis. In addition, the effects of blinding and total darkness on MSH-cell activity have been studied to determine the role of illumination and light perception by the eyes in the control of MSH synthesis and release.

Materials and Methods

Sexually mature males of the cichlid teleost *Sarotherodon mossambicus* with a length between 10 and 12 cm and a body weight varying from 9 to 12 g were used. Sixty fishes were divided into six groups of ten fishes each. During an adaptation period of fourteen days the fishes were kept separately in buckets containing 6 l of tap water. Feeding and water replacement were done every other day. The room temperature was about 24° C. The groups were placed under one of the following regimes:

Group W: white background with normal day-night rhythm (12 h light/12 h darkness).

Group B: non-reflecting black background, with normal day-night rhythm.

Group P: non-reflecting black background, permanently illuminated.

Group D: non-reflecting black background in total darkness.

Group Bl: animals of this group were blinded by severing the optic nerves directly behind the eyes; they were kept on a white background with normal day-night rhythm.

Group BlD: fishes blinded as described above and kept in total darkness.

Illumination was provided by 60W TL tubes suspended 50 cm above the water surface At the end of the adaptation period the fishes were anaesthetized with MS 222 (Sandoz) and killed Pituitary glands were quickly removed and fixed

For light microscopic examination five pituitaries of each group were fixed in Bouin-Hollande fluid for 24 h at room temperature After rinsing, dehydration and embedding in Paraplast, sagittal sections of 7 µm thickness were cut and stained in periodic acid-Schiff followed by Mac Connaill's lead haematoxyline (Mac Connaill 1947) Measurements of the MSH cells were made at a magnification of × 1000 by means of an ocular micrometer Cellular and nuclear volumes were calculated using the formula $4/3\ \pi\ l\ b^2$, in which l stands for the long and b for the short axis For each fish 25 randomly chosen cells and their nuclei were measured

For electron microscopy five pituitaries were prefixed in a 5% glutaraldehyde solution in cacodylate buffer (0 1 M, pH 7 2) for 15 min at room temperature They were subsequently transferred into a mixture of 1% OsO_4, 1 5% $K_2Cr_2O_7$, 1 5% cacodylate buffered glutaraldehyde for 1 h and 0° C After fixation the pituitaries were stained for 90 min at room temperature in an aqueous solution of uranyl acetate The tissue was dehydrated in ethanol and embedded in Spurr s resin (Spurr 1969) Ultrathin sections were post-stained in 2% lead citrate and examined in a Philips EM 301 electron microscope Micrographs of the sections at a final magnification of × 8000 were morphometrically analyzed with a Kontron Digiplan The analysis consisted of determining the following parameters

1 Total volume of the mitochondria as a percentage of the cytoplasmic volume
2 Total volume of the Golgi area as a percentage of the cytoplasmic volume
3 Total volume of the rough endoplasmic reticulum as a percentage of the cytoplasmic volume
4 Total length of the membranes of the rough endoplasmic reticulum per unit area of the cytoplasmic surface
5 Total volume of the granules as a percentage of the cytoplasmic volume
6 The number of secretory granules per unit area of the cytoplasmic surface

For each pituitary gland, cross-sections of at least 15 randomly chosen MSH cells were measured

For statistical analysis of light as well as electron microscopical data the Student's *t*-test was used

For the immunological identification of the MSH-producing cells, pituitaries were fixed in cacodylate buffered (0 1 M pH 7 2) glutaraldehyde for 24 h at 4° C The tissue was dehydrated in ethanol and embedded in Spurr's resin Ultrathin sections were mounted on nickel grids Immunological staining was based on the unlabeled antibody enzyme technique, using horseradish peroxidase- anti-horseradish peroxidase (PAP) complex as described by Sternberger et al (1970) The PAP complex was purchased from DAKO (Copenhagen) The rabbit anti-α-MSH serum was produced by New Zealand White rabbits by repeated injections of commercially available synthetic α-MSH (Peninsula) The anti-α-MSH sera were pre-reacted with ACTH 1–24 The ACTH 1–24 was a generous gift of Dr H Rigter (Organon, Oss) The purified anti-α-MSH serum was used in dilutions of 1 200 The PAP complex was used in a dilution of 1 50 Controls for this technique with normal rabbit serum showed no specific staining

Results

Fish generally adapted well to their new background, and groups W and B were fully adapted within a week and did not undergo appreciable fluctuations in skin colour during the remainder of the experimental period Group P behaved as group B At the end of the experiment the fishes of group D appeared a light grey, whereas those of group Bl were indistinguishable from those of group B Group BlD fishes were greyish, much the same as the group D animals

Pars Intermedia Two secretory cell types can easily be distinguished. The predominant cell type is PbH positive (Fig 1) and round to oval in shape. At the electron microscopic level, the cytoplasm shows numerous spherical secretory granules with a great variation in volume and electron density Indications of

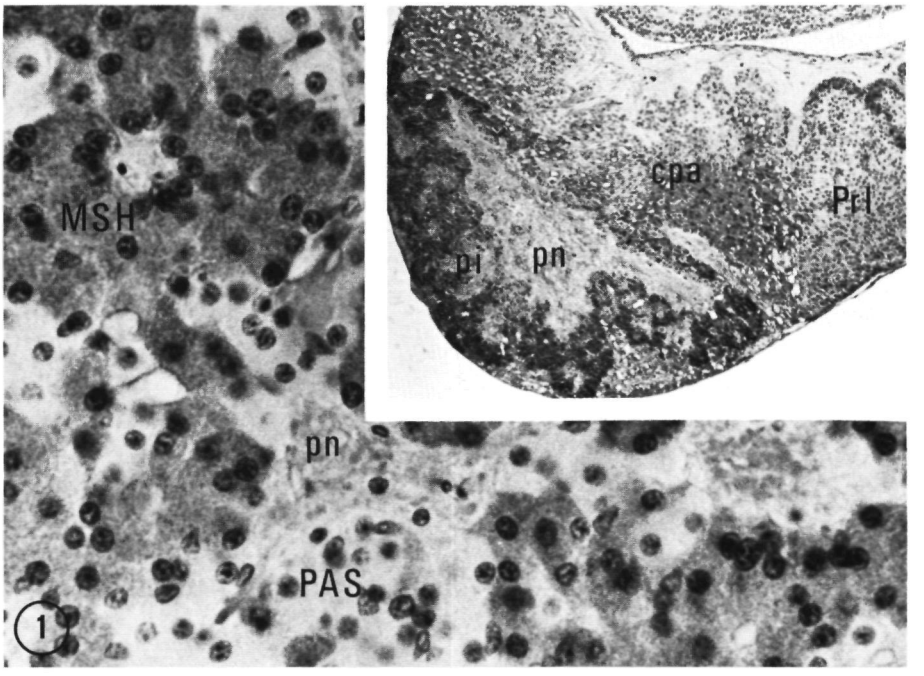


Fig. 1. L.S. of the pars intermedia. *MSH* PbH – positive cells; *PAS* PAS – positive cells; *pn* pars nervosa × 400. *Inset:* General view of the hypophysis. *pi* Pars intermedia; *pn* pars nervosa; *Prl* prolactin lobe; *cpa* caudal pars anterior

release of the contents of the granules by exocytosis are absent. The membranes of the rough endoplasmic reticulum (rER) are scattered throughout the cytoplasm, with small concentrations of cisternea around the nucleus and the mitochondria. The Golgi apparatus is well developed and consists of flat saccules often enclosing electron dense material (Fig. 2). Formation of secretory granules by budding off from the Golgi sacculi is commonly observed (Fig. 4).

The second secretory cell type is PAS positive and mostly situated along the recesses of the pars nervosa, which penetrate into the pars intermedia. The PAS positive differ from the PbH positive cells in their cytoplasmic organisation and the shape and electron density of their granules. In most of these cells the rER is well developed, with membranes arranged in parallel arrays. The granules are very electron dense and often oblong in shape (Fig. 2). The content of the granules of the PbH positive cells is strongly immunoreactive to anti-α-MSH (Fig. 3), and there is hardly any non-specific staining of the nucleus and the membranes of the rER, whereas the PAS positive cells and pars nervosa show no reaction at all.

Besides these two secretory cell types there are agranular supporting cells, few in number and lying between the secretory cells. Their nucleo-cytoplasmic ratio is high; there is so little cytoplasm that the shape of the adjoining cells determines the shape of the nucleus. Their cytoplasmic processes extend between the secretory cells and contain numerous mitochondria (Fig. 2).

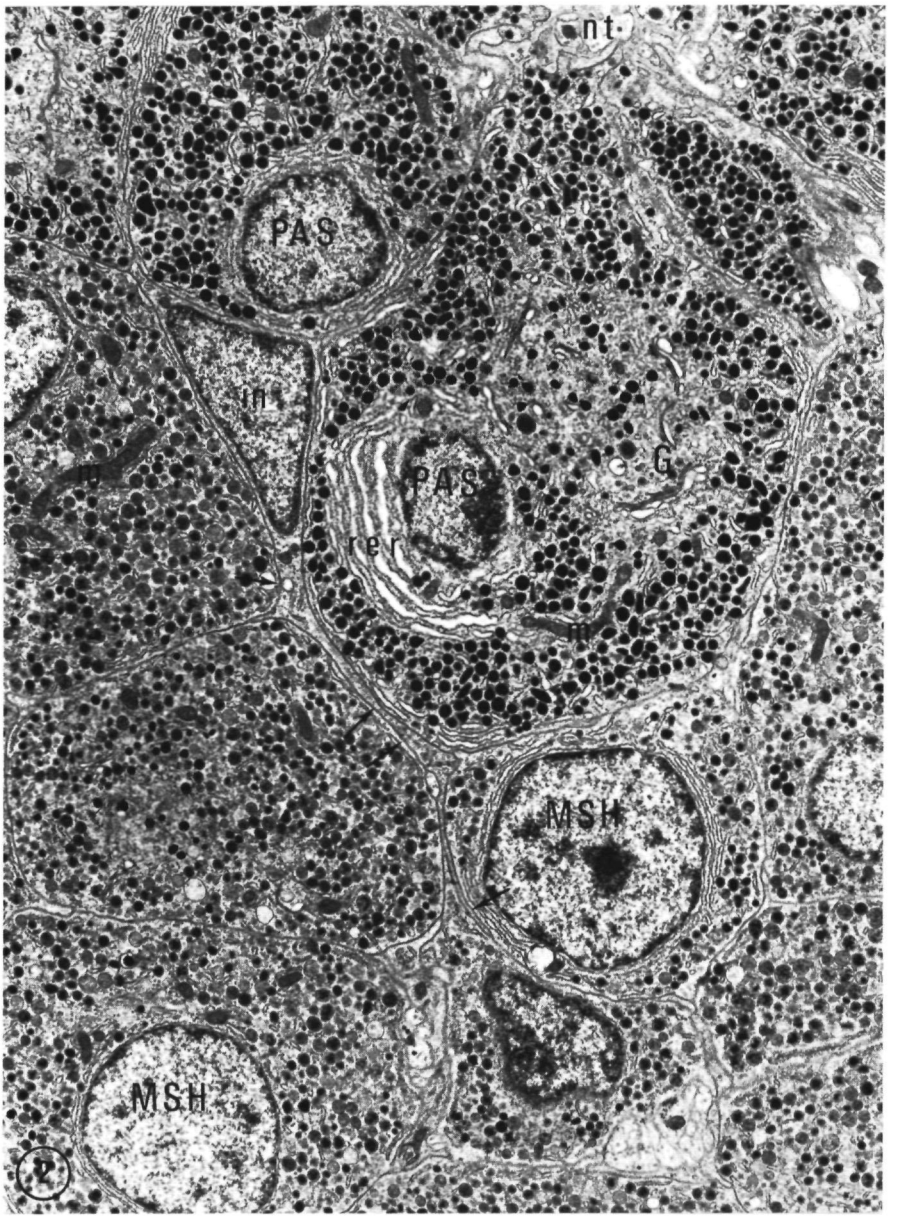
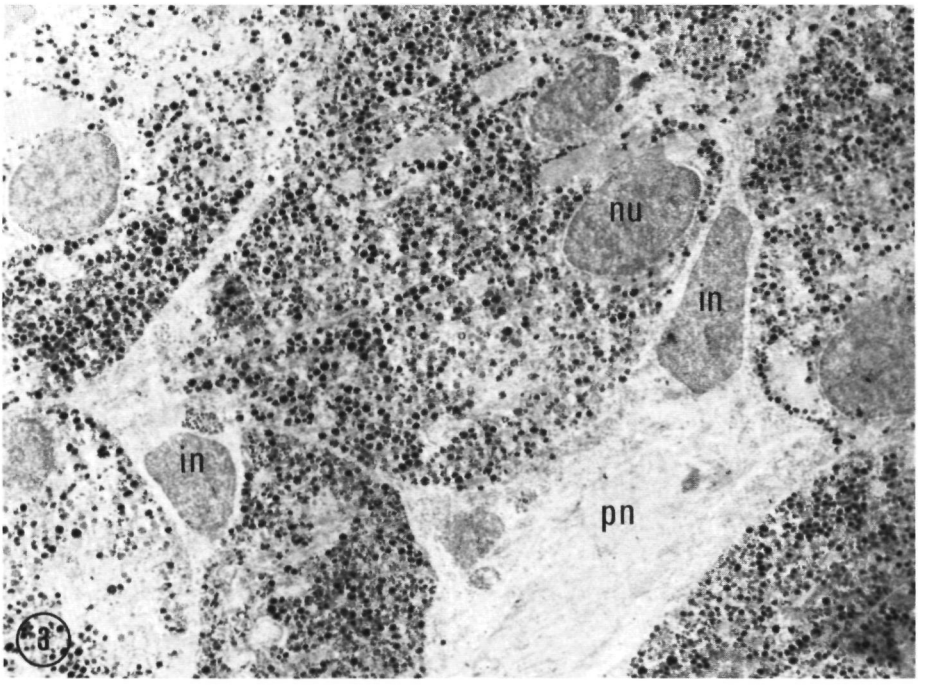


Fig. 2. Electron micrograph of the pars intermedia. Granules of the PAS positive cells (*PAS*) are more electron dense and show a greater variation in shape than those of the MSH producing cells (*MSH*). The rough endoplasmic reticulum (*rer*) is differently organized in the two cell types. The long processes of the interstitial cells (*in*) can be seen penetrating between the endocrine cells (*arrows*). *G* Golgi areas; *m* mitochondria; *nt* nervous tissue. × 6000



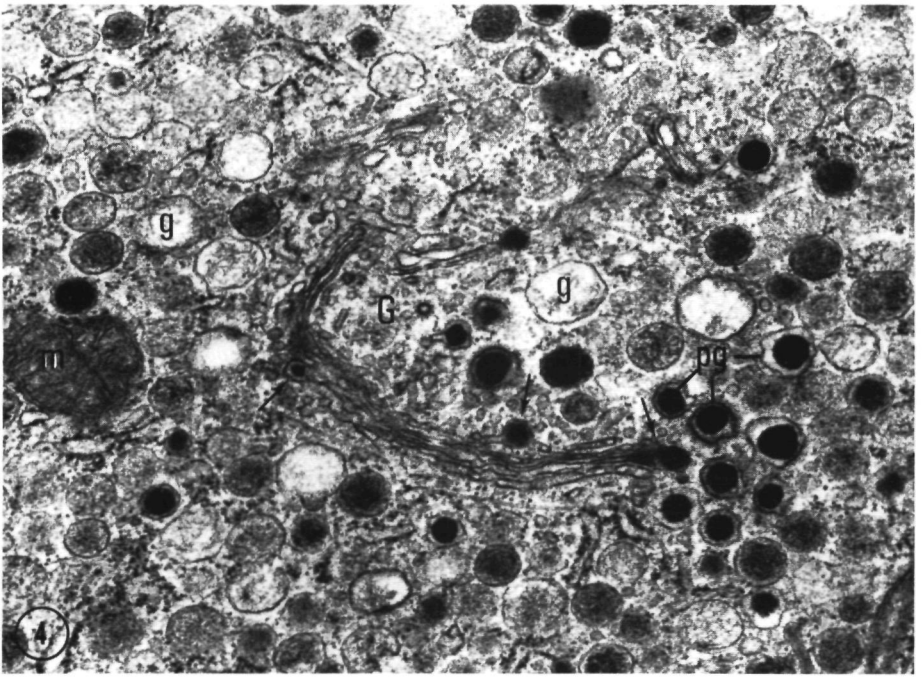


Fig. 3. Immunocytochemically stained pars intermedia. A positive response for α-MSH is only found in the secretory granules of the MSH cells. *nu* Nucleus; *pn* pars nervosa; *in* interstitial cells. ×4000

Fig. 4. Golgi area (*G*) in MSH cell of a blinded fish. The Golgi area is well developed and contains secretory material (*arrows*) which is condensed into presecretory granules (*pg*). *g* Mature secretory granules; *m* mitochondria. ×40,000

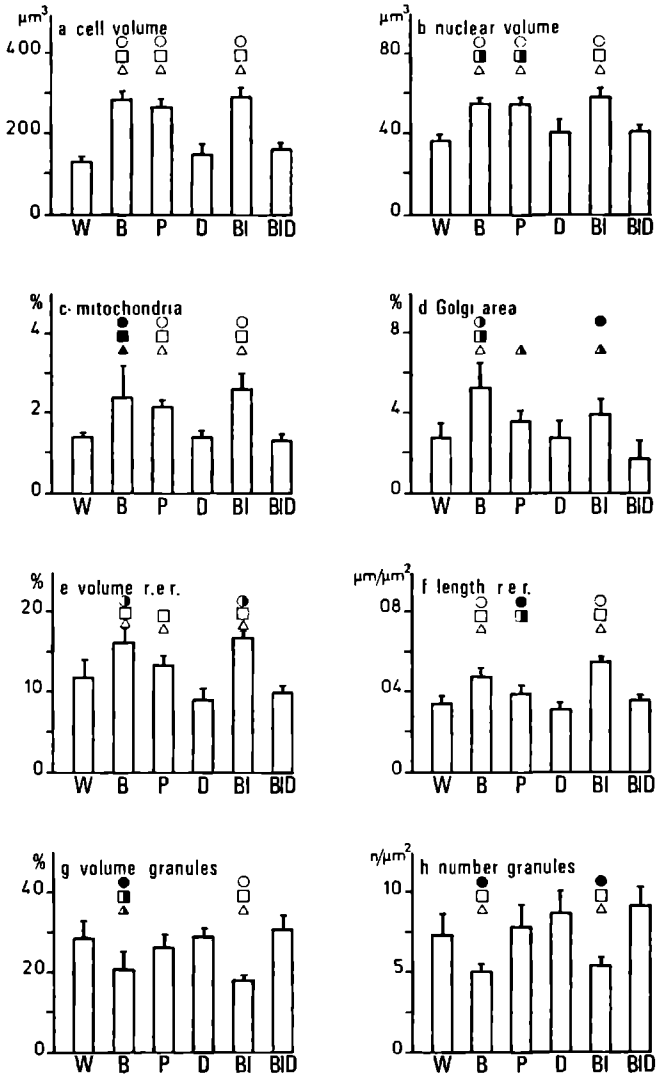
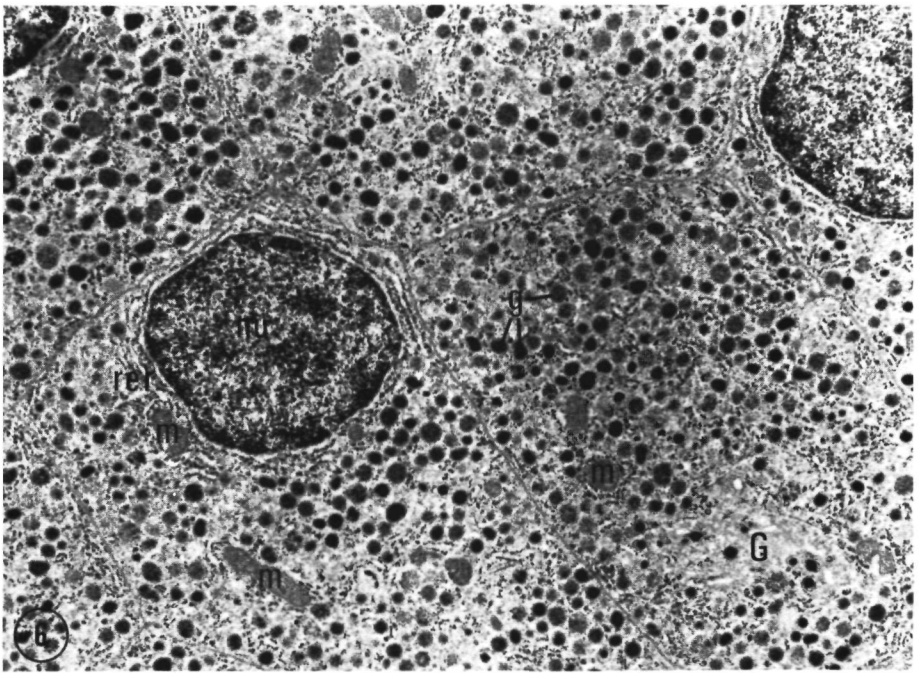


Fig. 5. Morphometrical analysis of the MSH cells of fishes adapted to a white (*W*) or black (*B*) background, under a normal day-night rhythm; *P* permanent illumination on a black background, *D* total darkness; *Bl* blinded fishes under a normal day-night rhythm; *BlD* blinded fishes in total darkness. Statistical significance is indicated by the symbols above the bars: Circles, significantly different from group W (○ $p<0.001$, ◑ · $p<0.01$, ● $p<0.05$). Squares, significantly different from group D (□: $p<0.001$, ◨: $p<0.01$, ■ $p<0.05$) Triangles, significantly different from group BlD (△: $p<0.001$, ▲ . $p<0.01$; ▲· $p<0.05$)

Effect of Experimental Manipulation on the Activity of the MSH Cells. Considerable differences in cell volume occur in the different experimental groups (Fig. 5). The volumes are small in groups W, D, and BlD, but are much larger in groups B, P, and Bl. Similar differences, although less pronounced, are also found in the nuclear volumes.



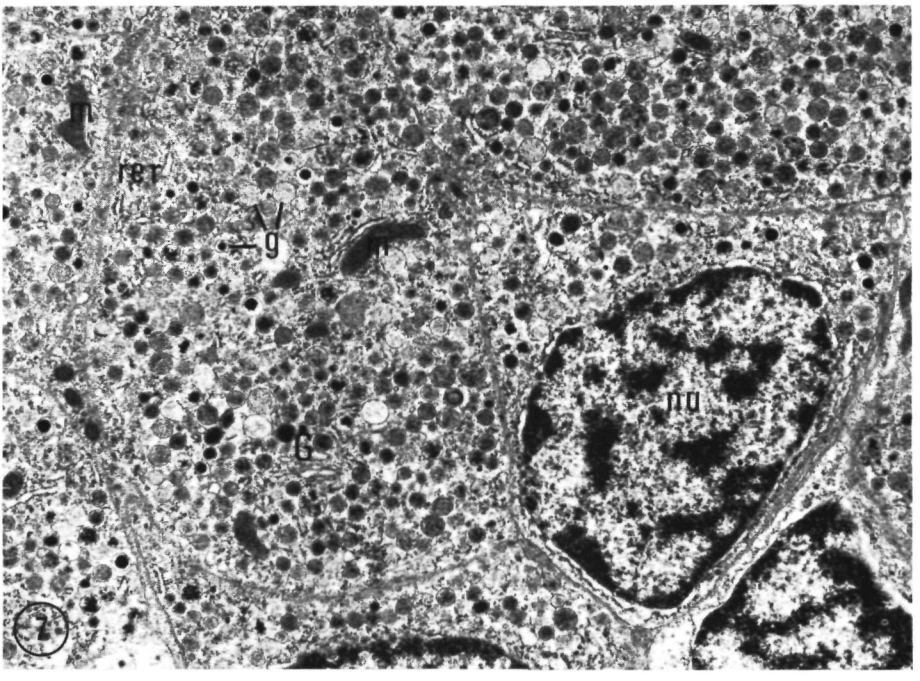


Fig. 6. MSH cells of a fish adapted to a black background under a normal day-night regime. There are numerous mitochondria (*m*), and active Golgi areas (*G*). The rough endoplasmic reticulum (*rer*) is relatively well developed, and the number of secretory granules (*g*) is relatively low; *nu* nucleus. × 8000

Fig. 7. MSH cells of a fish adapted to a white background under a normal day-night regime. There are relatively few mitochondria (*m*). The Golgi areas are small (*G*); the rough endoplasmic reticulum (*rer*) is little developed, and the cytoplasm is filled up with mature granules (*g*). × 8000

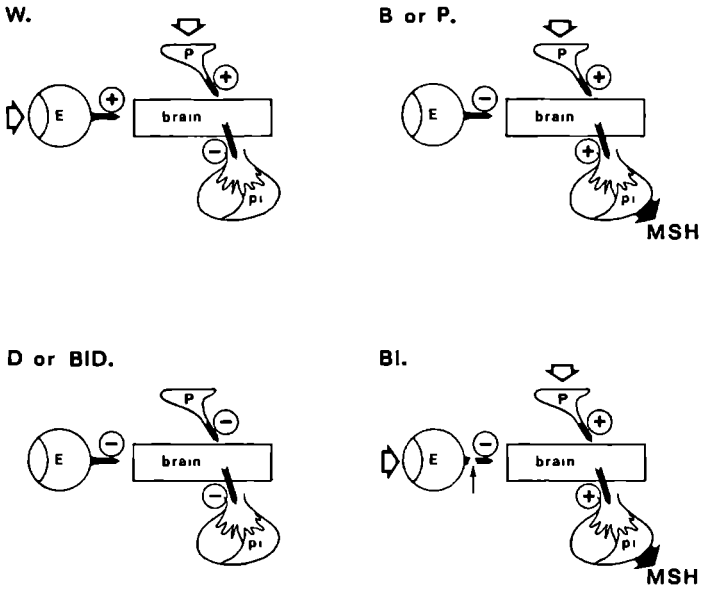


Fig. 8. Suggested regulatory mechanism of MSH cell activity On a white background (group *W*) light will be received by both the eyes and the extra-ocular receptor (*pineal*) MSH cells are inactive Inactive MSH cells are also observed when light is not received by the eye nor by the extra-ocular photoreceptor (groups *D* and *BlD*) When no light is received by the eye (groups *B* and *P*) or when the optic nerve is severed (group *Bl*), but light is received by the extra-ocular photoreceptor, the MSH cells are active These findings suggest that the absence of ocular light reception stimulates MSH production This stimulation can be inhibited by the absence of light reception by the extra-ocular photoreceptor *E* eye, *P* pineal gland (?), *pi* pars intermedia

Electron microscopical analysis reveals considerable differences between groups W, D, and BlD on the one hand, and groups B and Bl on the other. These differences, observed for all six cytoplasmic parameters, are highly significant (Fig 5). In the MSH cells of specimens from groups W, D and BlD, organelles like mitochondria, Golgi apparatus and rER are relatively poorly developed, but in specimens of groups B and Bl they are more abundant and their fractional volume is higher (Fig 5) The opposite holds for the granules (Figs. 6, 7). Group P occupies an intermediate position for all the six cytoplasmic parameters, and statistical values for the differences between group P and the groups W, D and BlD are included in Fig 5 In addition significant differences occur between group P and group B (Golgi apparatus: $p < 0\ 05$, number of granules: $p < 0\ 01$) and between P and Bl group (mitochondria. $p < 0.05$, volume and length of rER: $p < 0.001$, volume of granules· $p < 0.05$; number of granules· $p < 0.01$)

Discussion

Several investigations have indicated that the PbH positive cells of the pars intermedia in teleost fishes must be considered as MSH producing cells

(*Gasterosteus aculeatus:* Follenius and Dubois 1974; *Anguilla anguilla*: Olivereau et al. 1976a; *Boops salpa*: Malo-Michele et al. 1976; *Perca fluviatilis*: Follénius and Dubois 1976; salmonid spp.: Olivereau et al. 1976b; Follénius and Dubois 1978). These various studies used light microscopy and the indirect fluorescent antibody technique, a method which offers limited possibilities for discrimination between reacting and non-reacting cells. The present results, obtained by using the unlabeled antibody enzyme technique, clearly show that only the PbH positive cell type of the pars intermedia binds anti-α-MSH. In these cells the reaction is confined to the granules and, thus, it may be concluded that the granules contain α-MSH or related peptides.

The results of the measurements on the MSH cells of the W and B groups indicate that light reflected from the background influences the activity of these cells. Thus, in the black background adapted animals the cellular and nuclear volumes of the MSH cells were significantly larger than those of the white background adapted animals. Such increased cellular and nuclear volumes are indicative of a higher metabolic activity in these cells. The ultrastructural observations support this conclusion; greater relative volumes of mitochondria, Golgi apparatus and rER were noted in the group B fishes (Fig. 5). In group P fishes, permanently illuminated on a black background, the MSH cells also showed a high metabolic activity, as can be derived from their cellular and nuclear size and ultrastructural characteristics. These quantitative data are in line with observations on some other fish species (*Anguilla anguilla*: Thornton and Howe 1974; Baker 1972; Olivereau 1971, 1972, 1976; *Gasterosteus aculeatus*: Follénius and Dubois 1974; *Tinca tinca*: Romain et al. 1974; *Boops salpa*: Malo-Michele 1977). All these data show that reflected light decreases the activity of the MSH producing cells.

The difference in activity of the MSH cells of white and black background adapted fishes suggests that activity is enhanced when the amount of light reflected by the background is reduced. However, control of MSH secretion is apparantly more complicated than this since animals kept in total darkness (group D) had relatively inactive MSH cells. This suggests that the ratio of reflected and direct light may be involved in the regulation of the activity of MSH cells and, consequently, in background adaptation. Similar conclusions have been reached by other investigators based on observations of the melanophore index (review: Waring 1963). To explain such findings in *Xenopus leavis* Hogben and Slome (1936) introduced the "split-retina" hypothesis, suggesting that the retina consists of two different parts, one functioning as a receptor for direct light, the other as a receptor for reflected light. They concluded that the higher the ratio of direct/reflected light, the higher is the melanophore index. In the present investigation, however, the MSH cells of the blinded fishes or group Bl showed an activity state which, surprisingly, was almost as high as that of the fishes of group B, and in almost all structural aspects these cells were significantly different from those of groups W and D. Such results tend to refute the suggestion that the eyes are the only receptor involved in transferring information about reflected as well as direct light to the brain. The differences in reaction to their white background between the W and Bl groups does, however, confirm that light perceived by the eyes has some function in the regulation of the activity of the MSH cells. The results of group BlD (blinded fishes kept in total darkness) show that the higher activity in the Bl group is not due to a non-specific effect of the blinding.

The discrepancy between the D and BlD groups on the one hand, and the Bl group on the other hand, strongly suggests the existence of an extra-ocular photoreceptor The most likely candidate would appear to be the pineal. A similar conclusion has been drawn by Charlton (1966) as a result of measurements of the melanophore index. He suggests that the pineal gland produces a melanin concentrating factor If, however, the pineal is involved in the process of background adaptation, the differences observed between the groups B and W on the one hand and the groups D, Bl and BlD on the other, suggest that its effects are not exerted directly on the melanophores, as suggested by Charlton, but mediated by the MSH cells (Fig 8) Some such evidence of pineal influence on the pars intermedia has been shown by Oshima and Gorbman (1969), based on electrophysiological activity measurements of MSH producing cells after stimulation of the pineal region

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CHAPTER 2

Structural changes in the pars intermedia of the cichlid teleost Sarotherodon mossambicus as a result of background adaptation and illumination.

II. The PAS-positive cells.

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Structural Changes in the Pars Intermedia of the Cichlid Teleost *Sarotherodon mossambicus* as a Result of Background Adaptation and Illumination

II. The PAS-positive Cells

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Summary. The pars intermedia of *Sarotherodon mossambicus* contains two structurally different endocrine cell types The predominant cell type is assumed to synthesize MSH and related peptides The second cell type is PAS positive, its function and products are unknown In this second cell type changes occur in relation to background colour and illumination Thus, PAS positive cells of fish adapted to a white background are less numerous and metabolically less active than those of fish adapted to a black background, and are most active in fish kept in total darkness In blinded fish, whether in light or in darkness, the activity of the PAS positive cells is similar to that of the black background-adapted animals. The significance of these responses in relation to the control of background adaptation is discussed

Key words: Pars intermedia PAS-positive cells – Background Illumination Morphometric analysis

The pars intermedia of teleosts contains two endocrine cell types Apart from the predominant lead-haematoxylin positive MSH producing cells, a cell type is present that can be stained with periodic acid-Schiff (PAS) Physiological and immunocytochemical studies have not yet elucidated the function of this cell type and the nature of its product(s)

Although Rawdon (1979) recently found a positive reaction with an ovine anti-prolactin serum, the results of other studies indicate that the product of the PAS positive cells shares no immunological properties with any of the known pituitary hormones (Follénius et al. 1978)

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Studies on PAS positive cells have shown that these cells react to very different environmental stimuli. Some investigators have reported changes in their cellular and nuclear volume after variations in ambient salinity (Baker 1972; Leatherland 1970; Olivereau 1967, 1968, 1969), and Olivereau (1969) and Pang et al. (1973) have suggested that PAS positive cells may play a role in the endocrine control of calcium metabolism. Baker and Ball (1970) have shown that the cellular and nuclear volumes of the PAS positive cells from *Poecilia latipinna* and *Blennius spec.* change in response to changes in background colour, and are more active in fish on a black background. Malo-Michele (1977) has reached a similar conclusion for *Boops salpa,* and has further observed that permanent illumination stimulates the metabolic activity of these cells. Thus, it seems possible that, in addition to the MSH cells, PAS positive cells may also play a role in the adaptation of the skin to changes in background colour.

The present study is part of an investigation of the effect of background colour and illumination on the metabolic activity of the secretory cells of the pars intermedia in the cichlid fish *Sarotherodon mossambicus.* In a previous paper data were presented on the MSH cells (Van Eys 1980). The present report records changes in the metabolic activity of the PAS positive cells in response to direct and indirect light.

Materials and Methods

Sexually mature males of the cichlid teleost *Sarotherodon mossambicus* with a length of 10–12 cm and a body weight of 9–12 g. were divided into six groups of ten fish each. During an adaptation period of 14 days the fish were kept separately in buckets containing 6 l of tap water. Feeding and water replacement were done every other day. The room temperature was about 24° C. The groups were placed under one of the following regimes:

Group W: white background with normal day-night rhythm (12L. 12D).

Group B. non-reflecting black background, with normal day-night rhythm.

Group P. non-reflecting black background, permanently illuminated.

Group D: non-reflecting black background in total darkness.

Group Bl: animals of this group were blinded by severing the optic nerves directly behind the eyes; they were kept on a white background with normal day-night rhythm.

Group BlD: blinded and kept in total darkness.

Illumination was provided by 60W TL tubes suspended 50 cm above the water surface. At the end of the adaptation period specimens were anaesthetized with MS 222 (Sandoz), killed, and their pituitaries quickly removed and fixed. The methods used for fixation, and the procedures for measuring cellular and nuclear volumes and volumes of ultrastructural components, were as described by Van Eys (1980).

Results

Black background caused a darkening (groups B and P), and white background a paling of the skin (group W). Blinded animals under a normal day-night rhythm (group Bl) were as dark as the black background adapted specimens. In total darkness fish became greyish, whether they were intact (group D) or blinded (group BlD).

A detailed description of the general morphology of the pars intermedia of *S. mossambicus* can be found elsewhere (Van Eys 1980). Of the two secretory cell types

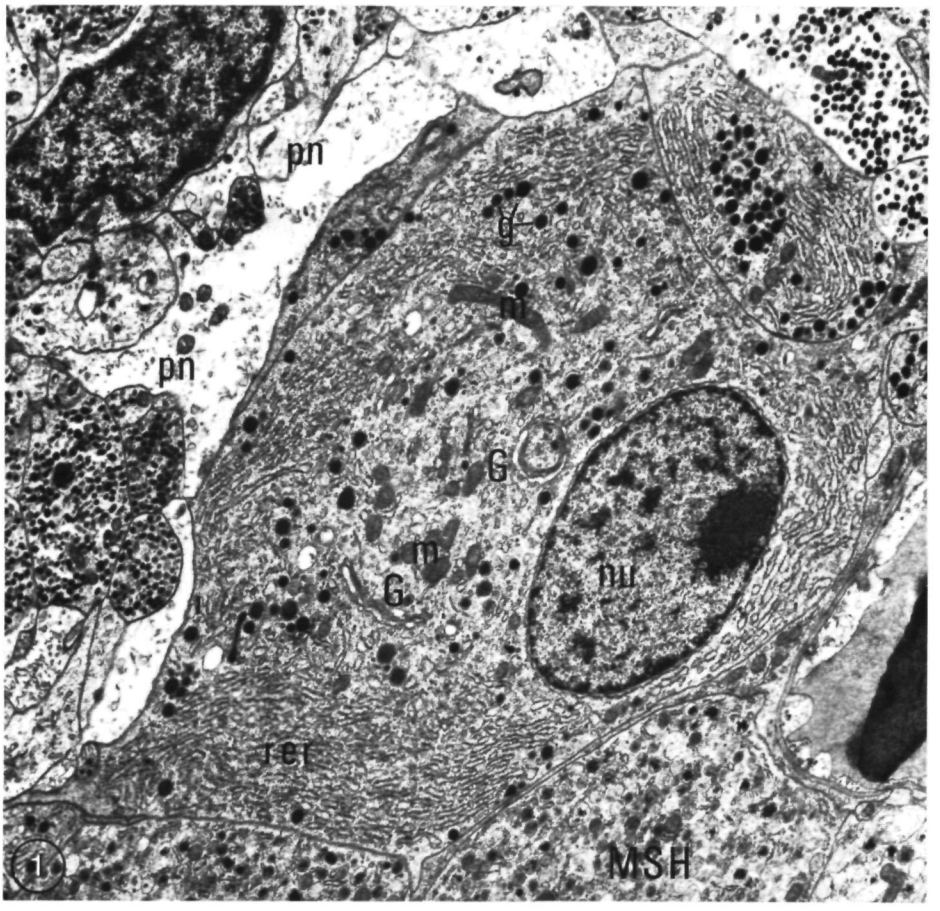


Fig. 1. Electron micrograph of a PAS positive cell, situated between a recessus of the pars nervosa (*pn*) and the MSH cells (*MSH*). *G* Golgi area; *g* granules; *m* mitochondria; *nu* nucleus; *rer* rough endoplasmic reticulum. ×8,000

of the pars intermedia, the MSH cell was predominant. The PAS positive cells are elongated or oval in shape and mostly situated along the recesses of the pars nervosa that penetrate into the pars intermedia. At the ultrastructural level these cells are easily distinguishable from the MSH cells by their cytoplasmic organisation and shape, and the electron density of the secretory granules (Fig. 1). The rough endoplasmic reticulum (rER) is well developed, with its membranes arranged in parallel arrays, mostly along the nucleus and the outer cell membrane. The Golgi apparatus generally is very extensive and consists of long, well defined saccules often enclosing electron dense material. There is no evidence of release of secretory products by exocytosis.

Fish from group W have fewer PAS positive cells than fish from any of the other groups (Figs. 2, 3, 4). The cellular and nuclear volumes of these cells are smallest in group W, indicating a state of low metabolic activity, whereas in group D, they are largest (Fig. 5). Electron microscopic analysis of the relative volumes of

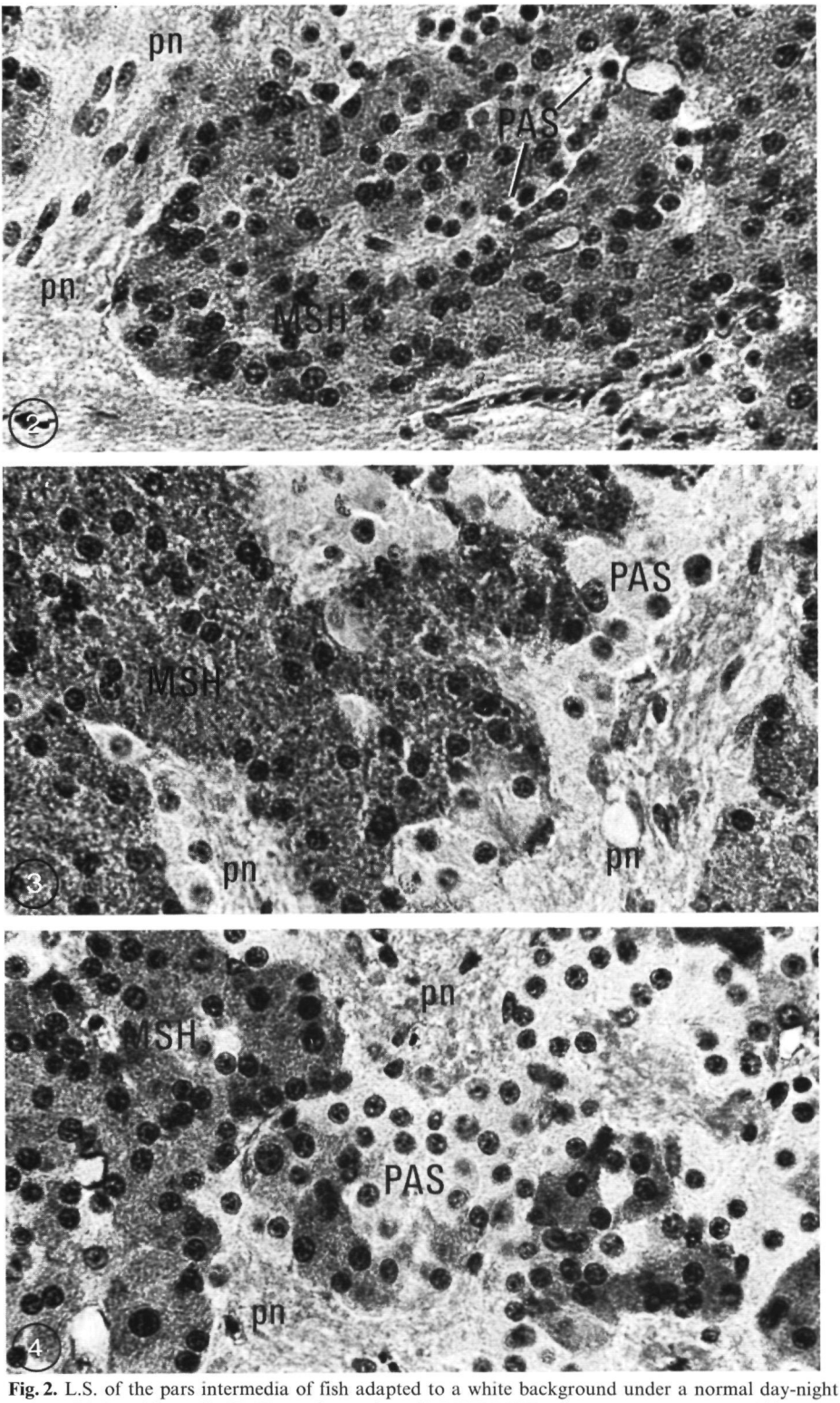


Fig. 2. L.S. of the pars intermedia of fish adapted to a white background under a normal day-night regime. PAS positive cells (*PAS*) are few and small. *MSH* MSH cells; *pn* pars nervosa. ×400

Fig. 3. L.S. of the pars intermedia of fish adapted to a black background under a normal day-night regime. ×400

Fig. 4. L.S. of the pars intermedia of fish kept in total darkness. ×400

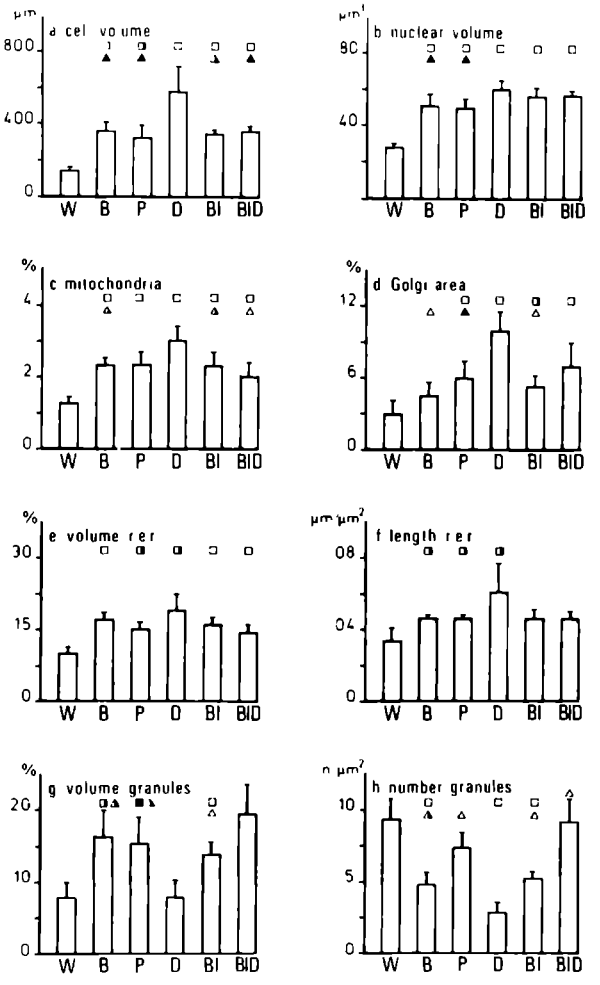
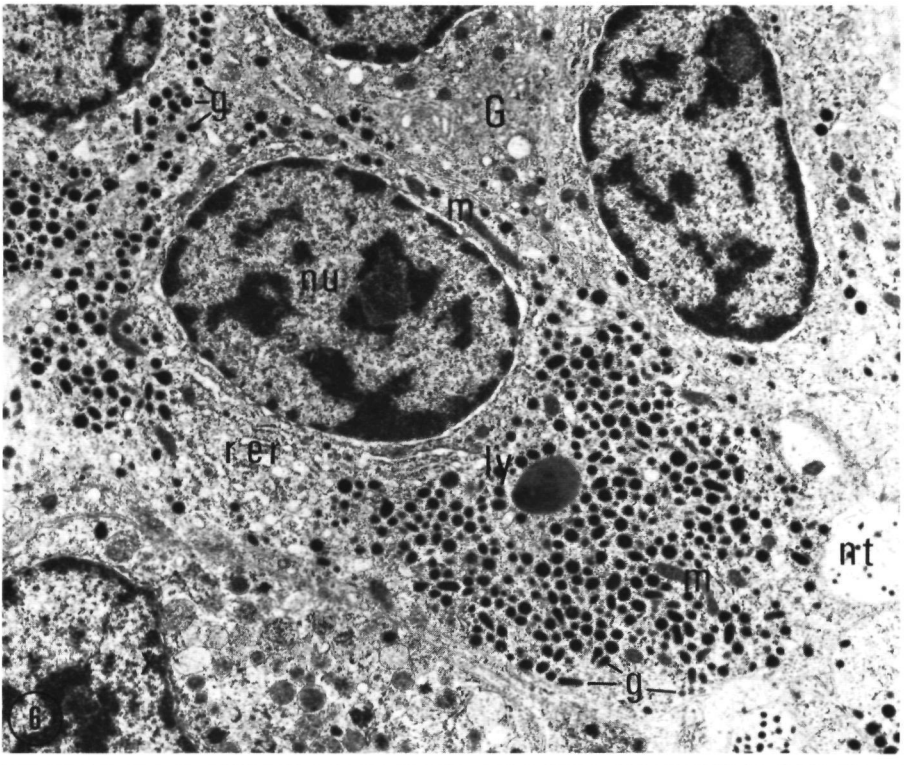


Fig. 5. Morphological analysis of the PAS positive cells of fishes adapted to a white (*W*) or black (*B*) background, under a normal day-night rhythm, *P* permanent illumination on a black background, *D* total darkness, *Bl* blinded fishes under a normal day-night rhythm, *BlD* blinded fishes in total darkness Statistical significance is indicated by symbols above the bars: *Squares*, significantly different from group W (□: $p<0.001$, ◨: $p<0.01$, ■: $p<0.05$) *Triangles*, significantly different from group D (△: $p<0.001$, ◭: $p<0.01$; ▲: $p<0.05$)

mitochondria, rER and Golgi apparatus reveal similar differences between the groups. Thus, these volumes are largest for group D and smallest for group W, whereas groups B, P, Bl and BlD show no significant differences (Fig. 5).

The size of the secretory granules in the PAS positive cells differs between the groups. Two cell categories can be distinguished. Cells of the first category contain secretory granules with a mean volume per granule about eight times larger than that of cells of the second category. In fish of group W all cells are exclusively of the second category, but fish from group P also have significant numbers of large



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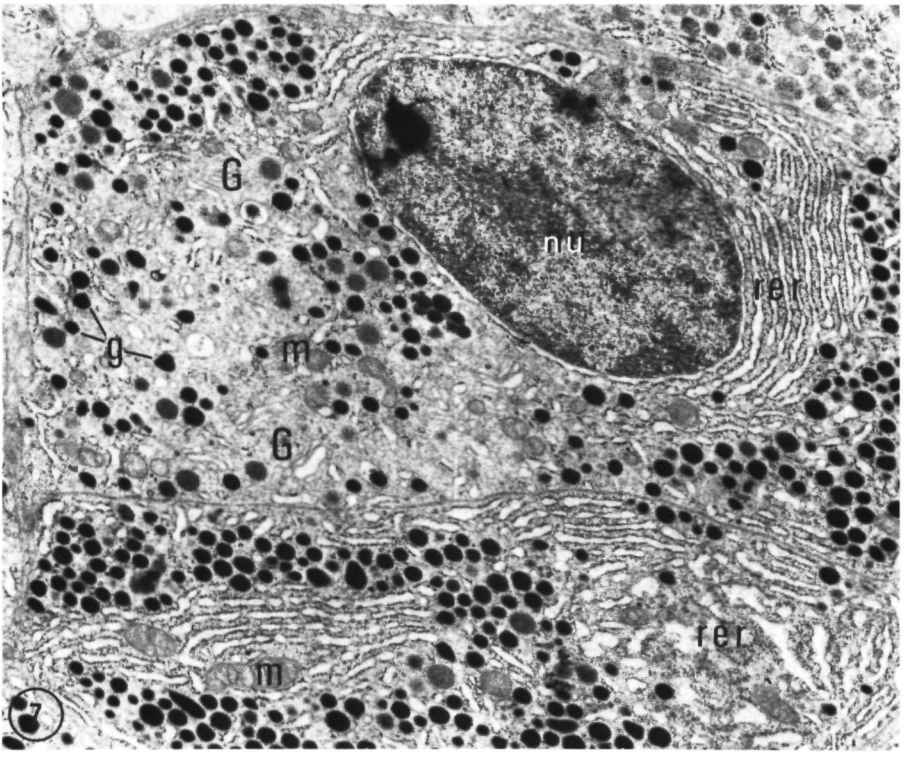

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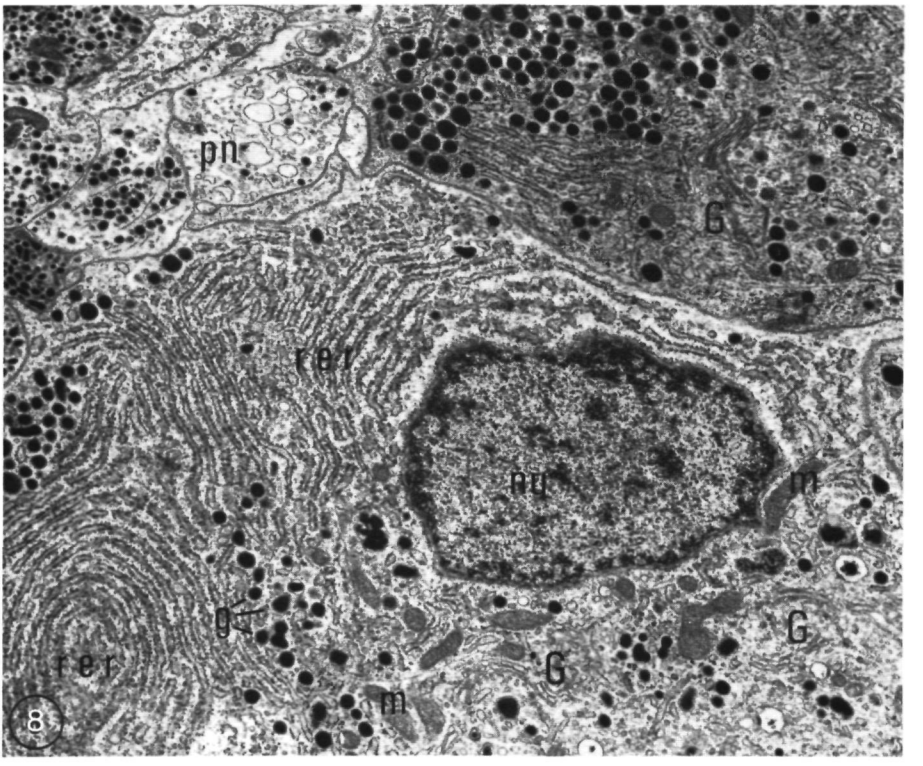

Fig. 8. PAS positive cells of a fish kept in total darkness. The rough endoplasmic reticulum as well as the Golgi areas are very extended, the number of granules in the cytoplasma is low. ×8,000

granule cells (about 10%). In the remaining groups these latter cells are only rarely seen. The total volume and number of the granules per unit area of cytoplasmic surface (Fig. 5g and h) do not correlate with data for the other cell parameters. The difference in granule size may be one of the factors responsible for this irregularity.

Discussion

The biochemical and physiological information on PAS positive cells is limited. Therefore determination of cellular and nuclear volumes and morphological analysis of the ultrastructure is an obvious approach to examine the function of this cell type. In this approach larger cellular and nuclear volume as well as higher

Fig. 6. PAS positive cells of a fish adapted to a white background under a normal day-night regime. Granules (*g*) and mitochondria (*m*) are small, the rough endoplasmic reticulum (*rer*) and Golgi apparatus (*G*) are relatively little developed; *ly* Lysosome; *nu* nucleus; *nt* nervous tissue. ×8,000

Fig. 7. PAS positive cells of a fish adapted to a black background under a normal day-night regime. Granules are relatively large. The rough endoplasmic reticulum and Golgi apparatus are well developed and the mitochondria have an active appearance. ×8,000

relative volume of mitochondria, rER and Golgi apparatus in the cytoplasm are considered to be indicative of a higher metabolic activity

The present results clearly show that the activity of PAS positive cells is much lower in group W than in all the other groups Groups W and B differ with respect to almost all parameters tested This suggests that background colour greatly influences the metabolic activity of these cells In a few light microscopic studies on other species similar effects of background colour on the activity of PAS positive cells have been reported (Ball and Baker 1970, Malo-Michele 1977) More widely examined and generally accepted, however, is the relationship between background colour and activity of the MSH cells (Baker 1965, 1972, Olivereau 1971, 1972, Thornton and Howe 1974) In *S. mossambicus* PAS positive cells and MSH cells show similar changes in cellular activity in relation to background colour (Van Eys 1980)

The results for groups B and P indicate that the effects of prolonged illumination, or a lack of day-night rhythm, are of minor, if any, significance in comparison with background colour These results are at variance with those of Malo-Michele (1977), who has reported an increase in cellular activity of PAS cells under permanent light conditions in *Boops salpa*

Fish of groups Bl and BlD cannot transfer information about the light reflected by the background The activity of the PAS positive cells of these groups is similar to those of the groups B and P Thus, the absence of reflected light and the inability to transfer information about the reflected light, result in a similar activity of the PAS positive cells

The data of group D show that absence of direct as well as reflected light causes the highest activity of the PAS cells, but only if the optical nerves are intact. Removal of the pineal organ does not change the activity of the PAS positive cells of animals kept in total darkness (unpublished results) The difference between the groups BlD and D points to a possible physiological involvement of the eyes in regulation of the activity of the PAS positive cells

The present data indicate that reflected light received by the eyes decreases the activity of the PAS positive cells Sensory information from the pineal organ (direct light) does not appear to influence these cells In contrast, the activity of the MSH cells has been found to be influenced by sensory information from both eyes and pineal organ (Van Eys 1980)

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CHAPTER 3

Structural changes in the pars intermedia of the cichlid teleost Sarotherodon mossambicus as a result of background adaptation and illumination.
III: The role of the pineal organ.

Summary

The MSH producing cells in the pars intermedia of Sarotherodon mossambicus have been shown to be involved in background adaptation processes. Reflected light received by the eyes affects the activity of these cells. In the present study the hypothesis was tested that also the pineal organ, as a second photoreceptor, is involved in regulation of the metabolic activity of the MSH cells. The pineal organ appeared to contain photoreceptor cells and is considered to be capable of transferring information about light conditions to the animal. Removal of the pineal organ of fish kept on a black background had no effect on activity of MSH cells, whereas the activity of these cells in fish kept in darkness was increased. Thus it seems that the pineal organ exercises its influence on MSH cells only in darkness and that this influence results in a reduced activity of these cells. It is therefore concluded that the metabolic activity of MSH cells is inhibited not only by reflected light received by the eyes, but also by the action of the pineal organ as a result of the absence of illumination.

No structural signs of secretory activity were observed in the pineal, which might indicate synthesis or release of substances like melatonin. However, administration of melatonin reduced the activity of MSH cells. Neither pinealectomy nor treatment with melatonin had influence on the second cell type of the pars intermedia, the PAS positive cells.

Introduction

The pars intermedia of teleosts contains two distinct endocrine cell types, the predominant MSH producing cells and the PAS positive cells. In Sarotherodon mossambicus the MSH cells are activated in fish on a black background and have low metabolic activity in fish on a white background. In darkness, the activity is also reduced. In blinded fish on a white background and under normal day-night conditions, the activity of the MSH cells is as high as in black background adapted fish, whereas activity is low when blinded fish are kept in darkness (Chapter 1). From these findings it was concluded that MSH cells are activated by a high ratio between reflected light (background) and direct light (illumination), rather than by the absence of reflected

light. The results obtained with blinded fish point towards the existence of a second photoreceptor involved in the regulation of metabolic activity of MSH cells. As such a second photoreceptor the pineal organ has been suggested (Chapter 1).

The PAS positive cells react to background colour in a similar way as the MSH cells. In darkness these cells are extremely active and in this respect they differ from the MSH cells. Blinding resulted in a state of activity similar to that of black background adapted animals, and a role of the pineal organ in the control of activity of these cells was considered unlikely (Chapter 2).

The pineal organ of several teleost species has been shown to contain photoreceptor-like structures. Electron microscopic observations provided indirect evidence for photoreceptivity (Herwig, 1976, 1980; McNulty, 1978a,b; Bergmann, 1971; Ueck et al., 1978). Morita (1966), working with Salmo irideus, provided electrophysiological evidence for photoreceptive properties of the pineal organ. In this last study it was demonstrated that, like in other lower vertebrates, light perception by the pineal organ reduced the activity of this organ.

In the present study the effects of pinealectomy on the activity of MSH and PAS positive cells of the pars intermedia of Sarotherodon mossambicus were investigated. On the basis of electrophisiological and biochemical studies that report an activation of the pineal organ in the absence of light (Morita, 1966; Smith and Weber, 1974, 1976; Joss, 1977; Vodicnik and De Vlaming, 1978), pinealectomy was performed in fish under different light regimes, namely complete darkness and normal day-night conditions. The effects of administration of melatonin were studied as it has been suggested that the pineal organ of teleosts contains melatonin (Fenwick, 1970). Moreover, in several reports a relationship between melatonin and melanophore aggregation was described (Ruffin et al., 1969; Reed et al., 1969). However, in adult teleosts colour change appears to be unaffected by treatment with melatonin (Abbott, 1973; Bagnara and Hadley, 1973; Kavaliers et al., 1980).

Materials and methods.

Sexually mature males of the cichlid teleost Sarotherodon mossambicus with a body length between 10 and 12 cm and a body weight varying from 9 to 12 g were used. Each experimental group consisted of five fish.

During an adaptation period of 14 days animals were kept separately in buckets containing about 6 l of tap water. Feeding and water replacement were done every other day. Room temperature was about 24°C. The groups were placed under one of the following regimes:

Group B: non-reflecting black background with normal day-night rhythm (12 h light/12 h darkness).

Group EXB: fish were pinealectomized by removal of a part of the meninges and the underlying pineal organ; the operated fish were kept on a black background as described under B. After killing the fish and dissection of the pituitary gland, the head was fixed and embedded in paraplast, and sections were examined with the light microscope to ascertain whether the pineal organ had been removed.

Group D: non-reflecting black background in total darkness.

Group EXD: pinealectomy as described for group EXB. Operated fish were kept in total darkness as described under D.

Group MB: non-reflecting black background as described for group B. An Alzet osmotic minipump, model 1701 (Alza, Palo Alto), was implanted in the abdomen. The minipump contained 170 µl of an aqueous solution of synthetic melatonin (1 mg/ml). The minipump released approximately 0.7 µl of fluid per hour over a period of ten days. The presence of hormone-free minipumps has no influence on the metabolic activity of MSH and PAS positive cells (Chapter 4).

At the end of the adaptation period fish were anaesthetized with MS 222 (Sandoz) and killed. Pituitary glands of all experimental fish were quickly removed and fixed. Method of fixation for electron microscopy as well as the procedure for morphometric analysis of the ultrastructure of MSH and PAS positive cells have been described in detail elsewhere (Chapter 1).

Results

Morphology of the pineal organ. Light microscopic observations on sagittal sections of the head showed the pineal organ as a long stalk with a discus-like pineal body attached to the meninges (Fig. 1). The stalk originates caudally from the telencephalon and extends almost vertically to the roof of the cranial cavity. At the place where the pineal organ is attached to the meninges a window-like thin area in the bony skull is observed. The pineal organ is surrounded

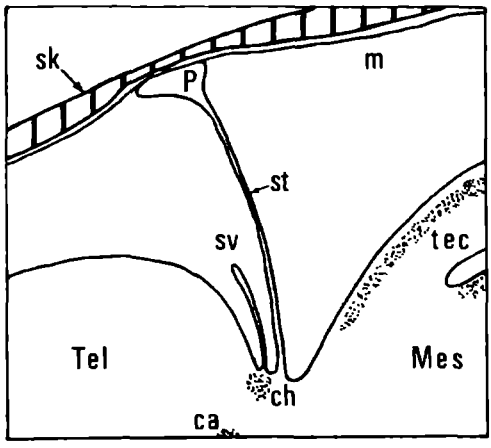

Fig. 1: Diagram of the position of the pineal organ in the head of Sarotherodon mossambicus. sk: skull; m: meninges; P: pineal organ; st: stalk of the pineal organ; s.v.: saccus vasculosus; tec: tectum of mesencephalon; Mes: mesencephalon; Tel: telencephalon; ch: commissura habenula; ca: commissura anterior.

by a capsule of connective tissue and a network of blood capillaries. Electron microscopic examination showed that the pineal organ is composed of three cell types: supporting cells, neurosensory receptor cells bearing an outer segment and nerve cells (Fig. 2). The supporting cells have a glia-like appearance. Their nucleus is often oval in shape and the electron translucent cytoplasm contains mitochondria, poorly developed endoplasmic reticulum (rER), Golgi areas and polysomes. The most remarkable part of the sensory cell is the outer segment. It consists of a stack of 28-38 parallel doubly folded membranes that are invaginations of the ciliary membrane (Fig. 3 and 4). This stack is connected to the head of the sensory cell by two cilia (Fig. 5). The cell body of the sensory cell contains many large mitochondria, rER consisting of short cisternae, and relatively large Golgi areas. Dense cored granules, that could be indicative of secretory activity were not observed in these cells. Nerve cells were scarce but when present were located in the periphery of the organ and were characterized by electron translucent cytoplasm. Nerve fibers were observed in the proximal part of the stalk.

Effects of experimental manipulation on the pars intermedia cells. Influence of background, pinealectomy and administration of melatonin on the metabolic activity of the pars intermedia cells were investigated by morphometric analysis. In MSH as well as PAS positive cells high relative cytoplasmic volume for mitochondria, rough endoplasmic reticulum and Golgi area as well as low relative cytoplasmic volumes for the

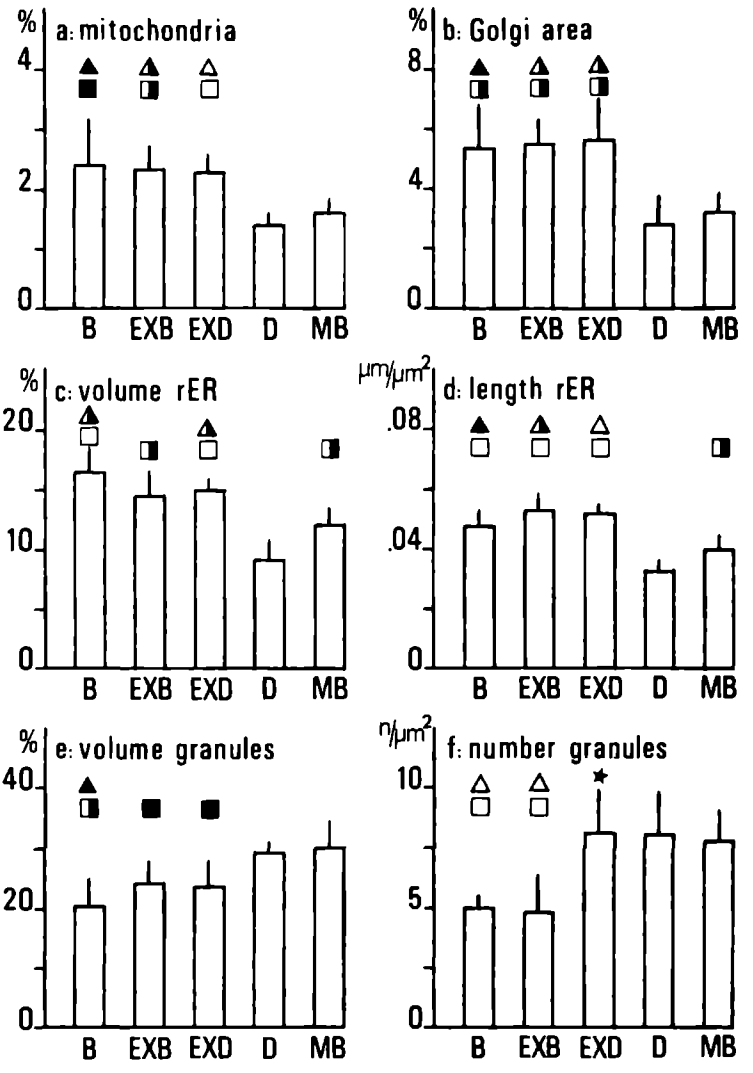

Fig. 6: Morphological analysis of MSH cells of fish adapted to a black background, under normal day-night rhythm (B); total darkness (D); pinealectomized on a black background (EXB); pinealectomized in total darkness (EXD); on a black background with melatonin treatment (MB). Statistical significance is indicated by symbols above the bars: Triangles, significantly different from group MB (△: $p<0.001$; ◭: $p<0.01$; ▲: $p<0.05$). Squares, significantly different from group D (□: $p<0.001$; ◨: $p<0.01$; ■: $p<0.05$). Star, significantly different from groups B and EXB ($p<0.01$).

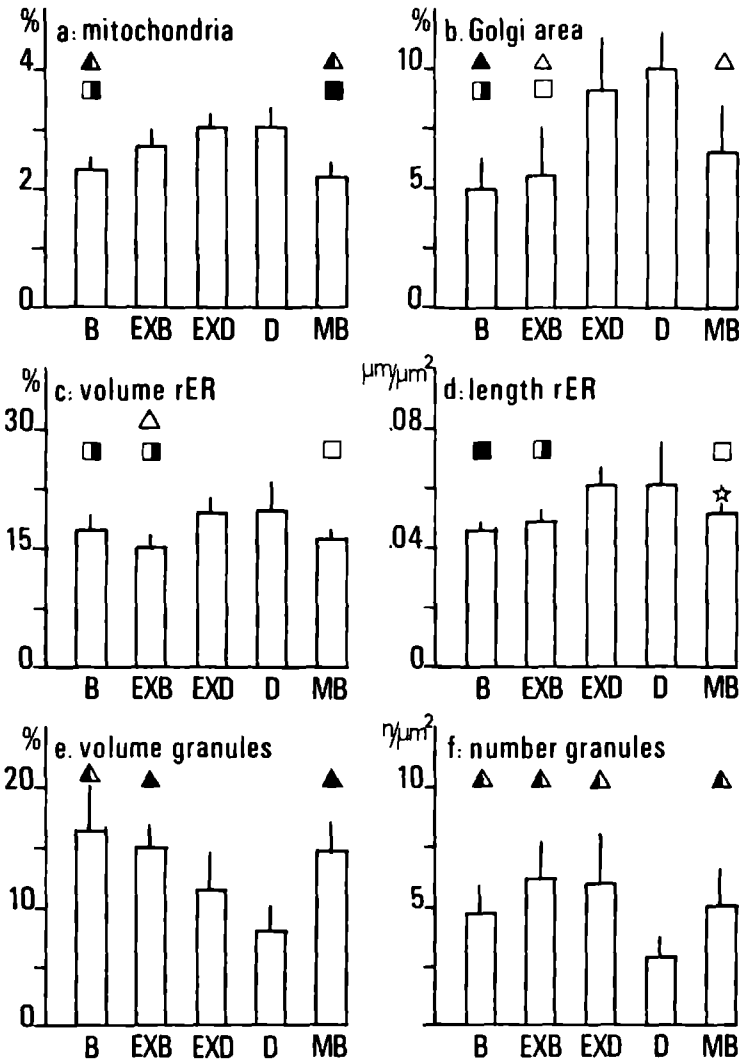

Fig. 7: Morphological analysis of PAS positive cells of fish adapted to a black background, under normal day-night rhythm (B); total darkness (D); pinealectomized on a black background (EXB); pinealectomized in total darkness (EXD); on a black background with melatonin treatment (MB). Statistical significance is indicated by symbols above the bars: Triangles, significantly different from group D (▲: $p<0.001$; ◭: $p<0.01$; △: $p<0.05$). Squares, significantly different from group EXD (■: $p<0.001$; ◨: $p<0.01$; □: $p<0.05$). Star, significantly different from group B ($p<0.05$).

granules were considered as indications for high activity.

Activity of the MSH cells of black background adapted fish was high, whether the fish were intact (group B) or pinealectomized (group EXB; Fig. 6). Activity of the MSH cells of fish kept in darkness (group D) was significantly lower than in black background adapted fish. However, pinealectomy of fish kept in darkness (group EXD) resulted in an activity of the MSH cells significantly higher than in fish of group D. The activity of group EXD was similar to that of fish of group B and EXB (Fig. 6).

Administration of melatonin to fish placed on a black background resulted in a decreased activity of the MSH cells when compared to untreated controls (group B).

Neither pinealectomy nor administration of melatonin influenced the activity of the PAS positive cells. The activity in group B did not differ noticeably from that in groups EXB and MB, similarly no significant differences were found between groups D and EXD (Fig. 7).

Discussion

In a previous chapter a hypothesis for the regulation of the activity of MSH cells was suggested which involved a second photoreceptor in addition to the eyes (Chapter 1). It was stated that the ratio between reflected light received by the eyes and the intensity of illumination perceived by a second photoreceptor was the factor determining the activity of the MSH cells. A low ratio was correlated with low activity and a high ratio with high activity. The pineal organ was suggested to be that second photoreceptor.

Our observations of the pineal organ of Sarotherodon mossambicus show that its general structure corresponds with that of most teleosts investigated so far (Bergmann, 1971; Herwig, 1976, 1980; Ueck et al., 1978; McNulty, 1980a,b). The presence of well developed photoreceptor cells indicates that the pineal organ is capable of converting received light stimuli into nervous or endocrine signals. The presence of nerve cells and the absence of dense-cored granules in the photoreceptor cells suggest a transfer of information by neural pathways.

Influence of the pineal organ on the activity of the MSH cells was only exercised in darkness. This may be deduced from the observed similarity in activity between intact and pinealectomized fish on a

black background on the one hand and the difference in activity between intact and pinealectomized fish in darkness. Such a conclusion is in line with considerable evidence in lower vertebrates, that shows a reduction of the pineal activity as a result of illumination (Morita, 1966; Smith and Weber, 1976; Firth et al., 1979; Vivien-Roels et al., 1979; Joss, 1977). The increased activity of the MSH cells observed after pinealectomy of S. mossambicus kept in darkness, demonstrates that the effect of the pineal organ on these cells is inhibitory.

Previously, it was reported that reflected light received by the eyes reduces the activity of the MSH cells (Chapter I). The present study shows that the pineal organ in the absence of light reduces the activity of these cells. Therefore, it is concluded that high activity of the MSH cells is the result of low signal transference from the eyes as well as from the pineal organ (Fig. 8). Activation of either the eyes by reflected light from a bright background, or of the pineal organ, by lack of illumination, results in a reduced activity of the MSH cells.

Melatonin reduces the activity of the MSH cells. This might indicate that the pineal influence on the MSH cells is mediated by melatonin. However, the electron microscopic observations revealed no signs of endocrine activity. This result is similar to that of other structural studies (Bergmann, 1971; Ueck et al., 1978; Herwig, 1976, 1980). Fenwick (1970) demonstrated the presence of melatonin in salmon pineal organ, a finding that is supported by some other studies that show a disruption of diurnal variations in plasma melatonin levels after pinealectomy (Delahunty et al., 1978; Vodicnik and De Vlaming, 1978). In addition, melatonin has been shown to have pigment aggregating properties in vitro and is suggested to control the changes between nocturnal and diurnal pigmentation patterns in fish (Reed et al., 1969; Kavaliers et al., 1980; Hafeez and Quay, 1970; Joss, 1973; Ruffin et al., 1969; Finnin and Reed, 1970). These investigations suggest that melatonin is able to influence skin melanophores directly. However, the present results suggest that the influence of melatonin during background adaptation is mediated by the MSH cells. This conclusion is supported by the lack of correlation between plasma melatonin levels and background adaptation observed in trout (Owens et al., 1978). In addit addition, in Fundulus heteroclitus pinealectomy as well as hypophysectomy abolish the rhythmic colour changes without affecting physiological

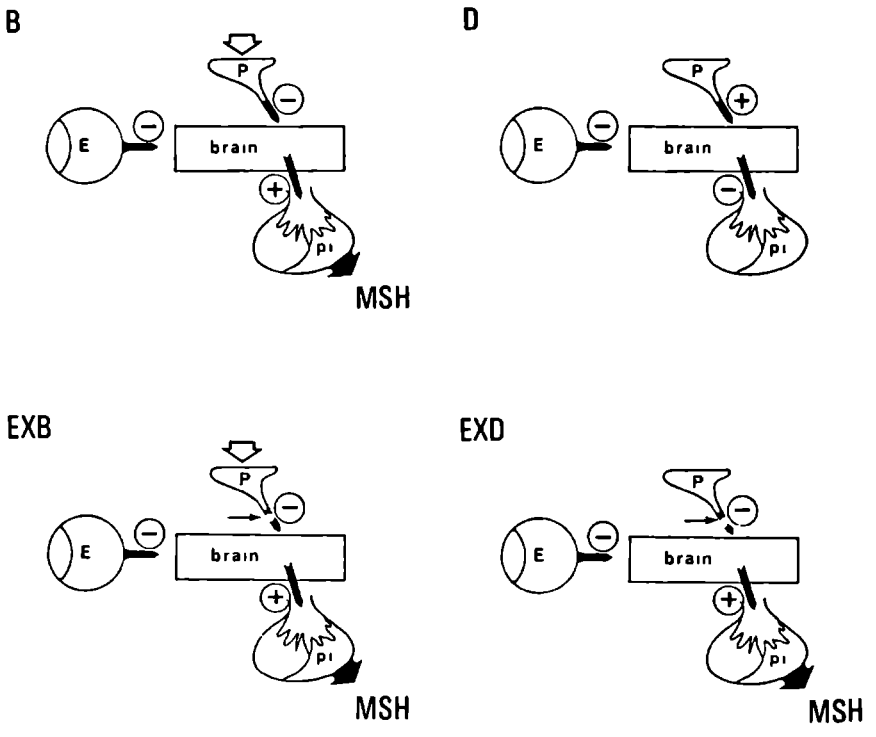

Fig. 8: Suggested regulatory mechanism of the MSH cell activity. On a black background light is received by the pineal organ but not by the eyes (B and EXB). MSH cells are active whether the pineal organ was present or not. In darkness light is not received by the eyes nor by the pineal organ (D and EXD). MSH cells are inactive when the pineal organ is intact, but active when the fish is pinealectomized. Thus the pineal organ exercises its influence on the MSH cells only in darkness. The influence has a negative effect on the activity of the MSH cells. In chapter 1 it was concluded that light received by the eyes (reflectance via a white background) has a negative influence on the activity of the MSH cells. Thus it is concluded that the absence of light reception by the eyes results in an activation of the MSH cells, but only when the activity of the pineal organ is reduced by the presence of direct light. E: eye; P: pineal organ; pi: pars intermedia.

background adaptation processes (Kavaliers and Abboth, 1977; Kavaliers et al., 1980).

Removal of the pineal organ had no influence on the activity of the PAS positive cells of Sarotherodon mossambicus. Thus, the effect of background and darkness on these cells is most likely mediated by the eyes only.

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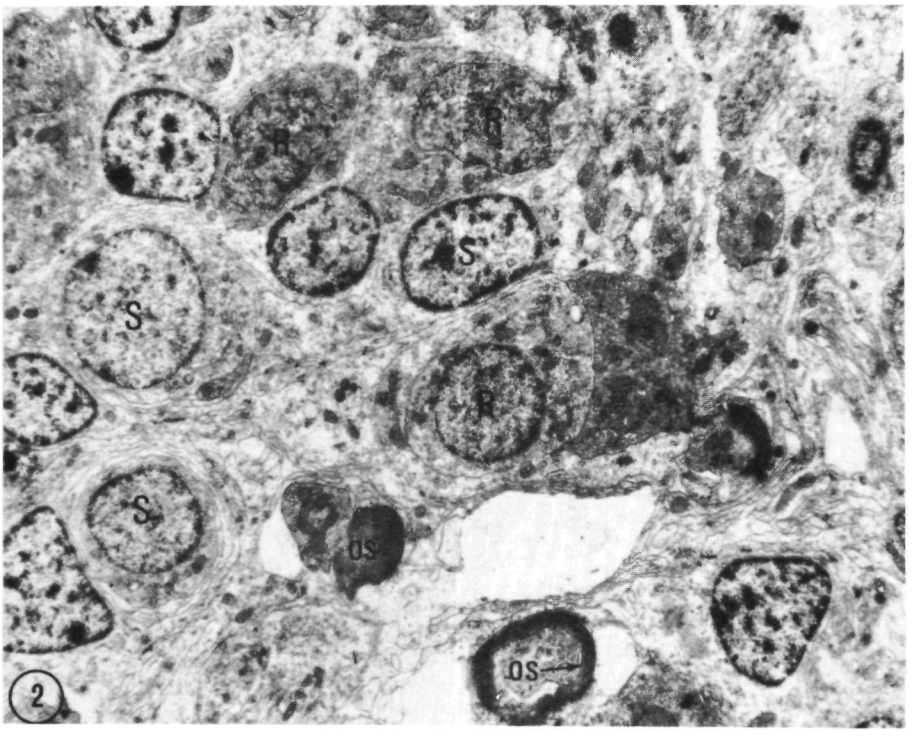
Fig. 2. Electron micrograph of the pineal organ of Sarotherodon mossambicus. S: supporting cells; R: neurosensory receptor cells; os: outer segment. x4000

Fig. 3. Electron micrograph of a neurosensory receptor cell. nu: nucleus; G: Golgi area; m: mitochondrium; os: outer segment. x8000

Fig. 4. Electron micrograph of the outer segment of a neurosensory receptor cell. x40.000.

Fig. 5. Electron micrograph of the cilia that connect the outer segment with the head of the neurosensory receptor cell. x40.000



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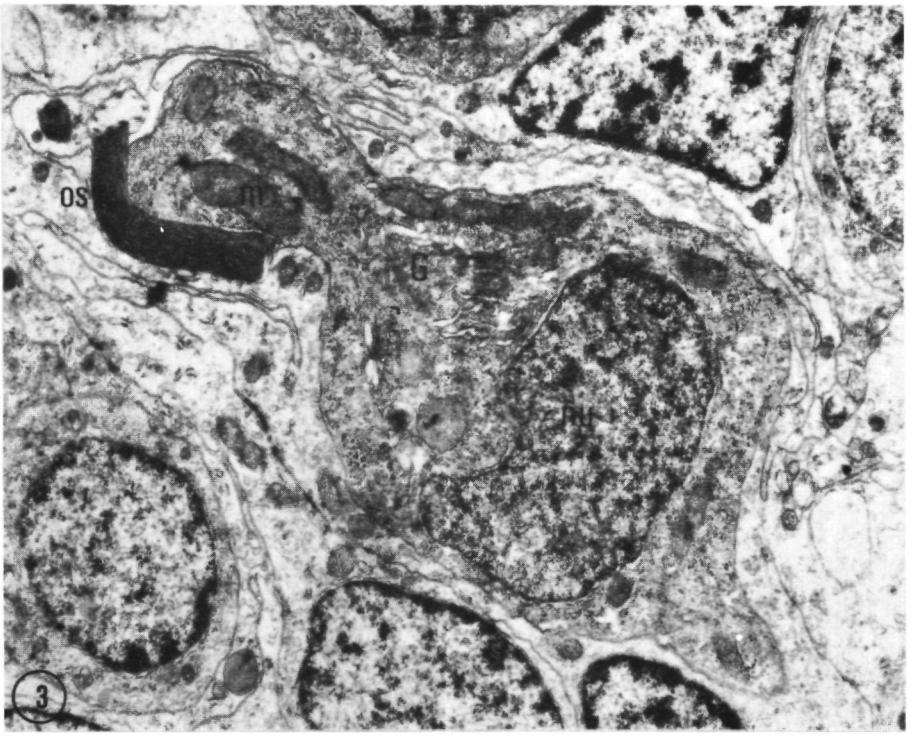

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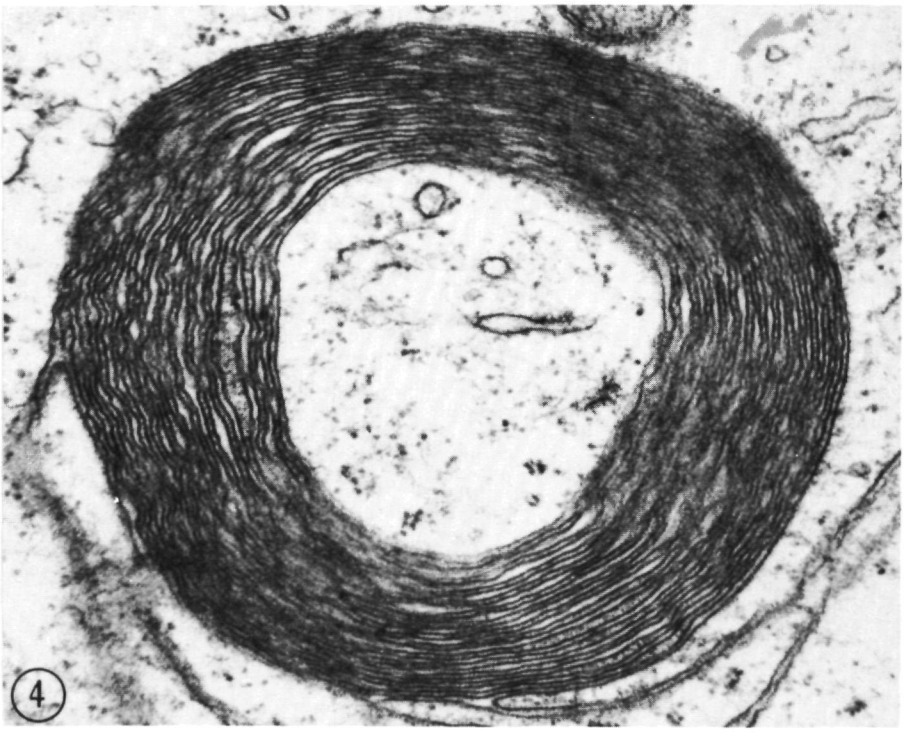

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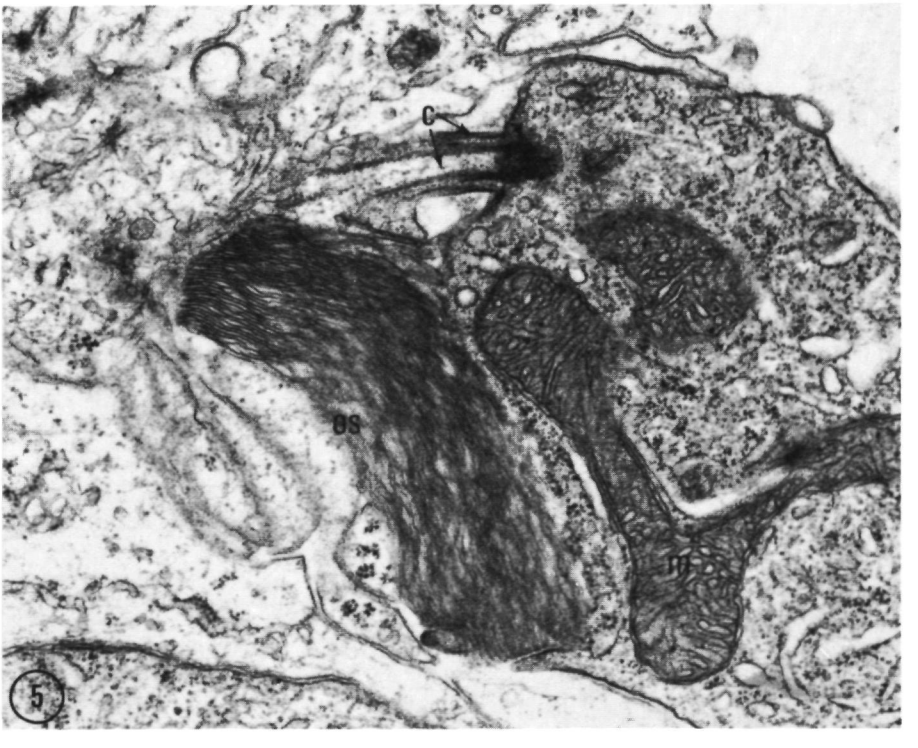

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CHAPTER 4

Evidence for a direct role of α-MSH in morphological background adaptation of the skin in *Sarotherodon mossambicus*.

Summary

The skin colour of the cihclid teleost Sarotherodon mossambicus adapted rapidly to changes in background colour. The physiological adaptation was associated with morphological changes in the dermis. Differences in the dermis were found between fish adapted to a black or white background for 14 days. Number and size of the melanophores as well as the amount of pigment in the cytoplasm of the melanophores were significantly increased in fish adapted to a black background. Changes in the dermis paralleled changes in the state of activity of the two endocrine cell types in the pars intermedia of the pituitary. Both the PAS positive cells and the MSH producing cells were more active when the fish were exposed to a black rather than a white background. Fish continuously infused with α-MSH, using an osmotic minipump, had more melanophore cytoplasm and pigment per dermis surface unit area than untreated fish. The activity of the MSH cells in MSH-infused fish exposed to a black background was reduced to a level comparable to the MSH cell activity of untreated fish on a white background. α-MSH treated fish that were exposed to a white background had many desintegrating MSH cells. These findings point to inactivation of these cells by exogenous α-MSH. The activity of the PAS positive cells was not influenced by treatment with α-MSH.

Introduction

Background adaptation in many teleosts is regulated by a dual control mechanism. The adaptation involves a physiological response, aggregation and dispersion of pigment, that is usually triggered by neural stimuli (Abboth, 1973; Novales, 1973). In addition, a morphological response, changes in the number of melanophores and the amount of pigment, is observed and appears to be under hormonal control (Pickford and Kosto, 1957; Odiorne, 1956; Waring, 1963). Thus, in teleosts, in contrast to amphibians, background adaptation is often regulated by both neural and hormonal processes. Therefore the melanophore index (Hogben and Slome, 1936) gives no adequate information about the metabolic activity of the pars intermedia.

The teleost pars intermedia contains two endocrine cell types. The lead haematoxyline positive, MSH producing cell type is predominant.

The nature and function of the secretory product of the other cell type, which is stainable with periodic acid-Schiff (PAS), is unkown. Changes in background colour have been reported to affect MSH cells (Baker, 1972; Olivereau, 1971, 1972; Thornton and Howe, 1974) as well as the PAS positive cells (Baker and Ball, 1970; Malo-Michele, 1977). Results from studies using injections of extracts of mammalian pars intermedia indicate that products of MSH cells stimulate melanogenesis and the formation of melanophores (Pickford and Kosto, 1957; Kosto et al., 1959).

In Sarotherodon mossambicus both MSH cells and PAS positive cells show a higher metabolic activity when the fish are exposed to a black rather than a white background (Chapter 1 and 2). In the present study we examined to what extent these higher activities are paralleled by morphological changes in the dermis. In addition the effects of exogenous α-MSH on dermis and pars intermedia endocrine cells were measured.

Materials and Methods

Sexually mature male cichlid teleosts (Sarotherodon mossambicus) with a length between ten and fifteen cm and a body weight between ten and twenty g were used. Forty fish were divided into four groups of ten fish each. During the adaptation period the fish were kept in buckets containing about six liter of tap water. Every two days, the animals were fed and the water was refreshed. Illumination was provided by 60W TL tubes suspended 50 cm above the water surface. Temperature of the water was about 24°C. The following experimental groups were used.

Group W: 14 days exposed to a white background with normal day-night rhythm (12 h light/12 h darkness).

Group B: 14 days exposed to a non-reflecting black background with normal day-night rhythm.

Group WP: 10 days exposed to a white background with normal day-night rhythm; at the start of the experimental period an Alzet osmotic minipump, model 1701, (Alza, Palo Alto) was implanted in the abdomen of the fish. The minipump contained 170 µl of an aqueous solution of synthetic α-MSH (Peninsula, San Carlos) (1 mg/ml). The minipump released approximately 0.7 µl of fluid per hour (see below).

Group BP: 10 days exposed to a non-reflecting black background with normal day-night rhythm. In animals of this group an Alzet osmotic minipump was implanted as described above.

At the end of the adaptation period the fishes were anaesthetized with MS 222 (Sandoz) and sacrificed. Pituitary glands and parts of the skin of the lower jaw were quickly removed and fixed. Skin parts and five pituitaries of each group were prepared for electron microscopy. The five remaining pituitaries of each group were fixed for light microscopy. A full description of the fixation procedures has been published elsewhere (Chapter 1).

For each animal micrographs of at least 15 randomly chosen MSH cells and 15 PAS positive cells (final magnification x8,000), as well as micrographs of 5 cross-sections of the dermis (final magnification x15,000), were morphometrically analyzed with a Kontron Digiplan. For cells of the pars intermedia the following parameters were taken:

1. Total volume of the mitochondria as a percentage of the cytoplasmic volume.
2. Total volume of the Golgi area as a percentage of the cytoplasmic volume.
3. Total volume of the rough endoplasmic reticulum as a percentage of the cytoplasmic volume.
4. Total length of the membranes of the rough endoplasmic reticulum per unit area of the cytoplasmic surface.
5. Total volume of the granules as a percentage of the cytoplasmic volume.
6. The number of secretory granules per unit area of the cytoplasmic surface.

For technical reasons, ultrastructural studies of the dermis were performed on the scale-less skin of the lower jaw. The dermis was cut in a plane perpendicular to its surface. The sections were used to determine the following parameters:

1. Total volume of the melanophores as a percentage of the dermis volume.
2. Total volume of the melanin granules as a percentage of the cytoplasmic volume of the melanophores.
3. Total volume of the melanin granules as a percentage of the dermis volume.
4. Total volume of the guanophores as a percentage of the dermis volume.

For the experimental groups the means of the above mentioned parameters of pars intermedia, as well as dermis, were statistically analysed by means of the two-tailed Student's t-test. No corrections were made for the Holmes effect because errors due to this effect were considered to

affect all experimental groups in the same way (Weibel and Paumgartner 1978).

To determine the rate and duration of release of the hormone solution at 24°C, Alzet osmotic minipumps, model 1701, were implanted in the abdomen of eight Xenopus laevis. Four of the minipumps contained a solution of synthetic α-MSH of the same concentration as used in fish of groups WP and BP. The other four minipumps were empty. The animals were exposed two by two, one experimental and one placebo animal, to a white background. The melanophore index (m.i.; Hogben and Slome 1936) was monitored over at least ten days. After ten days the remaining content of two minipumps was collected and subsequently analysed by means of a Spectra Physics high-performance liquid chromatograph (model SP 8000) equipped with a Spherisorb 10 ODS stainless-steel column.

Results

The pilot experiment done on Xenopus leavis, showed that implantation of the Alzet osmotic minipump has no influence on the melanophore index (m.i.) or behaviour of the animals. After implantation of placebo minipumps the m.i. did not change. Animals with a placebo minipump had a m.i. of either 1 or 2. Implantation of an α-MSH containing minipump resulted in a rise of the m.i. up to 5 within a few hours. During the next 12 days the m.i. did not change. High-performance liquid chromatography analysis showed that fluid collected from the minipumps after 10 days had a profile identical to that of a freshly prepared α-MSH solution (Fig. 1). These findings demonstrate that the minipumps released α-MSH for at least 10 days under the given experimental conditions and that α-MSH in the minipumps was stable over this period.

Skin Colour. After a change in backround, fish started to adapt their colour to the new background by a fast colour change in about 30 sec. Adaptation was complete within 1 week. When exposed to a white background (group W) fish became pale. Treatment with α-MSH (group WP) had only a slight effect on skin colour; animals of this group were somewhat greyish, especially at the rim of caudal, pectoral and dorsal fins. When exposed to a black background fish became dark in colour (group B). Under this condition, α-MSH had no visible effect on skin colour (group BP).

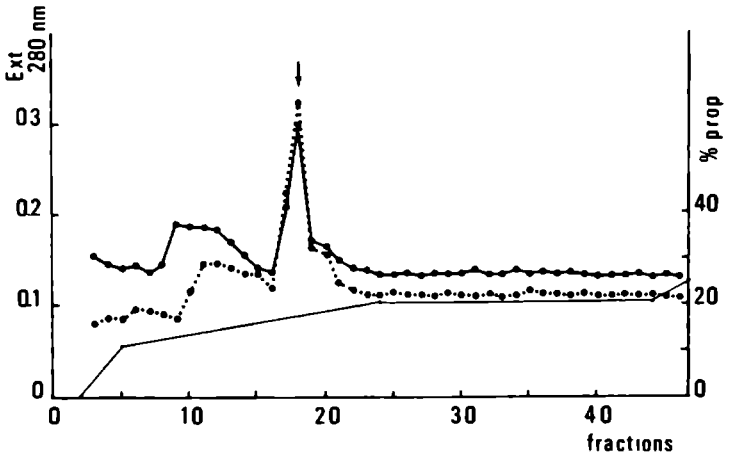

Fig. 1. High-performance liquid chromatogram of a freshly prepared α-MSH sample (—•—•—•—) and a sample of the remaining fluid from the mini-pump (••••••••••••••). Separation on Spherisorb 10 ODS with a flow rate of 2 ml/min; fractions were collected every 30 seconds. Primary solvent: 0.5 M formic acid -0.14 M pyridine (pH 3.0). Secondary solvent: 1-propanol, α-MSh peak is indicated by arrow.

Changes in the Dermis. Pigment disappeared completely from scales of fish of group W (Figs. 2, 3). Ultrastructural observations in the dermis of the lower jaw revealed that melanophores and guanophores are embedded between bundles of collagenous fibers, mostly close to the basal lamina (Figs. 5, 6). A number of chromatophores occur as melanophore-guanophore complexes (Fig. 4), in which the central part of the melanophore cell is surrounded by guanophores, while dendrite-like processes surround the guanophores. The results show that in black adapted animals (group B and BP) these processes are often filled with melanin granules while in fishes of group W and WP this is only rarely observed. Electron microscopic and morphometrical analyses revealed considerable differences in composition of chromatophores in the dermis between the experimental groups. The relative volume of melanophores and melanin was highest in fish of group BP, somewhat lower in the groups B and WP and significantly lower in group W (Figs. 5, 6, 7). The relative volume of the guanophores was high in the groups W and WP, but low in groups B and BP (Fig. 7).

Changes in the Pars Intermedia. A more detailed description of the pars intermedia of Sarotherodon mossambicus and its two endocrine cell types has been given in a previous paper (Van Eys 1980a). Cellular and nuclear volumes of both MSH and PAS positive cells were large in animals of group B. In this group the relative volumes of mitochondria, Golgi apparatus and rER were also high (Fig. 12). All these values were lower in animals from group W. In fish from group BP the MSH cells were similar to those of group W, while the PAS positive cells were similar to those of group B (Fig. 12). The PAS positive cells in the pars intermedia of animals of group WP looked like those in group W. Of the MSH cells from the WP group, however, a great number of cells (30 ± 3%) were in a state of disintegration. For this rason no random sample could be taken for morphometrical analysis. In the disintegrating cells the nucleus was reduced in size, and showed marked condensation of the nuclear chromatin; the cytoplasmic matrix had an increased electron density and the number of lysosomes was markedly higher. The MSH cells in animals of group WP that showed no signs of desintegration had an appearance similar to those of the groups W and BP.

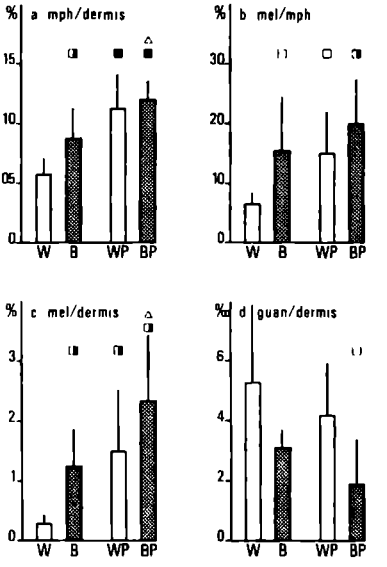

Fig. 7. Morphometrical analysis of chromatophores in the dermis of fish exposed to a white background (W) or black background (B) and treated with α-MSH (WP and BP). Statistical significance is indicated by the symbols above the bars: Triangles, significantly different from group B (△: $p<0.05$); Squares, significantly different from group W (■: $p<0.001$; ◨: $p<0.01$; □: $p<0.05$).
mph melanophores; mel melanin; guan guanophores.

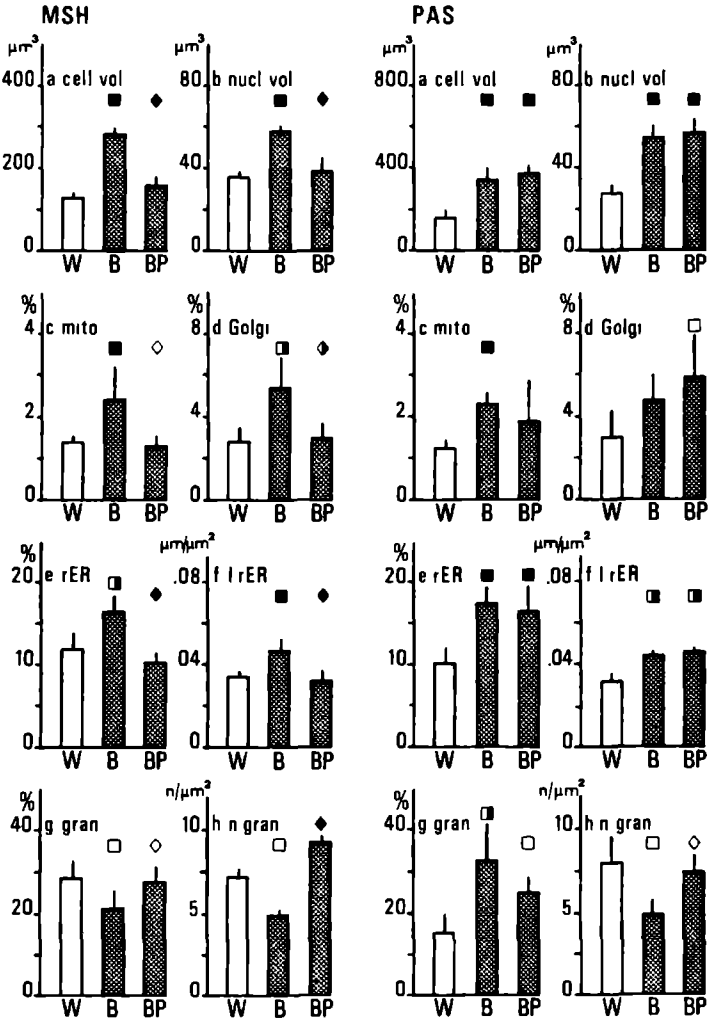

Fig. 12. Morphometrical analysis of the MSH and PAS positive cells of fish exposed to a white background (W) and to a black background without α-MSH treatment (B) and with α-MSH treatment (BP). Statistical significance is indicated by the symbols above the bars: Squares, significantly different from group W (■: $p<0.001$; ◨: $p<0.01$; □: $p<0.05$) Diamonds, significantly different from group B (◆: $p<0.001$; ◈: $p<0.01$; ◇: $p<0.05$).

Discussion

In Sarotherodon mossambicus change of background colour results in a rapid change in skin colour. The promptness of the adaptation process and the observation that, after a transverse cut in the caudal fin, this adaptation does not occur in the skin distal from the incision (unpublished results), are strong indications that physiological background adaptation is controlled by a neural mechanism. This conclusion is in line with findings in other teleosts, in which dispersion and aggregation of melanin in the melanophores have been reported to be under neural control (Fujii 1969; Abboth 1973). In this respect many teleosts differ from elasmobranchs (Waring 1963; Wilson and Dodd 1973) and amphibians (Hogben and Slome 1936; Fingerman 1970; Ide 1974) in which hormonal action is responsable for at least dispersion of pigment in the melanophores.

Apart from the well documented effect of background colour on physiological adaptation of skin colour in fish, a second, morphological adaptation process has been reported (Odiorne 1957; Waring 1963; Fujii 1969). The present study confirms, at an ultrastructural level, the existence of such a morphological adaptation process in Sarotherodon mossambicus, as evidenced by the observed quantitative changes in number of chromatophores and amounts of melanin and guanine in the dermis. A black background stimulates melanophore formation as well as melanogenesis. A white background results in more guanophore cytoplasm. Thus, development and breakdown of melanophores and guanophores are controlled by background colour. Our data further demonstrate that α-MSH is implicated in the process of morphological background adaptation, since treatment with α-MSH over a 10 day period results in an increase of melanophore cytoplasm and melanin, independent of background colour (Table 1). α-MSH has no significant effect on the guanophores.

As reported elsewhere (Chapter 1 and 2), MSH cells as well as PAS positive cells of the pars intermedia are most active in fish exposed to a black background. In most teleost species that have been studied, a relation has been demonstrated between background colour and the activity of the MSH cells (Baker 1972; Olivereau 1970; Thornton and Howe 1974). There are a few reports of a relation between background colour and the activity of the PAS positive cells (Baker and Ball 1970; Malo-Michele 1977). Results for groups WP and BP show that

Table 1. Summary of the main effects of background colour and administration of α-MSH on the secretory cells of the pars intermedia and on the chromatophores in the dermis of Sarotherodon mossambicus.

Group	Background	Administration -MSH	Amount melanin in the dermis	Amount guanine in the dermis	Activity MSH cells	Activity PAS cells
W	white	-	-	+	-	-
B	black	-	+	-	+	+
WP	white	+	+	+	--	-
BP	black	+	++	-	-	+

administration of α-MSH causes simultaneously an increase of melanophores and melanin in the dermis and a decrease in metabolic acitivity of the MSH cells in the pars intermedia. The activity of the PAS positive cells is not influenced by α-MSH (Table 1). This indicates that the MSH cells control melanophore formation and melanogenesis. The PAS positive cells are apparently not involved in these processes. However, the finding that the activity of the PAS positive cells was correlated with background colour in fish of all the experimental groups, indicates that a role of these cells in morphological background adaptation cannot be excluded. Therefore, the physiological significance of the influence of background colour on the PAS positive cells remains obscure.

A recent paper of Kawauchi and Muramoto (1979) showed that the amino acid sequence of α-MSH in the salmon Oncorhynchus keta is identical to the synthetic α-MSH used in this study. Synthetic α-MSH seems to have the same effect on melanophore formation and melanogenesis as the product of the MSH cells of Sarotherodon mossambicus, indicating a structural similarity of these compounds. Therefore, the inhibitory influence of exogenous α-MSH on the activity of the MSH cells in groups WP and BP may be due to the existence of a feedback control mechanism, responding to high levels of circulating α-MSH. Since α-MSH did not induce degeneration of the MSH cells of fish on a black background, the considerable number of degenerating cells in group WP must be contributed to the accumulative inhibitory effect of white background and exogenous α-MSH on these cells.

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Fig. 2. Light microscopic micrograph of a scale of fish exposed to a white background. No pigment can be seen. x280

Fig. 3. Light microscopic micrograph of a scale of a fish exposed to a black background. Melanophores are well developed and filled with pigment. x280

Fig. 4. Detail of a melanophore-guanophore complex in the dermis. Mel: melanophore; mel: melanin granules; Gua: guanophores; gua: guanine platelet; col: collageneous fibers; nu: nucleus; m: mitochondria. x13,500

Fig. 5. Dermis of the lower jaw of a fish exposed to a white background. Gua: guanophore; Mel: melanophore; col: collageneous fibers. x2,500

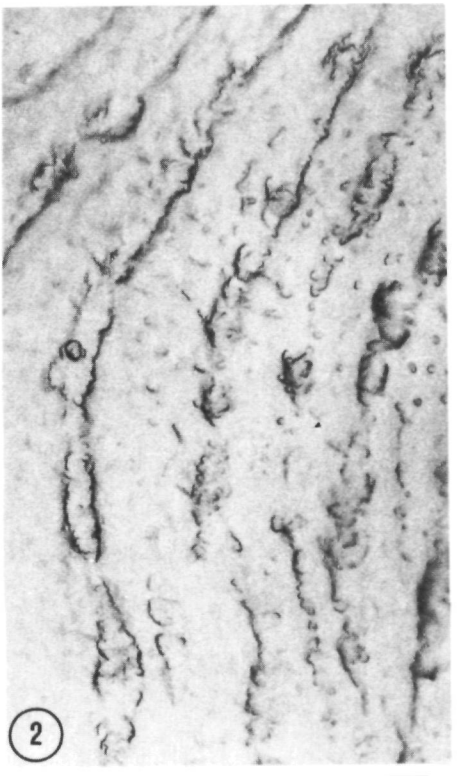
Fig. 6. Dermis of the lower jaw of a fish exposed to a black background and treated with α-MSH. Gua: guanophore; Mel: melanophore; col: collageneous fibers. x2,500

Fig. 8. MSH cells of a fish exposed to a black background and treated with synthetic α-MSH (group BP). Mitochondria (m) and Golgi area (G) are small, granules (g) are numerous. nu: nucleus; rER: rough endoplasmic reticulum. x7,200

Fig. 9. PAS cells of a fish exposed to a black background and treated with synthetic α-MSH (group BP). Rough endoplasmic reticulum (rER) and Golgi apparatus (G) are well developed and mitochondria (m) are numerous; c: centrioles; nu: nucleus; g: granula; l: lysosome; MSH: MSH cell. x7,200

Fig. 10. MSH cells of a fish exposed to a white background and treated with synthetic α-MSH. Many cells are in a state of disintegration (D). The intact cells are similar to those of fish of group BP and W. nu: nucleus; m: mitochondria; rER: rough endoplasmic reticulum; G: Golgi area; g: granula. x7,200

Fig. 11. PAS positive cell of a fish exposed to a white background and treated with synthetic α-MSH. The cells and the secretory granules are relatively small. nu: nucleus; m: mitochondria; G: Golgi area; g: granula; rER: rough endoplasmic reticulum; l: lysosome. x7,200



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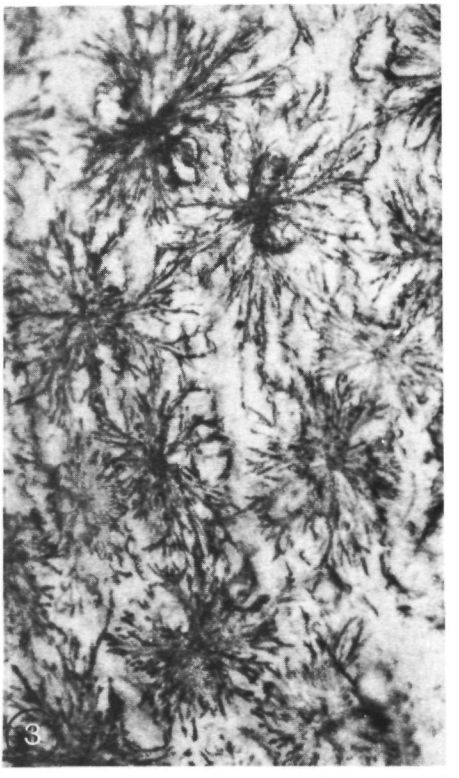

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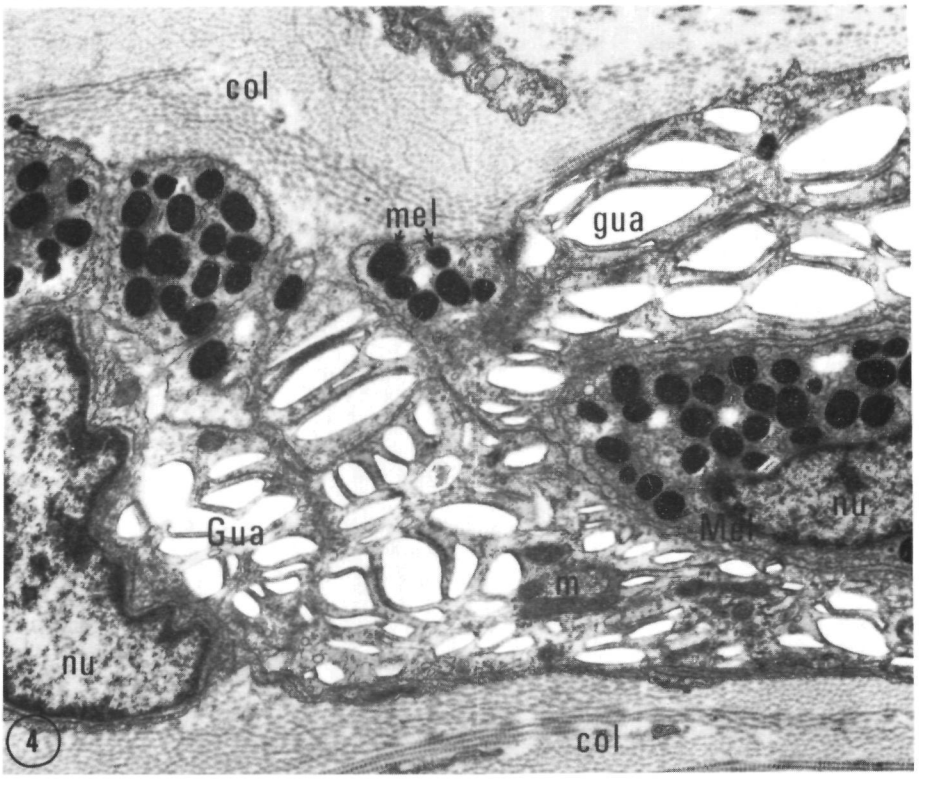

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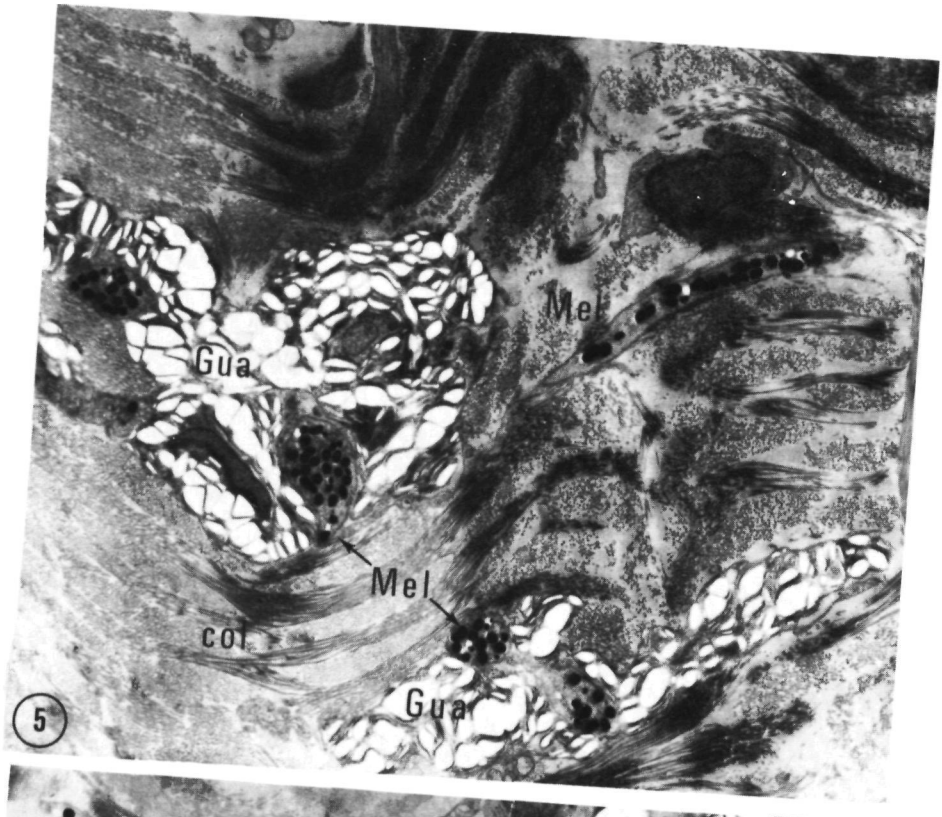
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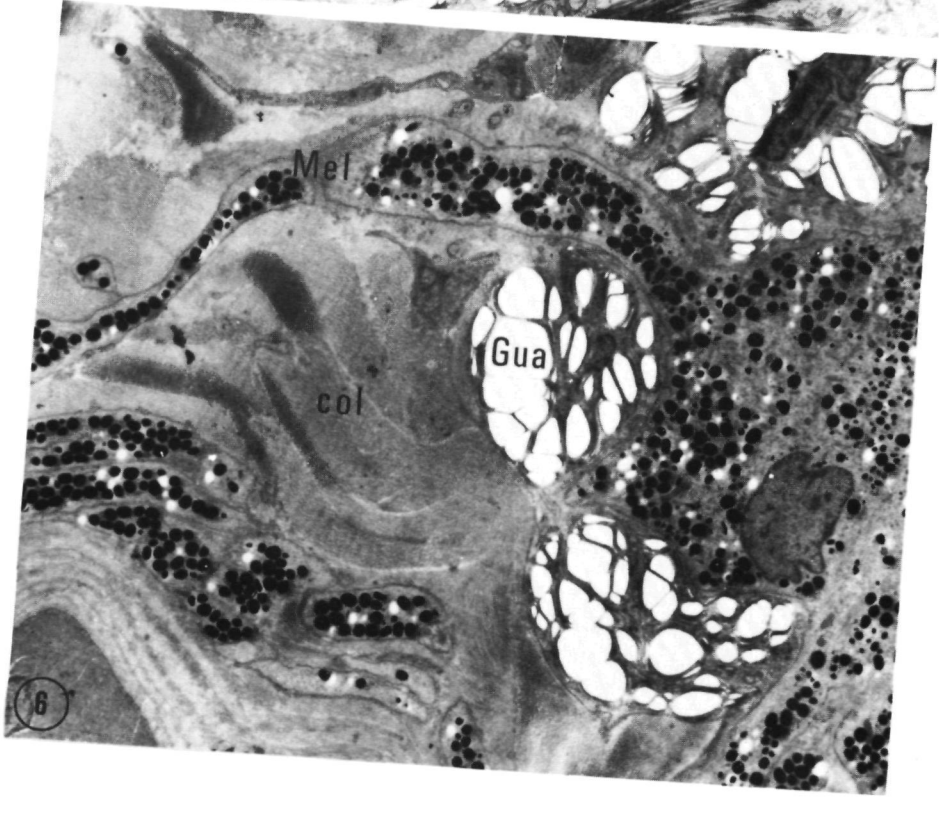
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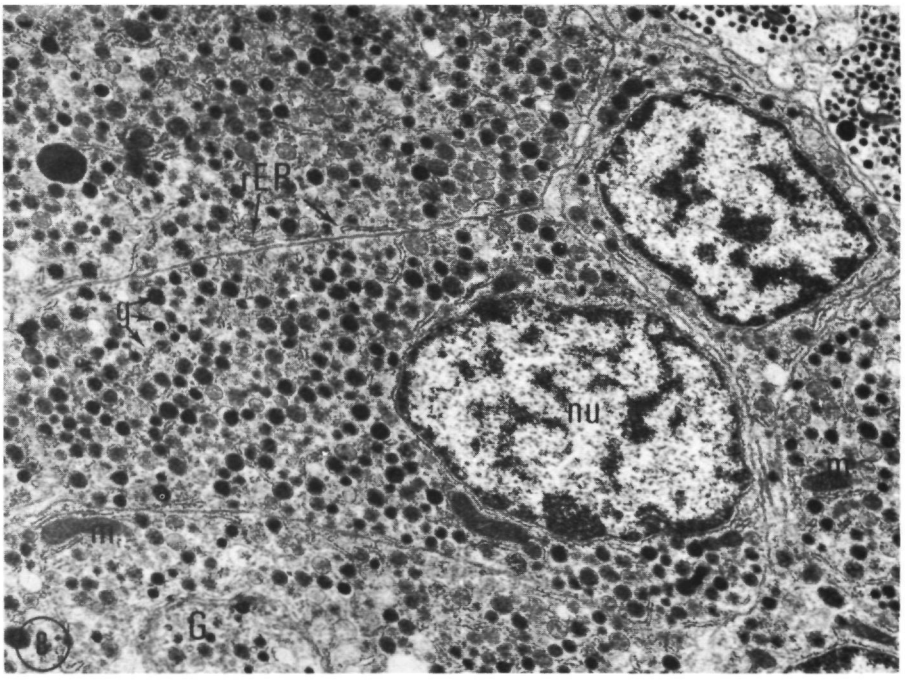
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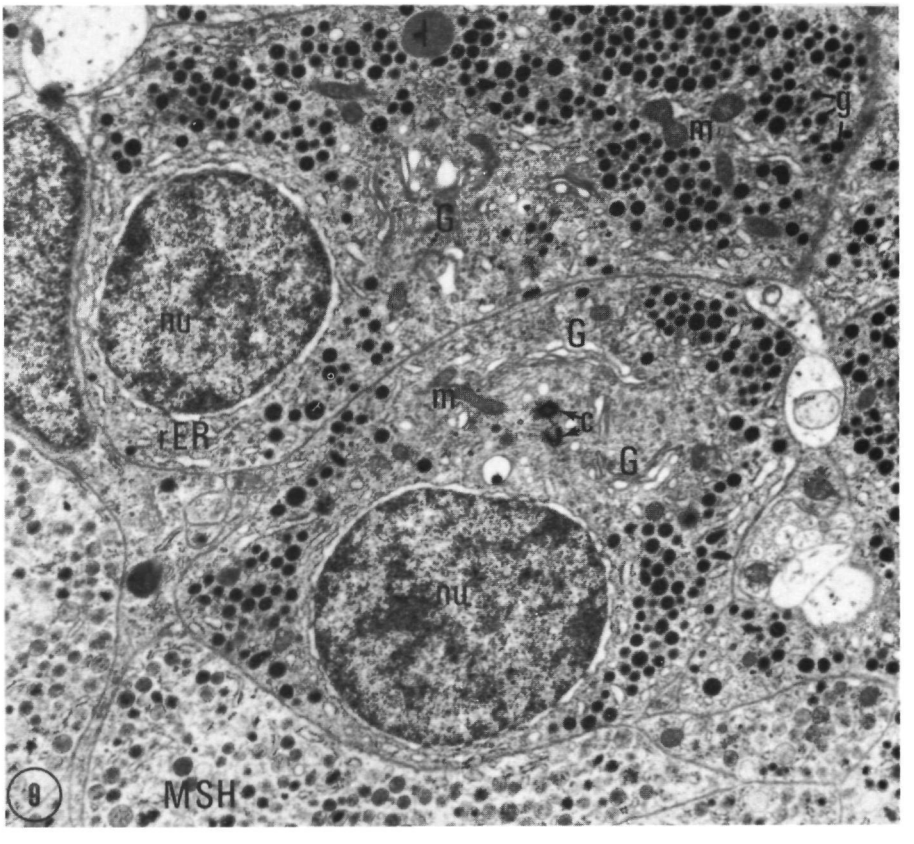
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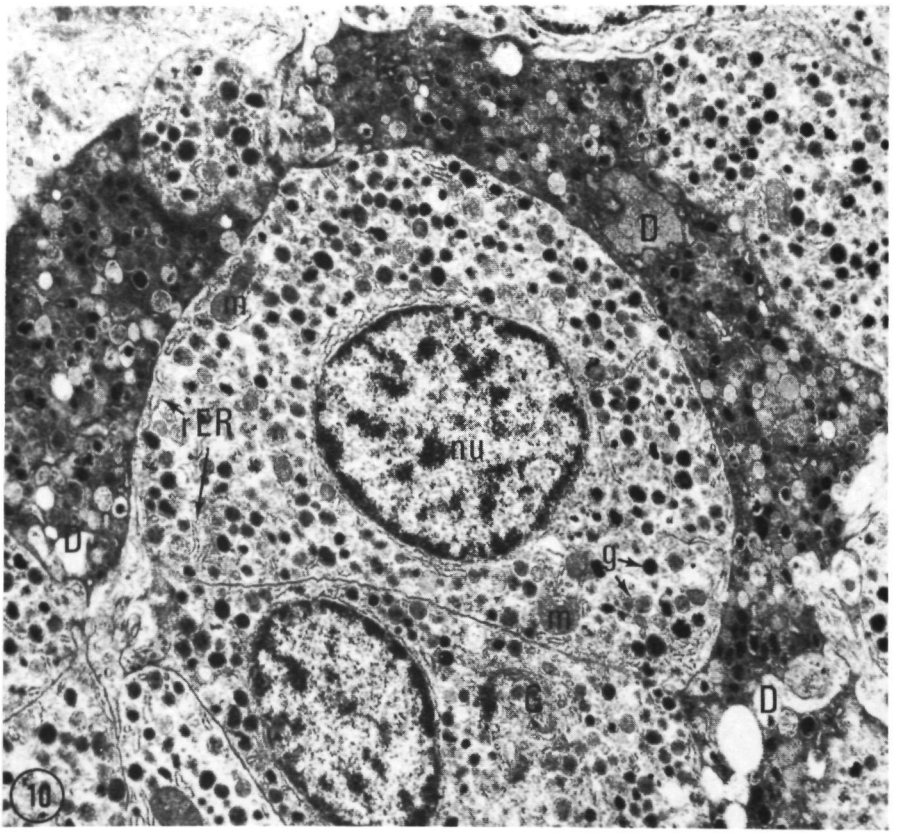

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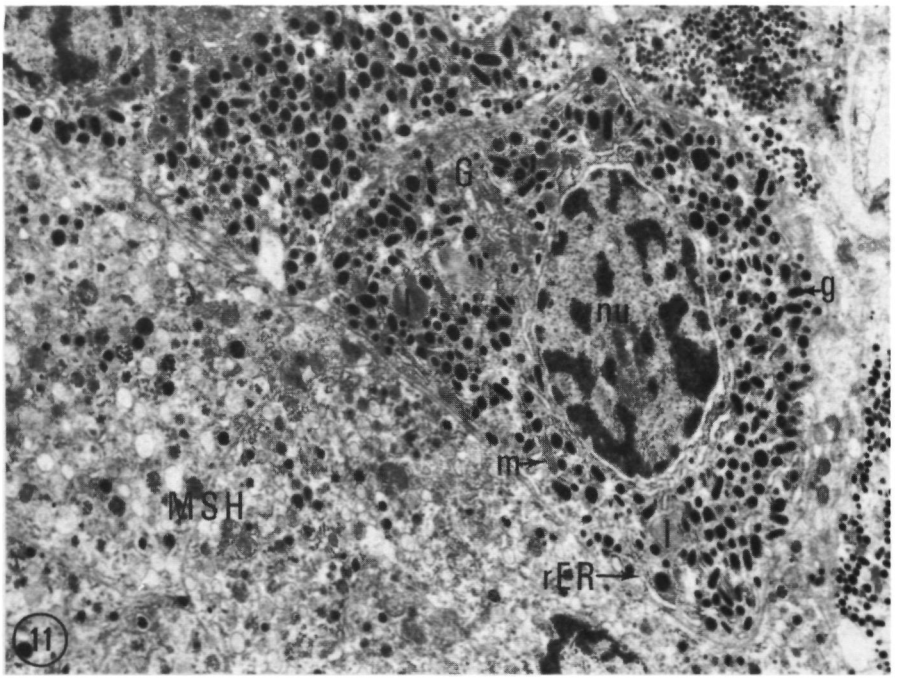

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CHAPTER 5

Cytological Localization of α-MSH, ACTH and β-Endorphin in the Pars Intermedia of the Cichlid Teleost *Sarotherodon Mossambicus*

Summary

The pars intermedia of S. mossambicus contains two different endocrine cell types. The predominant cell type is lead-haematoxyline positive and assumed to synthesize MSH and related peptides. The second cell type is PAS positive and its function and product(s) are unknown. Staining of light microscopic and ultrathin sections with antisera gainst α-MSH, ACTH 1-24 and human β-endorphin revealed that only the lead-haematoxyline positive cells of the pars intermedia reacted with these antisera, and that the secretory granules of these cells contained compounds that were immunoreactive to all three antisera. These findings are in line with the hypothesis that α-MSH, ACTH and endorphins are derived from the same precursor molecule. No specific reaction with one of the antisera could be detected in the PAS positive cells.

Introduction

In vitro studies in a number of non-teleostean vertebrate species have shown a breakdown of a precursor glycoprotein into peptides like β-LPH, endorphins, ACTH and α-MSH, and the release of these bioactive peptides in medium (Scott et al. 1973; Mains et al. 1977; Mains and Eipper 1979; Loh 1979; Jenks et al. 1979). The presence of peptides that are immunoreactive to antisera against α-MSH, ACTH-analogs and endorphins have been demonstrated in the cells of the pars intermedia of these species by immunohistological and immunocytochemical techniques. In addition, it has been demonstrated that antisera against different peptides react with the same cells, and even with the same granules (Bloom et al. 1977; Bugnon et al. 1971; Martin et al. 1979; Moriarty and Halmi 1972; Pellentier et al. 1977; Weber et al. 1978).

In contrast to what is seen for other vertebrates, the teleost pars intermedia contains two distinct endocrine cell types; the predominant lead-haematoxyline (PbH) positive cell type and the periodic acid-Schiff (PAS) positive cell type (Stahl 1958; Olivereau 1970; Baker 1972). Little is known about the synthesis and release of products by the teleost pars intermedia. The biochemical data reported so far concerned the PbH positive cells (Scott and Baker 1974, 1975; Pezalla et al. 1978). Immunological studies in fish are relatively scarce and have been performed mainly by the indirect fluorescent antibody technique (IFAT). The presence of α-MSH

or related peptides has been suggested by reactions of antisera raised against these peptides with the PbH positive cells in the pars intermedia of Anguilla anguilla (Olivereau et al. 1976a), Perca fluviatilis (Follenius and Dubois 1976), Tinca tinca (Romain et al. 1974), Gasterosteus aculeatus (Follenius and Dubois 1974), Boops salpa (Malo-Michele et al. 1976), Sarotherodon mossambicus (Chapter 1), and some salmonids (Olivereau et al. 1976b; Follenius and Dubois 1978; Dubois et al. 1979). None of the antisera used in these studies reacted with the PAS positive cells.

The present study investigates at light microscopic and ultrastructural level, whether the pars intermedia cells of S. mossambicus contain material immunoreactive to anti-α-MSH, anti-ACTH 1-24 and anti-β-endorphin. Special attention has been paid to the distribution of the different peptides over the secretory granules.

Materials and Methods

Sexually mature females of the cichlid teleost Sarotherodon mossambicus with a length between 10 and 12 cm and a weight of approximately 10 g were used. The fish were kept in fresh water in tanks with a non-reflecting black background. Water temperature was about 25°C. Animals were anaesthetized with MS222 (Sandoz) and killed by cutting the spinal cord.

Indirect Fluorescent Antibody Technique (IFAT). After killing the fish, pituitaries were quickly removed, fixed in Bouin-Hollande and dehydrated in ethanol and isopropanol. Toluene was used as an intermediate between isopropanol and paraplast, in which the pituitaries were embedded. For IFAT, sagittal sections (7 µm) were used. Sections were preincubated with normal porcine serum (1:10) for 10 min at 37°C. Thereupon sections were incubated with one of the antisera for 30 min and with goat-anti-rabbit fluorescein conjugate (1:20) (Nordic, Tilburg) for 30 min. Both incubations were performed at 37°C and were followed by rinsing in PBS (pH 7.4) for 20 min. Sections were mounted in PBS/glycerine (1:9) and examined with a Zeiss Universal fluorescence microscope. Dilutions of the antisera used for IFAT were: 1:10 for rabbit-anti-α-MSH; 1:5 for rabbit-anti-ACTH 1-24; 1:20 for rabbit-anti-β-endorphin. Specificity controls consisted of preabsorption of the antiserum by Sepharose 4B bound antigen (1 µg/5 µl antiserum). After such treatment none of the antisera showed specific immunoreactivity.

Unlabeled Antibody Enzyme Technique for Electron Microscopy. Pituitaries were fixed in 3% glutaraldehyde in 0.1 M sodiumcacodylate buffer for 24 h at 4°C. The tissue was dehydrated by submersion in progressively increasing concentrations of ethanol in water and three times 10 min in 100% ethanol. The pituitaries were embedded in Spurr's resin (Spurr 1969). Ultrathin sections were mounted on uncoated nickel grids. Immunocytochemical staining was performed with the unlabeled antibody enzyme technique, using horseradish peroxidase-anti-horseradish peroxidase (PAP) complex, as described by Sternberger et al. (1970). The PAP complex was purchased from DAKO (Copenhagen). Dilutions of antisera used for this technique were: 1:75 for anti-α-MSH; 1:25 for anti-ACTH 1-24; 1:200 for anti-β-endorphin. The immunocytochemically stained sections were examined with a Philips EM 300 electron microscope. Morphometric analysis of the granules was done on micrographs with a final magnification of 8,000.

Antisera. The rabbit anti-α-MSH was raised in New Zealand White rabbits by repeated intramuscular injections of commercially available synthetic α-MSH (Peninsula, San Carlos). Prior to the injection α-MSH was conjugated to bovine serum albumin and emulsified with complete Freunds adjuvant (Calbiochem, La Jolla). Crossreaction with ACTH was avoided by prereaction of the anti-α-MSH serum with ACTH 1-24 covalently linked to Sepharose 4B. In a radioimmunoassay crossreaction with ACTH was less than 0.1%.

The rabbit-anti-ACTH 1-24 was raised in the same way as the anti-α-MSH serum. It was pretreated with Sepharose 4B linked α-MSH. After this treatment crossreactivity to α-MSH was less than 0.1%. ACTH 1-24 was a gift of Dr. H. Rigter (Organon, Oss).

The rabbit-anti-β-endorphin was raised against human β-endorphin and was generously supplied by Dr. J.G. Loeber (RIV, Bilthoven). The antiserum did not crossreact with α-MSH or ACTH. Crossreactivity with related peptides is extensively reported by Loeber et al. (1979).

Results

At the light microscopic level all PbH positive cells reacted with anti-α-MSH, anti-ACTH 1-24 and anti-β-endorphin. No differences in the distribution of fluorescence were observed for the different antisera, although small variations in intensity of fluorescence occurred (Figs. 1, 2a, b). Preincubation of antisera with corresponding antigen abolished specific

immunostaining and resulted in a fluorescence level identical to that of the controls. No immunoreaction was observed for the pars nervosa and the PAS positive cells.

At the ultrastructural level all observed PbH positive cells reacted with each of the antisera (Figs. 3 and 4). The immunoreactivity was restricted to the granules. No specific staining deposits were observed on the rough endoplasmic reticulum and in the Golgi area. Morphometrical analysis showed that the relative volume of the granules in the PbH positive cells did not differ significantly for the used antisera (mean $\pm$ s.d.: 18.1 $\pm$ 5.7 for anti-β-endorphin; 15.7 $\pm$ 5.7 for anti-α-MSH; 17.3 $\pm$ 4.2 for anti-ACTH 1-24). Observations on serial sections revealed that the same granule reacted with different antisera (Figs. 5 and 6).

The granules of the PAS positive cells in the pars intermedia and in the pars nervosa showed some electron density. This electron density appeared to be due to the osmiophilic properties of the content of these granules, since a similar electron density was observed in the controls.

Discussion

By means of IFAT it was demonstrated that the PbH positive cells react with anti-α-MSH, anti-ACTH 1-24 and anti-β-endorphin. No such reaction was found in the PAS positive cells. These findings suggest that α-MSH, ACTH and β-endorphin, or related peptides, are present in the PbH positive cells, of the pars intermedia of S. mossambicus. Our observations agree with findings in some other teleost species, in which, mostly by IFAT, the presence of these or related peptides was demonstrated in the PbH positive cells of the pars intermedia (for a review: Follenius and Dubois 1980).

The results of the unlabeled antibody enzyme technique at the ultrastructural level indicate that the immunoreaction in the IFAT is limited to the secretory granules. No specific staining was found in the rough endoplasmic reticulum and the Golgi area. The absence of immunoreaction in these organelles is not due to the absence of secretory material, as this has been shown to be present by conventional fixation (Chapter 1). This indicates that in the Golgi area and rough endoplasmic reticulum α-MSH, ACTH and β-endorphin amino acid sequences are part of a large precursor molecule. Evidence for a common precursor in the MSH-cells, of Sarotherodon mossambicus is presented in chapter 7. Exposed antigenic

sites on such a precursor molecule, fixed and embedded in plastic, may be extremely scarce when compared to the number of exposed antigenic sites of α-MSH, ACTH and β-endorphin. The observed high immunoreactivity in the granules to each of the antisera points towards a conversion of the precursor into these smaller molecules. The simultaneous presence of reactivity against these three different peptides in the same granules suggest that these peptides might be released together by exocytosis.

Our observations support the scarce biochemical data that indicate that in teleosts the pars intermedia synthesizes and releases ACTH (Scott and Baker 1974, 1975) and β-endorphin (Pezalla et al. 1978). Our observations are also in line with immuno-electron microscopic results in mammals (Martin et al. 1979; Moriarty and Halmi 1972; Weber et al. 1978).

The PAS positive cells did not react with any of the antisera. This means that the product(s) of these cells do not share immunological properties with the products of the PbH positive cells, in spite of some resemblances in the physiological response of the two cell types to environmental stimuli (Chapter 1 and 2).

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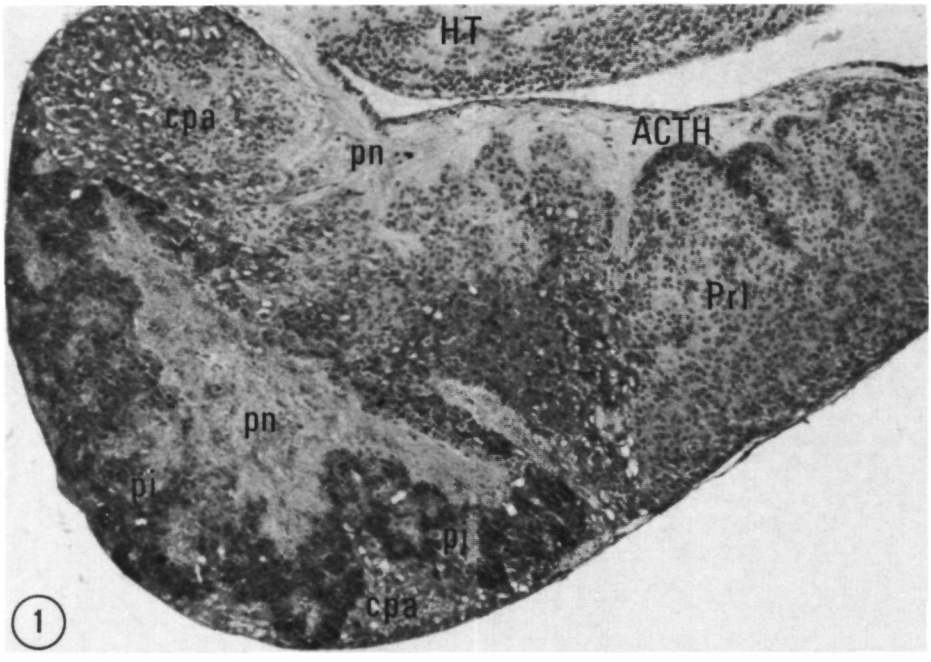
Fig. 1. L.S. of the pituitary. pi: pars intermedia, pn: pars nervosa, cpa: caudal pars anterior, Prl: prolactin lobe, HT: hypothalamus. x120

Fig. 2. Immunostaining of two consecutive sections of the pituitary with anti-α-MSH (a) and anti-$ACTH_{1-24}$ (b), IFAT. x50

Fig. 3. Electron micrograph of pars intermedia cells immunocytochemically stained (PAP) technique) with anti-β-endorphin. in: interstitial (stellate) cell, MSH: MSH cell, Gon: gonadotrophic cell, G: Golgi area, rER: rough endoplasmic reticulum. x5,700

Fig. 4: Electron micrograph of the same cells as shown in Fig. 3, immunocytochemically stained with anti-$ACTH_{1-24}$. Symbols as in Fig. 3. x5,700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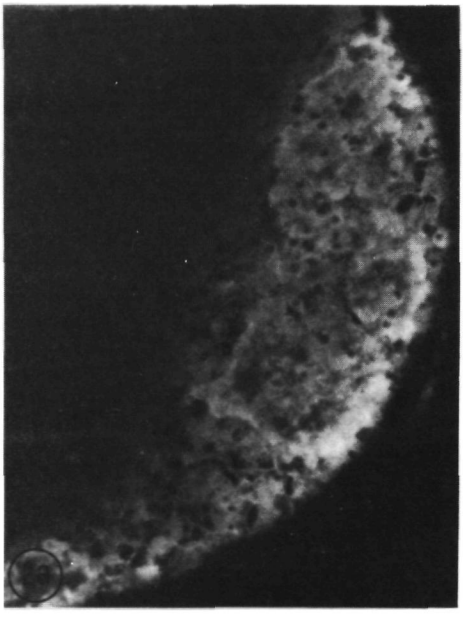
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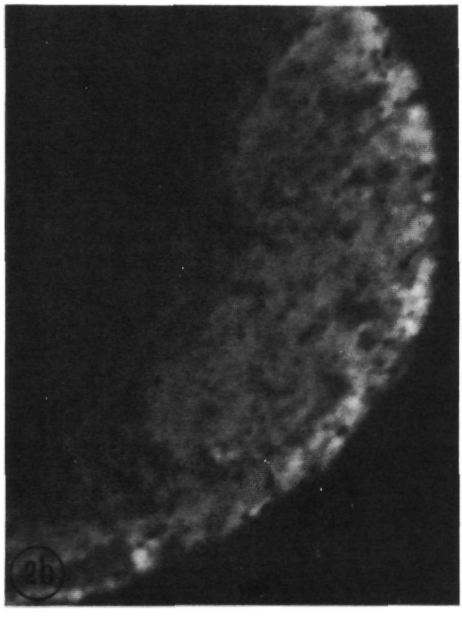
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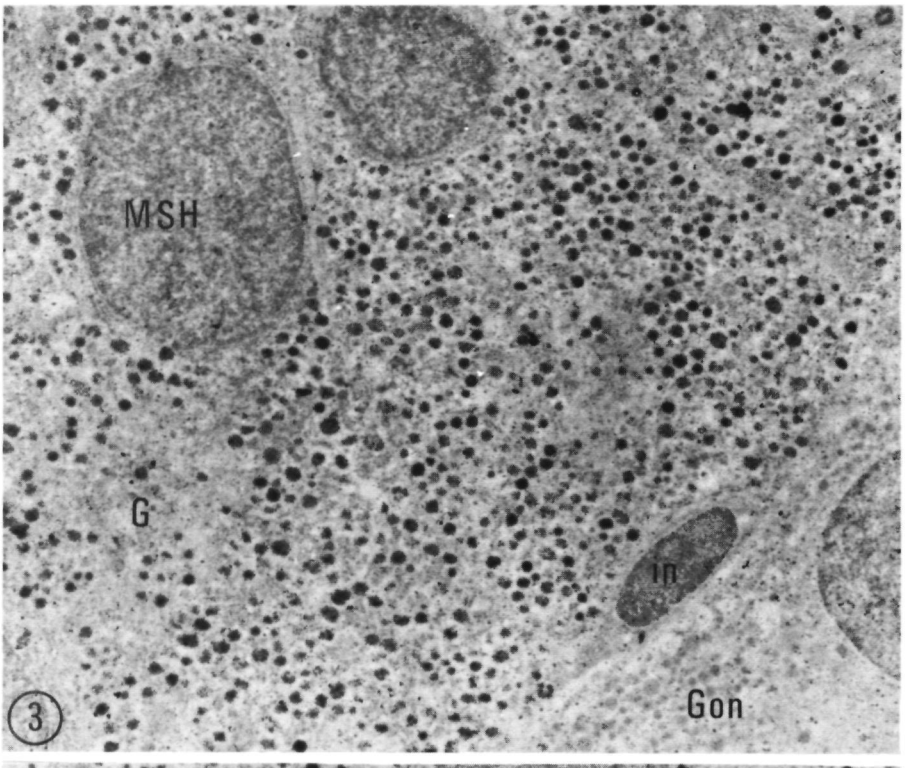
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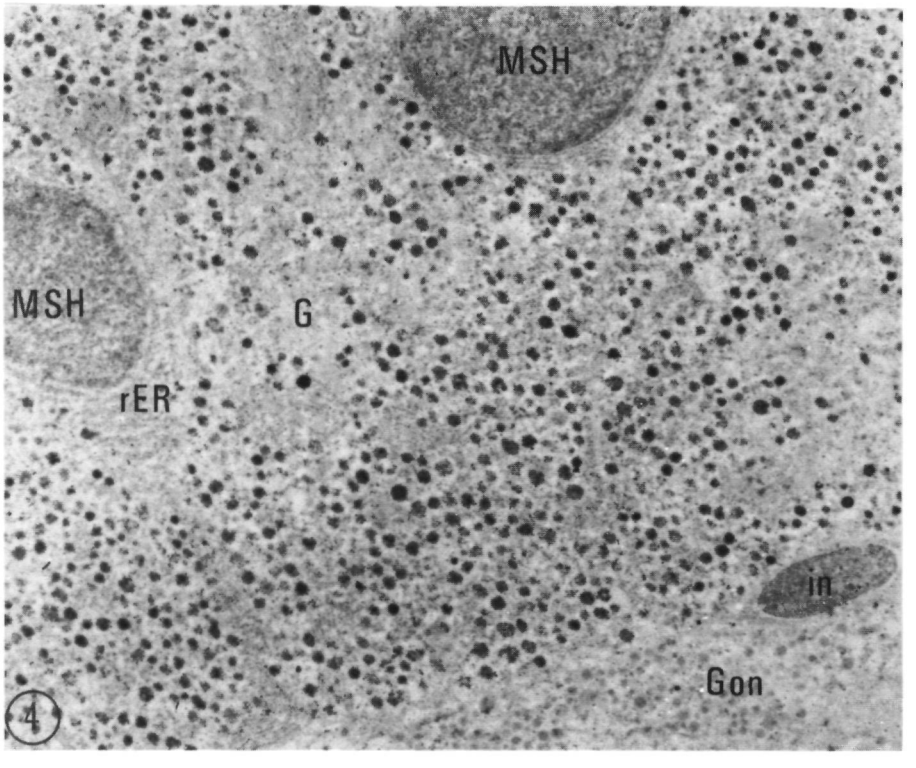
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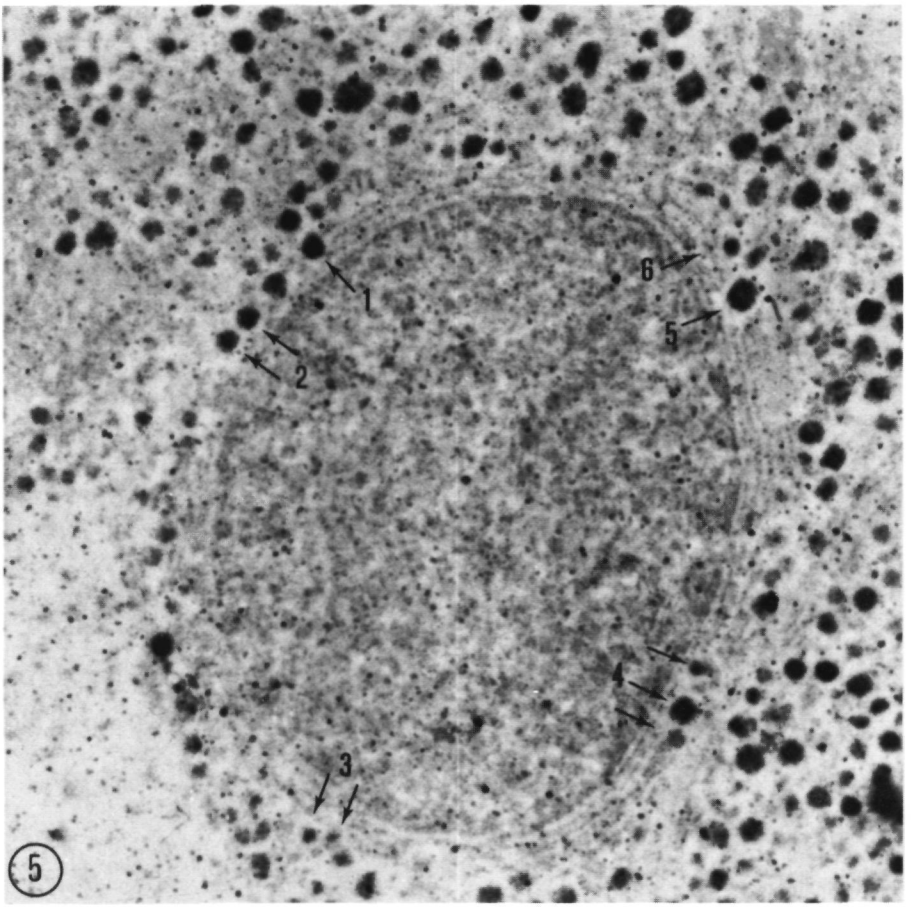


Fig. 5. Detail of PbH positive cell immunocytochemically stained with anti-α-MSH serum. Numbered arrows show identical granules in two consecutive sections (Fig. 6). x25,000

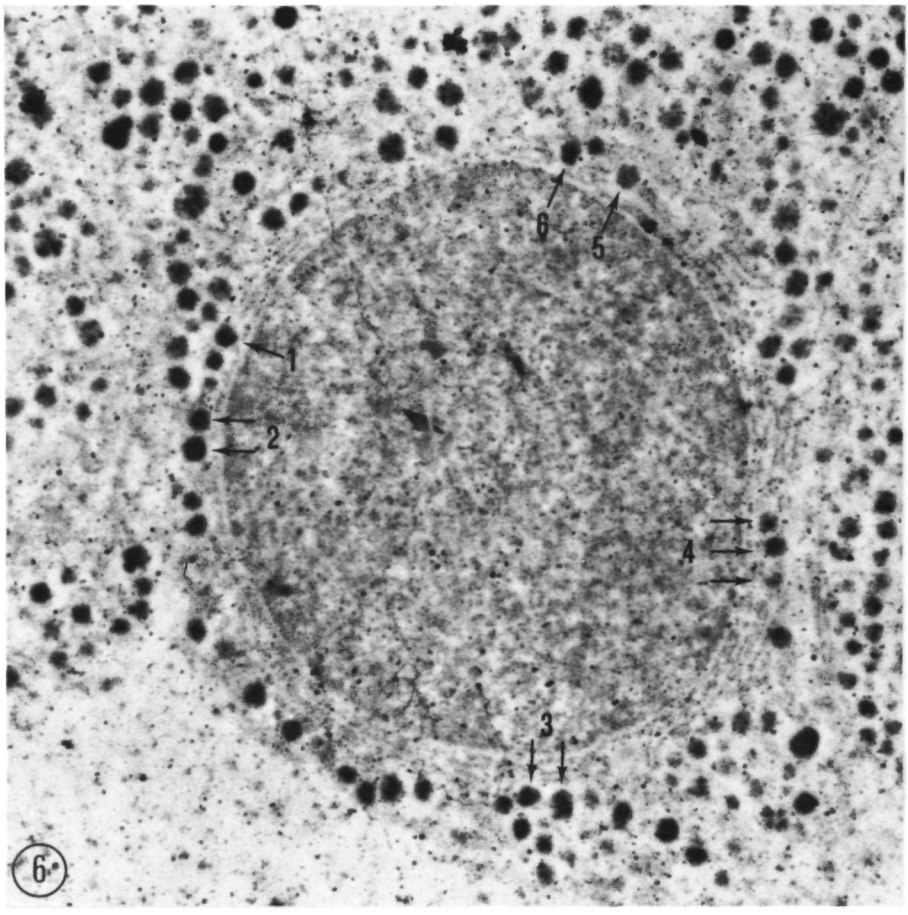


Fig. 6. Detail of the same cell as shown in Fig. 5, in this case stained with anti-β-endorphin. x25,000

Chapter 6

Analysis of biosynthetic products of the MSH cells and PAS positive cells of the pars intermedia of the cichlid teleost Sarotherodon mossambicus.

Summary

The two endocrine cell types, present in the pars intermedia of Sarotherodon mossambicus, the MSH cells and PAS positive cells, incorporated radioactive labeled amino acids during in vitro incubation. To identify the products synthesized by both cell types, labeled products of the pars intermedia were analysed by SDS polyacrylamide gel electrophoresis and high pressure liquid chromatography. During pulse incubations first observable product was a 30K molecule. This was followed almost immediately by the appearance of 22K and 26K products. The synthesis of the 22K and 26k products appeared to be correlated with the number of PAS positive cells present in the pars intermedia. In addition, (as was indicated by pulse-chase experiments) these two products are unlikely to be involved in precursor-product conversion. These experiments, nowever, did indicate a processing of the 30K product into a number of smaller compounds, including melanotropic, ACTH-like and endorphin-like substances. Processing of the 30K precursor seems to be similar to that described for the MSH cells of other vertebrate species. It was concluded that the 22K and 26K products were synthesized by the PAS positive cells and that the 30K product and the compounds probably derived from this product were synthesized in the MSH cells.

Introduction

From physiological and immunohistochemical studies we have concluded that the predominant cell type in the teleost pars intermedia is comparable to the endocrine cells of the pars intermedia of other vertebrates and therefore represents the MSH cells (Chapter 4 and 5). Studies on amphibians and mammals have shown that the biosynthetic processes in the MSH cells are of a complicated nature. Evidence has accrued to suggest a precursor-product relationship between the various compounds synthesized by these cells (Roberts and Herbert, 1977a,b; Mains and Eipper, 1976, 1979; Mains et al., 1977; Loh and Gainer, 1977; Gianoulakis et al., 1979; Jenks et al., 1979; Crine et al., 1979; Eipper and Mains, 1978; Pezalla et al., 1978). Investigations on the teleost pars intermedia have shown the presence of substances with melanotropic, corticotropic and opiate-like activity (Baker, 1972; Scott and Baker, 1975; Hunter and Baker, 1979; Carter and Baker, 1980).

The primary structure of some of these substances has been elucidated (Kawauchi and Muramoto, 1979; Kawauchi et al., 1979; Kawauchi et al., 1980a,b,c,d). The results of these studies suggested that the biosynthetic processes in the teleost MSH cells are comparable to those of other vertebrates.

The second cell type of the teleost pars intermedia, which is PAS positive, has been implicated in the endocrine control of a variety of physiological processes, including background adaptation, osmoregulation and the control of calcium metabolism (Baker and Ball, 1970; Olivereau, 1969; Olivereau et al., 1980, 1981; Malo-Michele, 1975, 1977; Pang et al., 1973). The techniques used to study the function of the PAS positive cells have been limited so far to the measurement of changes in nuclear and cellular volumes. Nothing is known about the chemical nature of the products of these cells, which hampers further investigations concerning their function.

In this chapter the results of an analysis of the biosynthetic products of the pars intermedia of Sarotherodon mossambicus are described. The main purpose of this analysis is to differentiate between the products of the MSH cells and the PAS positive cells. Isolation of the products of the PAS positive cells is severely hindered by the fact that these cells are intermingled with the MSH cells. Therefore, cell separation seems to be the method of choice to distinguish between the products of the two cell types. An attempt was done to separate the PAS positive cells from the MSH cells using Percoll gradient centrifugation, but the results were disappointing. Therefore, we decided to adopt a different strategy, based on the results of previous studies. Morphometrical analysis has shown that the MSH cells and PAS positive cells respond differently to changes in background colour and illumination to which the fish are exposed (Chapter 1 and 2). From these studies it was concluded that fish adapted to a black background had active MSH cells as well as active PAS positive cells. In darkness, MSH cells appeared to be inactive whereas PAS positive cells were highly active. In fish adapted to a white background activity was low for both cell types, although to a different extent. The reduced activity of the PAS positive cells in white background adapted fish was accompanied by a dramatic reduction in the number of PAS positive cells. Accordingly it was assumed that in the pars intermedia of white background adapted fish there would be a reduced synthesis of products of the PAS positive

cells, compared with that in black background and darkness adapted fish. Thus, it was expected that pars intermedia lobes of fish adapted to these different conditions would, when incubated with labeled lysine, display differences in incorporation pattern. Further, it was hypothesized that such differences might be correlated with differences in biosynthetic capacity of the PAS positive cells, and, therefore, lead to identification of the products of these cells.

Materials and methods

Animals. For the adaptation experiment sexually mature males of the cichlid teleost Sarotherodon mossambicus with a body length of 10-12 cm and a body weight of 9-12 g were used. Sexually mature females of the same length and weight were used for pulse and pulse-chase experiments. The fish were kept in fresh water. Water temperature was about 25°C. The fish were divided into groups, which were then placed under one of the following regimens:

Group W: white background with a day-night rhythm of 12 h light alternating with 12 h darkness.

Group B: non-reflecting black background, with lighting conditions as described for group W.

Group D: non-reflecting black background in total darkness. Animals were anaesthetized with MS 222 (Sandoz) and killed by cutting the spinal cord.

For light microscopic examination five pituitaries of each group were fixed in Bouin-Hollande fluid. Sections were stained in periodic acid-Schiff (PAS) followed by Mac Connaill's lead haematoxyline. The ratios of the volumes occupied by PAS positive cells and MSH cells were estimated by measuring the areas occupied by PAS positive cells and MSH cells in sagittal sections of the pars intermedia at a final magnification of x400 by means of a drawing prism. The drawings were morphometrically evaluated with a Kontron Digiplan. The data were tested for statistical significance with a Student's t-test.

Pulse incubations. After the fish were killed, pituitary glands were quickly dissected and the pars distalis was removed. The pars intermedia lobes were transferred into slightly modified Dulbecco's Modified Eagle's Medium (MDM). This medium differed from normal Dulbecco's

Modified Eagle's Medium by the absence of L-valine and L-cysteine, by the presence of 212 mg/l $CaCl_2$ instead of the prescribed 265 mg/l, and by replacement of Na_2HCO_3 by 20 mmol Hepes (Sigma). The final osmotic value of the medium was 310 mosm and the Ca^{2+} concentration was about 2.5 meq/l. These values are similar to the osmolality and Ca^{2+} concentrations found in the blood plasma of Sarotherodon mossambicus.

Pars intermedia tissues were placed in 100 µl MDM and preincubated for 1 h and 30 min in a metabolic shaker at 22°C. After the preincubation period the tissues were transferred to MDM containing 40 µCi ^{3}H-lysine (New England Nuclear, sp.act. 90 Ci/mmol). In pulse-chase experiments, pulse labeling was followed by a chase period in MDM containing 5 mM L-lysine at 22°C. The pars intermedia tissues were homogenized in 500 µl 0.1 M acetic acid in an all glass homogenizer. The homogenate was centrifuged at 10,000 g for 5 min in a Beckman Microfuge and the supernatant was stored at -20°C for later high pressure liquid chromatography or freeze-dried for later gel electrophoretic analysis.

High pressure liquid chromatography. The 500 µl samples were analysed with a Spectra Physics SP 8000 high pressure liquid chromatograph (Spectra Physics, Eindhoven, The Netherlands) equipped with a stainless-steel column packed with Spherisorb 10 ODS (Chrompack B.V., Middelburg, The Netherlands). The linear gradient was based on the one described by Martens et al. (1980), and consisted of a 0.5 M formic acid, 0.14M pyridine mixture (pH 3.0) and 1-propanol. The flow rate over the column was 2 ml/min, and 0.5 min fractions were collected with a fraction collector (LKB Redirac, model 2112). Four ml of Aqua Luma (Baker Chemicals) was then added and the samples were counted in a Philips liquid scintillation analyser (model PW 4540). Synthetic α-MSH (Peninsula, San Carlos) was used as a marker.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. For estimation of relative molecular weights of the pars intermedia products the homogenates were analysed by SDS polyacrylamide gel electrophoresis. Analysis was performed according to Leammli (1970) with the exception that a slabgel was used instead of gel rods. The separating gel was 10 cm long and contained 15% acrylamide (Serva), 0.4% methylene bisacrylamide (Biorad) and 0.1% SDS (SERVA). A stacking gel was applied. On polyacrylamide gels 25 µg of $ACTH_{1-39}$ (Organon BV) and

25 µg human β-endorphin (generous gift of Dr. Rigter, Organon BV) were used as markers. Staining was performed in an aqueous solution of methanol (25 ml/l) and acetic acid (40 ml/l), containing 2.5 g/l Coomassie Blue (Serva). Destaining in the same aqueous solution of methanol and acetic acid was followed by processing for autoradiography according to Bonner and Laskey (1974) in combination with the drying procedure described by Berns and Bloemendal (1974).

Autoradiography. For autoradiography pars intermedia lobes were incubated in MDM containing 20 µCi/100 µl ^{3}H-lysine for 2 hours. After incubation the lobes were fixed in Carnoy's fixative for 24 h and embedded in JB_4 (Meyvis). Autoradiography was performed with Ilford K-5 emulsion with an exposure time of 7 days.

Results

Analysis of newly synthesized products of the pars intermedia. Autoradiography at the light microscopic level of the pars intermedia from black background adapted fish showed that during in vitro incubations MSH cells and PAS positive cells incorporated comparable amounts of labeled lysine. Little of incorporation of label was observed in the neurohypophysial processes penetrating the pars intermedia.

The rate of incorporation of labeled lysine by the pars intermedia lobes was measured by counting samples of the supernatant of homogenates by LSA. Incorporation of label in the pars intermedia over a period of 240 min, was almost linear (Fig. 1).

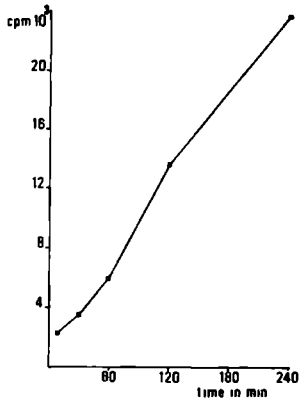


Fig. 1: Total incorporation of ^{3}H-lysine in pars intermedia secretory material of black background adapted fish as a function of time (1 lobe).

SDS polyacrylamide gel electrophoresis and high pressure liquid chromatography were used to separate the newly synthesized products from the pars intermedia of Sarotherodon mossambicus. The former technique has advantages over HPLC for the separation of large molecules, whereas HPLC can give excellent separation of relatively small molecules. HPLC analysis showed 14 peaks (Fig. 2). Further analysis of these peaks

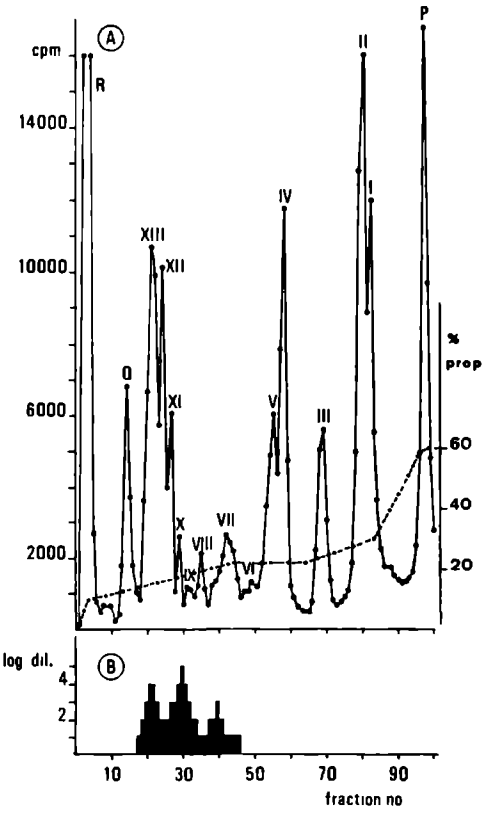


Fig. 2a. High pressure liquid radiochromatogram of pars intermedia products of 3 black background adapted fish. The tissue was pulse incubated for 4 hours with ^{3}H-lysine. Flow rate was 2 ml/min. Fractions were collected every 30 sec. Primary solvent: 0.5 M formic acid-0.14 M pyridine (pH 3.0), secondary solvent 1-propanol. (Peaks Q and R represent freeze-dry contamination and ^{3}H-lysine respectively.)

Fig. 2b. Melanotropic activity of HPLC fractions of the same pars intermedia tissue as in Fig. 2a, as determined by Anolis carolinensis skin bioassay. The relative potency of the material in the fraction is reflected by the ultimate dilution factor, whereby the green colour of the Anolis skin was changed into brown within 15 min.

by SDS gel electrophoresis revealed that peak P, the last peak eluting from the column, contained at least four products (Fig. 3). Two products were found only in small amounts (16K and 13K). The other two products were present in large amounts and appeared to be 22K and 26K molecules. Peaks I and II of the HPLC analysis were shown to be 30K and 9K molecules, respectively. Peak III represented a 3.2K molecule and IV and V appeared on the SDS gel as 3.5K and 6.5K molecules respectively. Products represented by other HPLC peaks were not found on SDS gel.

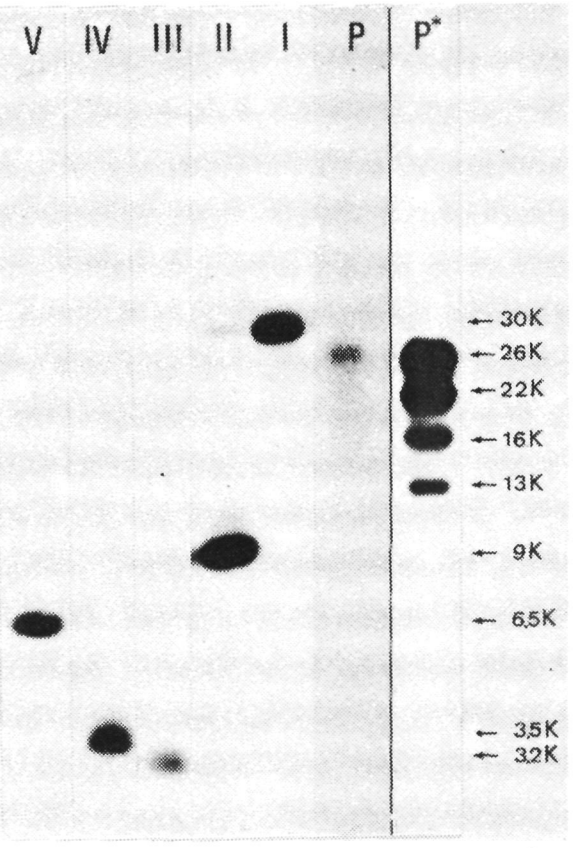


Fig. 3. Autoradiograph of a sodium dodecyl sulphate gel electrophoretic analysis of the HPLC peaks P, I, II, III, IV and V. HPLC fractions were freeze-dried and further treated for SDS gel electrophoresis as described in Materials and Methods. Peak P is from tissue of non-adapted fish; peak P* is from tissue of black background adapted fish but not from the same gel as the other samples.

Analysis of the pars intermedia of fish adapted to different conditions. Morphometrical analysis of light microscopic sections of the pars intermedia revealed statistically significant differences in relative total volume for the PAS positive cells and for the MSH cells between fish adapted to white background, black background or to darkness (Table 1). The percentage of volume occupied by PAS positive cells in the pars intermedia of fish adapted to a white background (group W) was significantly lower than that in the pars intermedia of fish adapted to a black background (group B) or to darkness (group D). Such differences were less pronounced between fish of group B and D.

SDS gel electrophoresis demonstrated that the pars intermedia homogenates of the experimental groups showed considerable differences with regard to newly synthesized 22K and 26K products. These products were found in much smaller amounts in the pars intermedia from white

Table 1. Volume of the pars intermedia occupied by PAS positive cells and relative metabolic activity of MSH cells and PAS positive cells as concluded from analysis by morphometric techniques (see Chapters 1 and 2).

Group	PAS pos. as % of PI tissue	Act. PAS pos. cells	Act. MSH cells
W	7.55 ± 2.62	-	-
B	22.71 ± 7.93*	+	+
D	30.12 ± 6.34**	++	-

Significantly different from group W (Student t-test). *: $p<0.01$; **: $p<0.001$. (-: low activity; +: high activity; ++: very high activity)

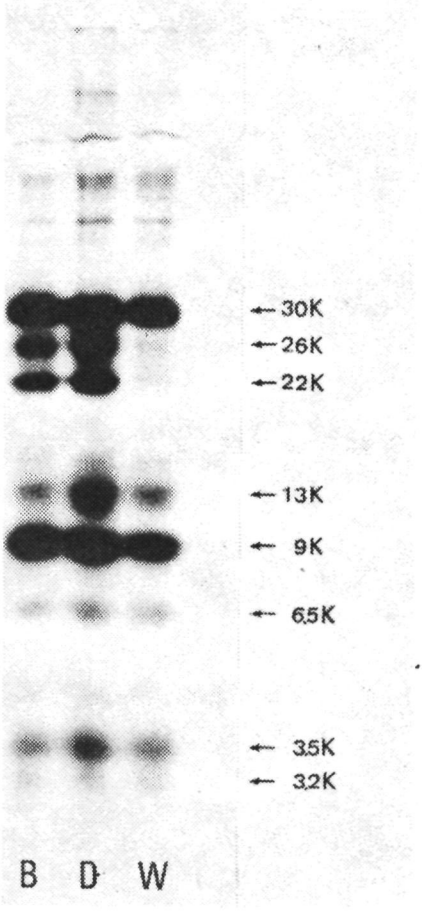


Fig. 4. Autoradiograph of SDS gel electrophoretic analysis of pars intermedia tissue of 3 black background adapted fish (B), 4 white background adapted fish (W) and 4 darkness adapted fish (D). Note that virtually no newly synthesized 22K and 26K products are present in the pars intermedia tissue of white background adapted fish. Tissue was incubated in 40 µCi/100µl ^{3}H-lysine for 4 h at 22°C.

background adapted fish, than in fish of the other two experimental groups, suggesting that these products were synthesized by the PAS positive cells (Fig. 4). For the other products no noticeable differences were observed on the SDS gel. HPLC analysis of homogenates of pars intermedia lobes from fish of groups W, B and D showed differences concerning peak P (Fig. 5). This peak was high for group D, and low for group W. Amongst the other peaks major differences were only seen between peaks I and II, which were relatively high for fish of group W.

Analysis of pulse and pulse chase experiments. Pars intermedia lobes of black adapted fish, incubated with labeled lysine for different periods of time and analysed by SDS polyacrylamide gel, showed that the appearance of newly synthesized products followed a definite temporal order (Fig. 6). A 30K product was observed after ten min incubation, whereas the 22K and 26K products appeared simultaneously only after 30 min.

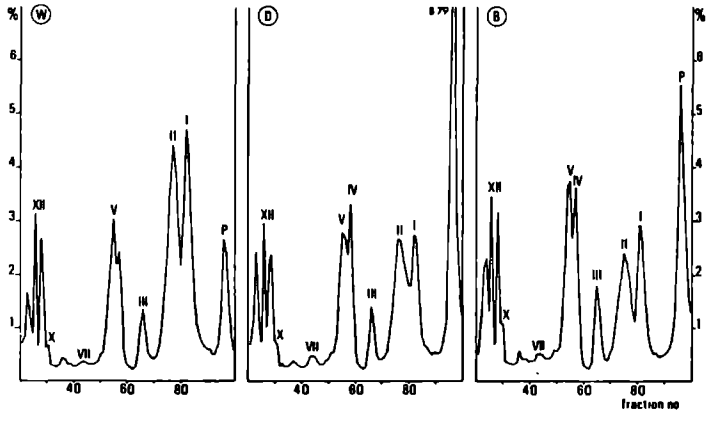


Fig. 5. High pressure liquid radiochromatograms of pars intermedia tissue of fish adapted to white background (W), black background (B) and darkness (D). Incorporation of ^{3}H-lysine for each fraction is given as the percentage of total incorporation (all fractions minus those under peaks Q and R). Pulse-labeling conditions as described in Fig. 4. Chromatographic conditions as given in Fig. 2a.

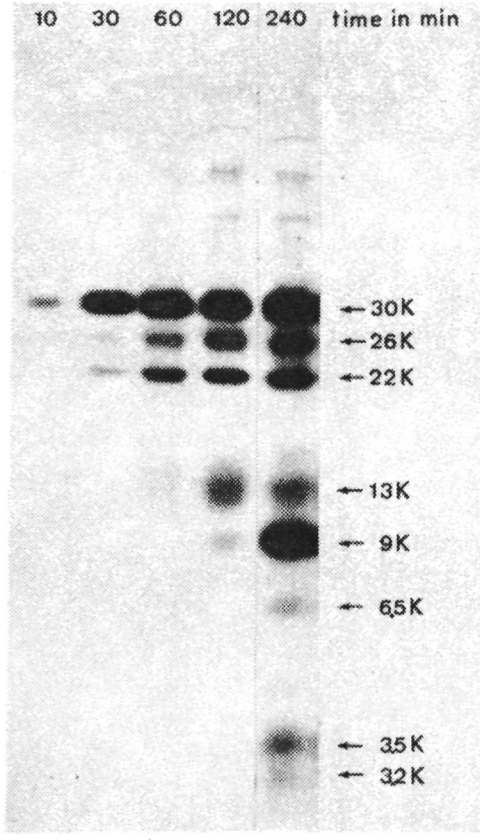


Fig. 6. Autoradiograph of SDS gel electrophoretic analysis of pars intermedia lobes incubated in MDM containing ^{3}H-lysine, for periods of 10, 30, 60, 120 and 240 min. (40 µCi/100 µl MDM; 22°C). Each group contained 3 lobes.

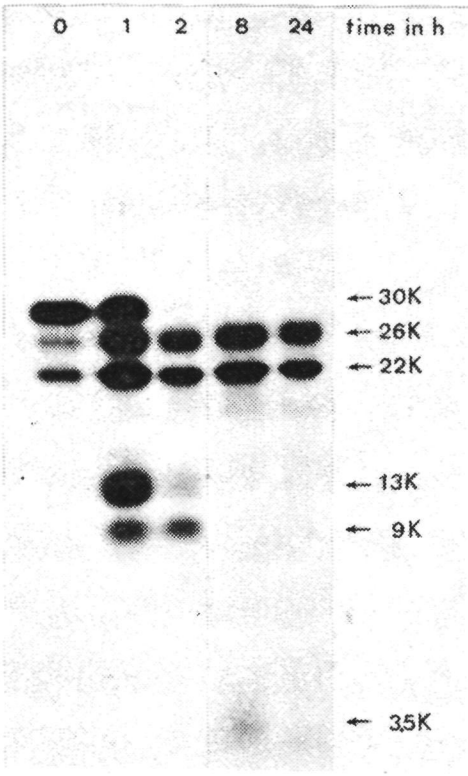


Fig. 7. Autoradiograph of SDS gel electrophoretic analysis of pars intermedia lobes pulse labeled for 30 min in MDM containing 40 µCi/100 µl ^{3}H-lysine and subsequently chased for 1, 2, 8 and 24 hours in MDM containing 5 mM L-lysine. Note the presence of 22K and 26K products in samples of all chase periods (3 lobes per group).

Samples of incubations longer than one hour also showed the presence of 13K, 9K, 6.5K, 3.5K and 3.2K products.

A pulse labeling of 30 min followed by chases of increasing duration showed the appearance of 13K and 9K products after a one hour chase (Fig. 7). After a two hour chase period the 30K product had almost disappeared. The 6.5K and 3.5K products were only found with chase periods longer than 2 hours. The 22K and 26K products were present in all samples taken during the 24 hour chase period. Moreover, these products

were the only ones found on the SDS gel after 24 hours chase. Analysis of pulse-chase samples by HPLC revealed that during the entire chase period peak P showed only minor fluctuations: 33-38% of total label incorporation (Table 2). Therefore, it was concluded that the 22K and 26K products, i.e. peak P, were not processed during chase incubation, and peak P was therefore not further taken into account in the HPLC analysis of the pulse-chase experiments.

Table 2: Percentage of label incorporated in peak P of pars intermedia of black background adapted fish during pulse chase experiment.

pulse time in min	chase time in h	max. value peak P (%)	integrated value peak P (%)
30	-	8.78	36.19
30	0.5	7.78	33.25
30	1	8.69	34.91
30	2	9.18	37.53
30	4	8.96	33.21
30	8	10.37	37.87
30	24	10.17	36.51

Values are calculated as the percentage of the sum of all fractions minus those under peaks R and Q.

This HPLC analysis showed that during pulse labelling in addition to peak P, only one product was synthesized, represented by peak I (Fig. 8) which decreased during the chase period. After a 30 min chase three new peaks appeared: II, V and VII. Peak II was maximal after a 60 min chase period whereas peaks V and VII started to diminish after a 240 min chase period. The amounts of newly synthesized products represented by peaks III, IV, XI, XII and XIII increased during the whole chase period. Label incorporation in products represented by peaks VI, VIII, IX and X was low for all chase periods and noticeable quantitative differences were not observed.

HPLC analysis revealed no newly synthesized products in the chase media of 30, 60 and 120 min. After 240 min a very small amount of products represented by peak P was found. In the media of the 8 and 24 hours chase periods small amounts of products represented by peaks P, III, IV, V, XII and XIII occurred. On the SDS gel no products could be detected in chase media.

Analysis of the identity of some products of the pars intermedia. Peak IV and V of the HPLC analysis, which appeared on SDS gels as 3.5K and 6.5K molecules, comigrated with human-β-endorphin and $ACTH_{1-39}$, respectively.

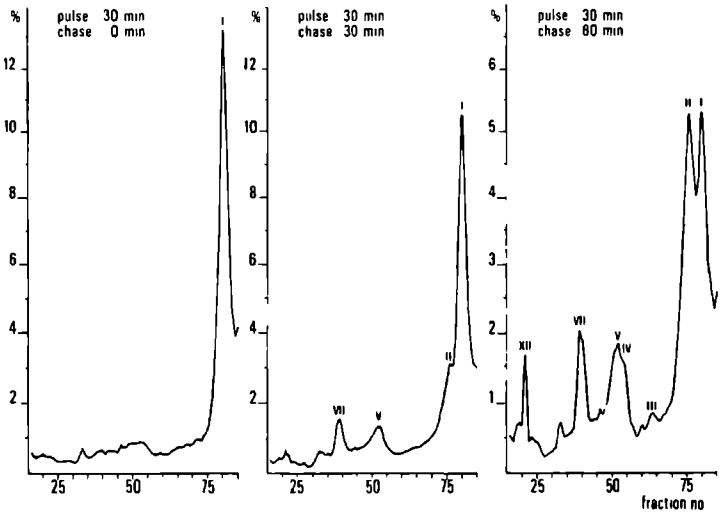
All fractions obtained by HPLC analysis were tested for melanotropic activity in the Anolis carolinensis skin bioassay according to Tilders and coworkers (1975). The product represented by samples of peak VII had weak, and the products represented by samples of peaks X and XIII had potent melanotropic activity in this bioassay (Fig. 2). HPLC elution time of peak X was similar to that of synthetic α-MSH.

Discussion

Autoradiography of the pars intermedia showed that both the MSH cells and the PAS positive cells incorporated labeled lysine under in vitro conditions but pars nervosa tissue did not. Thus, it seems reasonable to conclude that in the pars inermedia, incubated as described, products of both cell types will be synthesized. Therefore, labeled products of MSH cells as well as PAS positive cells will be present in homogenates used for SDS gel electrophoresis and HPLC analysis.

To differentiate between products of MSH cells and PAS positive cells, advantage was taken of differences in the number of PAS positive cells, that occur as a result of background colour and illumination. The sharp decline of the number and activity of the PAS positive cells in the pars intermedia of fish adapted to a white background was, on SDS gels, paralleled by a marked reduction of the 22K and 26K products of the pars intermedia tissue of white background adapted fish. This indicates that these products are produced by the PAS positive cells. The results of the HPLC analysis of pars intermedia homogenates of the experimental groups supported this conclusion, since peak P, which contains the 22K and 26K products was much lower for fish adapted to a

Fig. 8. HPLC analysis of pulse-chase experiment. Pars intermedia lobes (3 each group) were pulse-labeled in MDM containing ^{3}H-lysine (40 μCi/100 μl MDM) for 30 min and subsequently chased for 30, 60, 120, 240, 480 and 1440 min (24 h). Values given in the radiochromatogram represent the percentage of totally incorporated label minus the label recovered in fractions of peaks Q, R and P. The percentage of label incorporated in material of peak P is given separately in table 2. Chromatographic conditions were the same as given in Fig. 2a.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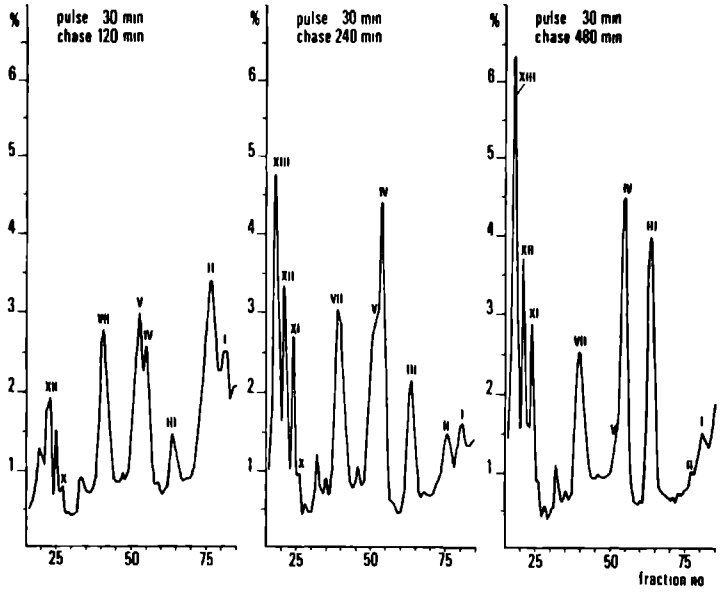
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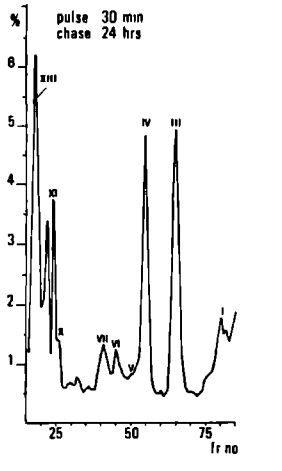
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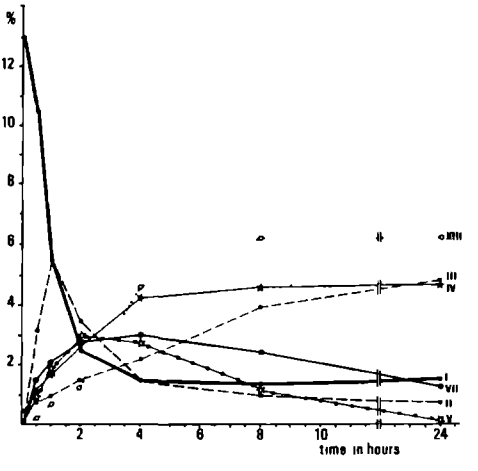
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white background than for fish of the other two groups. However, interpretations of differences observed for peak P should be made with some reservation, since this peak appeared to contain products other than just the 22K and 26K products. The cellular origin of the other two products remains to be elucidated.

The results of pulse and pulse-chase analysis on SDS gels indicate that the 22K and 26K products are not involved in a precursor-products processing. The observation that the amount of label incorporated in material represented by peak P remains constant throughout the chase period supports this conclusion. The occurrence on the SDS gel of two major products, likely synthesized by the PAS positive cells, does not necessarily imply that these cells produce two different secretory substances. It cannot be excluded that both products arise from cleavage of a larger molecule during preparatory procedures prior to application on the SDS gel. However, the occurrence of two forms of one product has been observed for the precursor molecule of the pars intermedia cells of frogs and rats (Loh, 1979; Crine et al., 1979). Crine and coworkers (1979) attributed this phenomenon to a difference in glycosylation. Such a difference in glycosylation between two forms of one product may be responsible for the appearance of the two products derived from the PAS positive cells.

Of the other products of the pars intermedia, a number may be tentatively identified. Peak I, a 30K product, was the first to appear during pulse labeling. It was apparently processed during chase incubations as it disappeared simultaneously with the appearance of a number of other products in the homogenates. No such products were found in the chase media. The 30K product, therefore, is likely to function as a precursor. On the SDS gels the 3.5K product (peak IV) and 6.5K product (peak V) comigrated with β-endorphin and $ACTH_{1-39}$ respectively. The products represented by peaks X, XIII and VII had melanotropic activity. Of these three products the one represented by peak X had the same elution time as synthetic α-MSH. This indicates that the product represented by peak X is at least similar to acetylated α-MSH. We suggest that the above mentioned products are biosynthetically interrelated and originate from the MSH cells.

In a number of vertebrate species it has been shown that ACTH and β-endorphin originate from a common precursor (Mains et al., 1977; Eipper and Mains, 1978; Mains and Eipper, 1976, 1979; Roberts and Her-

bert, 1977a,b; Nakanishi et al., 1979; Loh and Gainer, 1977; Loh, 1979; Gianoulakis et al., 1979; Crine et al., 1979). Conversion of the precursor in ACTH and β-endorphin occurs via intermediate products like the ACTH biosynthetic intermediate and β-LPH. An increasing amount of evidence points towards melanotropic and endorphin-like peptides as the final products of the pars intermedia cells (Scott et al., 1976; Mains and Eipper, 1979; Eipper and Mains, 1978; Crine et al., 1979; Loh, 1979; Gianoulakis et al., 1979; Jenks et al., 1979).

The teleost pars intermedia has been shown to contain substances similar to those found in other vertebrate species. For example, melanotropic activity was found in homogenates of pars intermedia of Anguilla anguilla and Salmo gairdneri (Baker, 1972; Baker and Ball, 1975) and Scott and Baker (1975) using a radioimmunoassay system, also reported material immunoreactive to anti-α-MSH and two products immunoreactive to anti-ACTH: one similar to human $ACTH_{1-39}$, and a high molecular weight form that was referred to as "big" ACTH. Recent investigations have also demonstrated the presence of substances with endorphin(opiate)-like activity in the eel and trout pars intermedia (Hunter and Baker, 1979; Carter and Baker, 1980). Such findings are confirmed by immunohistochemical studies (for review: Follenius and Dubois, 1980) and isolation of a number of melanotropic and endorphin-like peptides from whole pituitaries by Kawauchi and coworkers (1979a,b; 1980a,b,c,d). In Sarotherodon mossambicus we have demonstrated the presence of substances immunoreactive to anti-sera directed against α-MSH, $ACTH_{1-24}$ and β-endorphin in the same granules of the MSH cells (Chapter 5). Analysis of pars intermedia tissue on SDS gels and by HPLC also suggest the synthesis of these peptides in the pars intermedia. The ACTH-like and melanotropic products represented by peaks V and VII, respectively, seem to function as intermediates, whereas the endorphin-like and melanotropic products represented by peaks IV, X and XIII are likely final products. The biosynthetic interrelationship between the products found in the MSH cells of S. mossambicus as mentioned above, and the properties of some of the final products, suggest that biosynthesis within the MSH cells in teleost pars intermedia is similar to that in other vertebrates.

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SUMMARY

Summary

The pars intermedia in teleosts is unique compared to that of other vertebrate groups because of the presence of two distinct endocrine cell types. This investigation deals with various aspects of function, control of synthesis and the nature of the products of both of these cell types from the pars intermedia of the cichlid teleosts Sarotherodon mossambicus.

In many ectothermic vertebrates the pars intermedia has been implicated in the endocrine control of background adaptation processes. Therefore the present study was initiated by analysing the influence of background colour and illumination on the MSH cells and PAS positive cells. Chapter 1 describes the results of a morphometrical analysis of the effects of background and illumination on the activity of the MSH cells. Black background stimulated whereas white background decreased metabolic activity. Blinding the fish produced a similarly high degree of activity as that observed in the black background adapted fish, but the activity of the MSH cells of fish kept in darkness was as low as those of fish kept on a white background. From these data it was concluded that, in addition to the eyes, a second photoreceptor is involved in the control of the MSH cells. The pineal organ was demonstrated to be that second photoreceptor (Chapter 3). Therefore a new hypothesis is here presented to account for the regulation of the activity of the MSH cells. According to this hypothesis, high activity of the MSH cells results from a reduced light perception by the eyes because of a dark background and an inactivation of the pineal organ by light received by the pineal photoreceptors. Stimulation of either the eyes by light reflected from a white background or of the pineal organ by lack of illumination, results in a reduction of the activity of the MSH cells.

The PAS positive cells react in a similar fashion to background colour as do the MSH cells: that is they are active in fish on a black background and inactive in fish on a white background (Chapter 2). However in darkness they are highly active, contrary to the MSH cells. It was concluded that the stimulation of the PAS positive cells in darkness was not mediated by the eyes or by the pineal organ, since blinding and pinealectomy had no effect on these cells. Nevertheless, a role of the eyes in the control of the PAS positive cells cannot be ruled out, as blinding of fish kept on a white background resulted in

an increased activity compared with intact fish on a white background.

Differences in the activity of the MSH cells and PAS positive cells, resulting from differences in background colour, were associated with changes in the chromatophore composition of the dermis (Chapter 4). Black background initiates an increase in the number of melanophores and the amount of the pigment melanine. A similar change can be induced by prolonged treatment with synthetic α-MSH, indicating that this hormone is responsibel for the changes occurring in the dermis, as a result of adaptation of fish to a black background. Administration of α-MSH has an inhibitory effect on the activity of the MSH cells, but no effect on the PAS positive cells. On the basis of these data we concluded that the MSH cells are directly involved in morphological background adaptation processes, whereas the correlation between background colour and activity of the PAS positive cells may be only indirectly connected or unrelated to these processes. Recent investigations in our laboratorium indicate a relation between light intensity, direct or reflected, and calcium levels of blood and several tissues, and other investigators have suggested an influence of external calcium on the activity of the PAS positive cells. Therefore, the influence of background colour and darkness on the activity of the PAS positive cells may be a reflection of changes in calcium metabolism as a result of variations in light intensity.

Investigations on the physiological significance of the PAS positive cells are greatly hampered by the lack of knowledge about the products of these cells, whereas studies on the biosynthesis of the teleost MSH cells are hindered by the presence of the PAS positive cells in the pars intermedia. Therefore, an attempt was made to identify the products of the PAS positive cells. By means of immunohistochemical techniques it was demonstrated that a similarity of products between MSH cells and PAS positive cells is unlikely. Antisera, directed against α-MSH, ACTH and β-endorphin, reacted with material in the MSH cells but not with products of the PAS positive cells. In addition, it was demonstrated that antigenic determinants for all three antisera are present within the same granules (Chapter 5). Morphometrical analysis showed that in white adapted fish the number of PAS positive cells is significantly lower than in black background or darkness adapted fish (Chapter 6). SDS gel electrophoresis revealed that the low number of PAS positive cells in white background adapted fish is paralleled by

a low rate of synthesis of the 22K and 26K product. Thus, we concluded that the 22K and 26K products are synthesized by the PAS positive cells. This conclusion is supported by the fact that these two products are not involved in a precursor-product processing, like most of the other products. Analysis by gel electrophoresis and high pressure liquid chromatography showed that several other products are derived from a 30K precursor molecule. Processing of this precursor molecule may be similar to that found in the pars intermedia cells of other vertebrates, since melanotropic, ACTH-like and β-endorphin-like peptides were found in teleost pars intermedia tissue. Pulse-chase analysis revealed that ACTH may function as an intermediate in the synthesis of melanotropic peptides, which together with the endorphin-like substance, seem to be final products of the MSH cells. The biochemical and immunohistochemical findings suggest that the biosynthesis in the MSH cells in the teleost pars intermedia is comparable to that of the pars intermedia cells in other vertebrate groups.

Curriculum vitae

Guillaume van Eys werd geboren in Schin op Geul (gemeente Valkenburg-Houthem) op 18 maart 1948. Het HBS-B diploma werd in 1968 behaald op het St. Maartenscollege te Maastricht. In september 1969 begon hij met de studie biologie aan de Katholieke Universiteit Nijmegen. Het Kandidaatsexamen B4 werd afgelegd in februari 1973. Het doctoraalexamen met hoofdvak Dierfysiologie (Prof. dr. A.P. van Overbeeke: practische stage in Mwanza, Tanzania) en bijvakken Farmacochemie (Prof. dr. J.M. van Rossum. practische stage bij Organon B.V. te Oss) en Mariene Ecologie (Dr. H. Oomen: practische stage op Carmabi, Curaçao N.A.) werd afgelegd in september 1976 (cum laude). In december 1976 werd begonnen met het hier beschreven onderzoek op de afdeling Dierfysiologie (Prof. dr. S.E. Wendelaar Bonga) van de Katholieke Universiteit Nijmegen. Naast onderzoek werd een bijdrage geleverd aan het onderwijs aan biologie studenten.

1

De waarneming dat beta-endorfine de hormoonafgifte door de pars intermedia cellen stimuleert, lijkt niet in overeenstemming met de hypothese dat beta-endorfine een eindproduct is van de pars intermedia cellen.

Van Wimersma Greidanus et al. (1979) Life Sci. 24, 579-583.

Celis (1980) Can. J. Physiol. Pharmacol. 58, 326-329.

2

De term "chorion" voor het vlies dat het ei van beenvissen omgeeft, dient vervangen te worden door "zona radiata".

Tesoriero (1978) J. Ultrastruct. Res. 59, 315-326.

Riehl (1978) Riv. It. Pisc. Ittiop. A. XIII, 113-121.

3

Immunologische technieken dienen eerder gebruikt te worden ter bevestiging van de aanwezigheid van een bepaald molecuul dan ter identificatie van zo'n molecuul.

Julliard et al. (1980) Science 208, 183-185.

4

Gezien de momenteel beschikbare technische hulpmiddelen wordt in de opzet van farmacologische experimenten nog steeds te weinig rekening gehouden met het dag-nacht ritme van de proefdieren.

5

Bij gedragsonderzoek aan proefdieren van hogelijk ingeteelde stammen mag men niet zonder meer aannemen dat het materiaal genetisch uniform is.

Bailey (1978) In: Origins of inbred mice pp.197-216; ed. H.C. Morse III, Academic Press, NY.

6

Aan de hand van chemische karacteristieken van intermediaire filamenten van het cytoskelet is het mogelijk op snelle en betrouwbare wijze tumoren en hun afgeleide metastasen te karakteriseren.
Bannasch (1980) Proc. Natl. Acad. Sci. USA 77, 4948-4952.

7

De fysiologische betekenis voor de calciumregulatie van het calcitonine-achtige molecuul, wiens synthese plaats vindt via het ACTH/beta-endorfine precursor gen, is dubieus.
Nakanishi et al. (1980) Nature 287, 752-754.
Keutmann et al. (1981) In: Hormonal control of calcium metabolism p.395; eds. D.Cohn, R.V.Talmage, J.Les Matthews, Excerpta Med. A'dam.

8

Met het oog op de Wet op de dierproeven verdient het aanbeveling om waar mogelijk biochemische door morfometrische experimenten te vervangen.

9

Ontwikkelingshulp die gericht is op de verbetering van de gezondheidszorg dient gekoppeld te zijn aan een programma voor de regulatie van de bevolkingsgroei.

10

Met de opheffing van de Tegenpartij verdween de helderheid en slagvaardigheid uit de nederlandse politiek.

11

De oprichting van de Medische Faculteit in Maastricht heeft de Maastrichtenaren grandeur gegeven, en de hollanders werk.

GJJM van Eys, 1981