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BIOSYNTHESIS OF MELANOTROPINS AND ENDORPHINS BY THE LEAD–HAEMATOXYLIN POSITIVE CELLS IN PARS INTERMEDIA OF THE CICHLID TELEOST *SAROTHERODON MOSSAMBICUS*

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Abstract—1. Biosynthesis in the lead–haematoxylin positive pars intermedia cells of *Sarotherodon mossambicus* was analysed by SDS and acid–urea electrophoresis and high pressure liquid chromatography.

2. Pulse and pulse-chase experiments revealed that a non-glycosylated 30K-precursor was processed into a number of smaller products.

3. Melanotropic and endorphin-like peptides appeared to be the final products of this precursor–product processing.

4. Our results indicate that the biosynthetic pathways in the lead–haematoxylin positive cells in the teleost pars intermedia are similar to those described for pars intermedia cells in other vertebrate groups.

INTRODUCTION

Physiological and immunohistological investigations in a number of teleosts have indicated that the predominant, lead–haematoxylin positive, cell type in the teleostean pars intermedia is similar to the pars intermedia cells in other vertebrate groups, whereas the second, periodic acid–Schiff (PAS) positive, cell type is unique to teleosts (Baker, 1972; Olivereau, 1971, 1972; Thornton and Howe, 1974; Follenius and Dubois, 1980; Van Eys and Van den Oetelaar, 1981; Van Eys and Peters, 1981). Biochemical assays on the teleostean pituitary have shown the presence of substances with melanotropic, corticotropic and opiate-like activity (Baker, 1972; Baker and Ball, 1975; Hunter and Baker, 1979; Carter and Baker, 1980). Furthermore in salmon the primary structure of a number of peptides with such an activity was elucidated and shown to be similar to those found in other vertebrates (Kawauchi and Muramoto, 1979a; Kawauchi *et al.*, 1980a, b, c, d). In addition, McLean and Lowry (1981) showed that the primary structure of MSH-like peptides in the more primitive dogfish is similar to that in higher vertebrates.

In vitro studies on amphibian and mammalian pars intermedia tissue revealed that biosynthesis in this lobe is of a complicated nature. Evidence has accrued to suggest that in the pars intermedia cells a precursor, pro-opiomelanocortin, is processed via a number of intermediates into α-MSH and β-endorphin (Mains and Eipper, 1979, 1980; Gianoulakis *et al.*, 1979; Crine *et al.*, 1979, 1980; Loh, 1979; Loh *et al.*, 1981; Jenks *et al.*, 1979). Recently Martens *et al.* (1983) were able to show that the above mentioned *in vitro* results correlated very well with their *in vivo* findings.

Studies on the biosynthetic processes in the teleost pars intermedia have been precluded thus far, due to the complex intermingling of PAS positive and lead–haematoxylin positive cells. The recent identification of the products of the PAS-positive cells in *Sarotherodon mossambicus* (Van Eys *et al.*, 1983), offered the possibility to investigate the nature of the biochemical processes in the most predominant, lead–haematoxylin positive, (further in this paper referred to as MSH cells) and to compare the results with findings in other vertebrate groups.

MATERIALS AND METHODS

Animals

Sexually mature females, 10–12 cm long with a bodyweight of 9–12 g were used. Fish were kept on a black background in fresh water of 25°C under a 12 hr light/12 hr dark regimen. Fish were kept under these conditions for at least 2 weeks before the start of the experiment.

Pulse and pulse-chase incubations

Pars intermedia lobes were directly after dissection transferred into slightly modified Dulbecco's Modified Eagle's Medium (MDM), which differs from normal DMEM by the absence of L-valine and L-cysteine and by the replacement of Na_2HCO_3 by 20 mmol HEPES (Sigma). The final osmotic value of the medium was 310 mosm and the Ca^{2+} concn was about 2.5 meq/l (212 mg $CaCl_2$/l instead of the prescribed 265 mg/l). These values are similar to the osmolality and the Ca^{2+} concn found in the blood of *Sarotherodon mossambicus*.

Pars intermedia lobes were preincubated in 100 μl MDM for 90 min. All incubations were done in a metabolic shaker at 24°C. After preincubation the lobes were transferred into 100 μl MDM containing 40 μCi ^{3}H-lysine (New England Nuclear, sp. act. 90 Ci/mmol). In pulse-chase experiments 30 min of pulse labeling was followed by chase periods of increasing duration in MDM containing 5 mM L-lysine at 24°C. At the end of the incubation period the pars intermedia lobes 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 subsequent high pressure liquid chromatography (HPLC) or freeze-dried for later gel electrophoretic analysis.

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High pressure liquid chromatography

The 500 μl supernatant samples were analysed with a Spectra Physics SP 8000 high pressure chromatograph equipped with a stainless steel column packed with Spherisorb 10 ODS (Chrompack BV). The linear gradient consisted of a 0.5 M formic acid–0.14 pyridine mixture (pH 3.0) and 1-propanol. The flow rate over the column was 2 ml/min, and 1 ml fractions were collected. Four ml Aqua Luma (Baker Chemicals) were added and the fractions were counted in a Phillips liquid scintillation analyser (model PW 4540). Synthetic α-MSH run under identical chromatographic conditions served as a marker. From the HPLC fractions that were used for further characterization of the peaks, 100 μl was taken from each fraction for counting in the liquid scintillation analyser. The remaining 900 μl were frozen in liquid nitrogen and freeze-dried. They were stored at $-20°C$ until analysis was performed (not longer than 2 weeks). Freeze-dried fractions were tested for melanotropic activity and endorphin immuno-crossreactivity. Melanotropic activity was estimated by means of the *Anolis carolinensis* skin bioassay (Tilders *et al.*, 1975). Endorphin immuno-crossreactivity was measured by radio-immunoassay using anti-salmon-β-endorphin II antiserum as described by Rodriguez and Sumpter (in press).

To determine apparent mol. wt and *Rf* values on acid urea gels, HPLC fractions under each peak were pooled, freeze-dried and subsequently electrophoresed.

Sodium dodecyl sulphate and acid urea polyacrylamide gel electrophoresis

For estimation of relative mol. wt of pars intermedia products, the homogenates were analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis according to Laemmli (1970) with the exception that a slab gel was used instead of gel rods. The separating gel contained 15% acrylamide (Serva), 0.4% methylene bisacrylamide (Biorad) and 0.1% SDS (Serva). A stacking gel was applied. Labeled marker molecules were purchased from New England Nuclear. In addition, unlabeled ACTH 1-39 and human-β-endorphin (generous gifts of Dr Rigter, Organon BV) were used as markers.

Acid urea gel electrophoresis was performed according to Davis *et al.* (1972) on a 10% polyacrylamide gel, pH 2.7. The samples were dissolved in 0.9 M acetic acid/10 M urea. Cytochrome *c* was used as a marker reference to which the relative mobilities (*Rf*) of bands were calculated. ACTH 1-39 and human-β-endorphin were used as markers. For both gel types staining was done in an aqueous solution of methanol (25 ml/l) and acetic acid (40 ml/l), containing 2.5 g/l Coomassie Brilliant Blue (Serva). The gels were destained in the same aqueous solution of methanol and acetic acid, processed for autoradiography according to Bonner and Laskey (1974) and dried following the procedure described by Berns and Bloemendal (1974).

RESULTS

Analysis of the newly synthesized products of the MSH cells

HPLC analysis revealed the presence of at least 13 newly synthesized products in incubated pars intermedia lobes (Fig. 1). Further analysis of HPLC peaks by SDS and acid urea gel electrophoresis showed that peak I represents a 30 K dalton protein (*Rf* : 0.66). Incubations with tunicamycin did not alter the apparent mol. wt of this 30 K product (Fig. 2), and incubations with ^{3}H-glucosamine did not result in the incorporation of label (data not shown). Peak II contains two products, 19 K and 16 K respectively (*Rf* : 0.90 and 0.98). Apparent mol. wts of the products under peaks III, IV and V are estimated to be 3.2 K, 3.5 K, and 13 K respectively (*Rf* values are 1.02, 1.22 and 1.34 respectively). Peaks IV and V were, on both gel types, found to comigrate with human-β-endorphin and ACTH 1-39, respectively. All HPLC fractions were investigated for melanotropic activity by *Anolis carolinensis* skin bioassay. Products represented by peak VII had weak melanotropic activity, whereas those represented by peaks X and XIII had potent activity in this bioassay (Fig. 1b). In addition HPLC elution time of peak X was identical to that of synthetic α-MSH. Radioimmunoassay of the HPLC fractions revealed that the immuno-crossreactivity towards anti-salmon-β-endorphin II is largely restricted to peaks III (29%) and IV (55%) while minor reactivity is found in the fractions under peaks II (8%), VIII and IX (5%) and R (3%) (Fig. 1c).

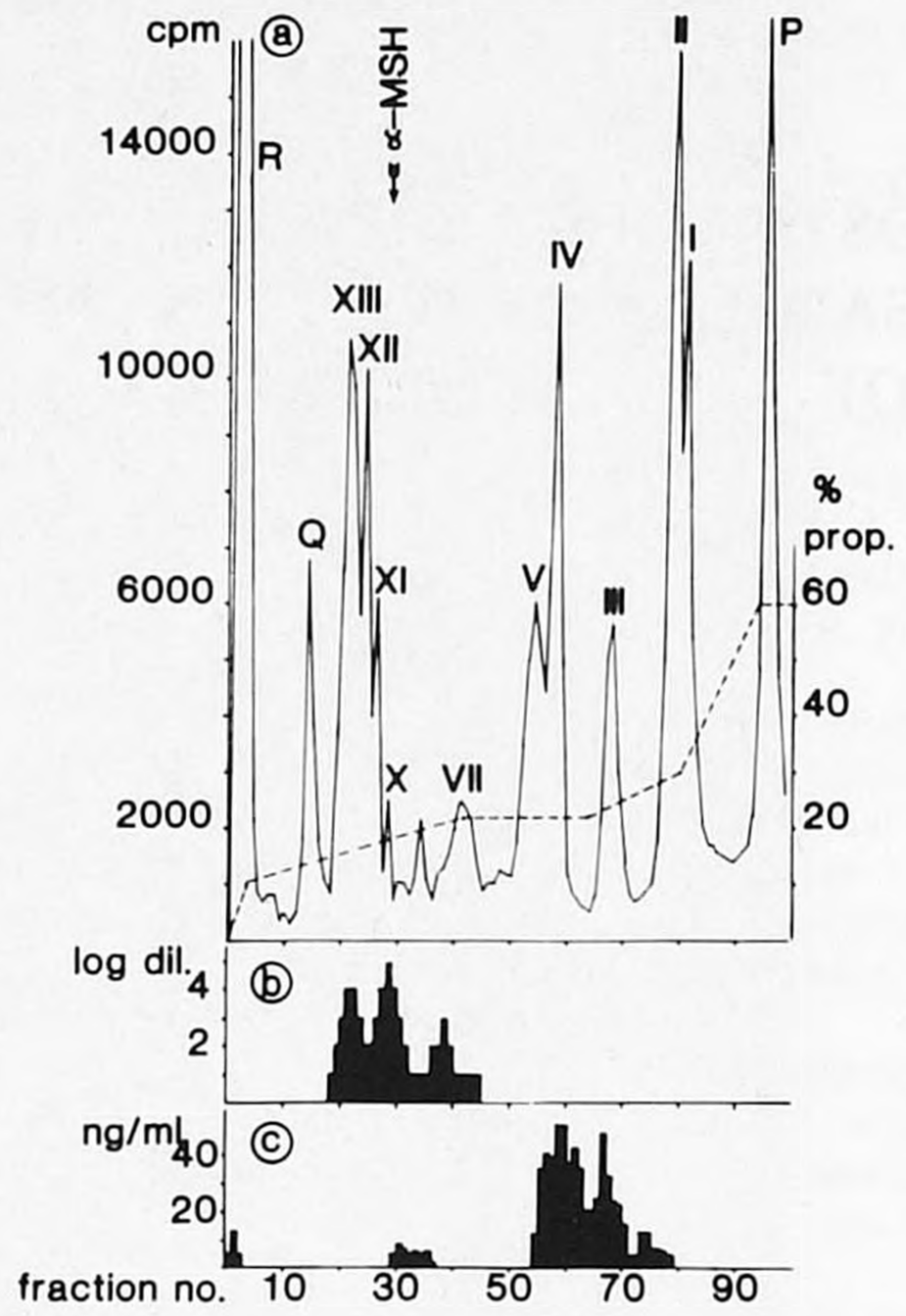

Fig. 1(a). High pressure radiochromatogram of the pars intermedia products of black background adapted fish. The lobes were pulse incubated for 4 hr with ^{3}H-lysine. The supernatant of the homogenates was applied to the HPLC. Flow rate was 2 ml/min and fractions were collected every 30 sec. Primary solvent: 0.5 M formic acid–0.14 M pyridine (pH 3.0), secondary solvent 1-propanol (dotted line). (Peaks Q, R and P contain freeze-dry contamination, ^{3}H-lysine and products of the PAS positive cells respectively). (b) Melanotropic activity of HPLC fractions of the same pars intermedia lobes as in Fig. 1a, as determined by *Anolis carolinensis* skin bioassay. The relative potency of the material in the fractions is reflected by the ultimate dilution factor. (c) Immuno-crossreactivity with anti-salmon-β-endorphin II antiserum. The pars intermedia lobes used for the radio-immunoassay were from fish treated similarly to those used for determination of melanotropic activity. The HPLC pattern for these lobes was the similar to that given in Fig. 1a.

Analysis of biosynthetic processing

Pars intermedia lobes of black background adapted fish, incubated with ^{3}H-lysine for different periods of time and analysed by SDS gel electrophoresis, showed that the appearance of newly synthesized products followed a definite temporal order (Fig. 3). The 30 K

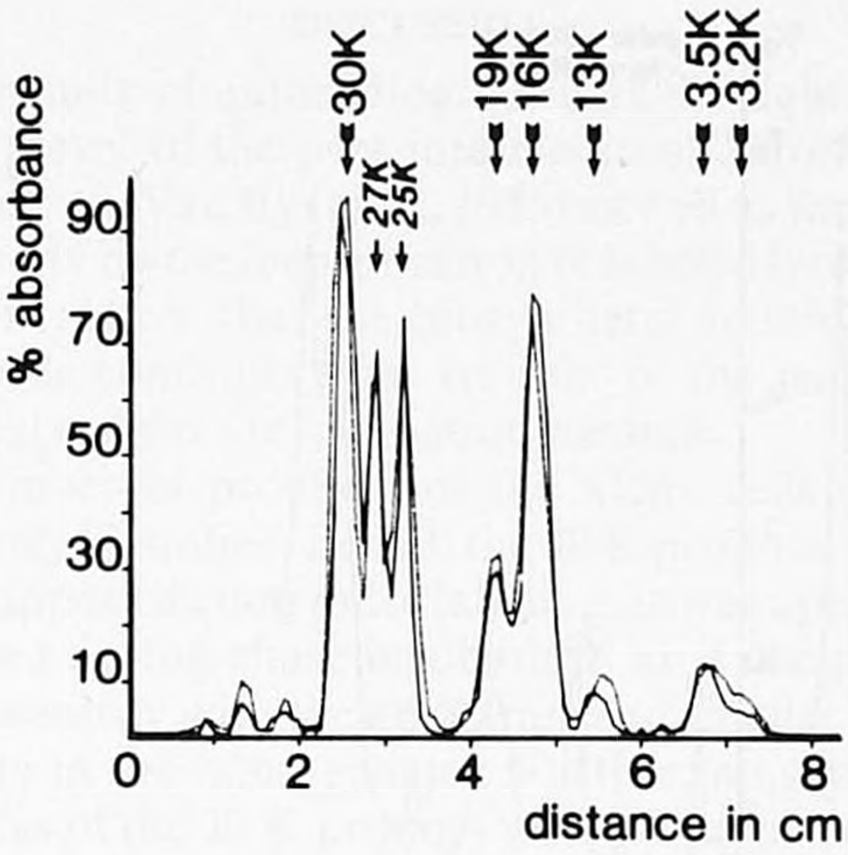

Fig. 2. Spectrophotometric scan of autoradiograph from SDS gel electrophoretic analysis of pars intermedia lobes (3 each lane) incubated for 4 hr in MDM containing 40 μCi ^{3}H-lysine with (solid line) or without tunicamycin (dotted line) (10 μg/ml). Incubation conditions as described under Materials and Methods.

product was observed as soon as 5 min after the start of the incubation, whereas the other products appeared one after another in samples taken after prolonged incubation. The 27 K and 25 K products are not taken

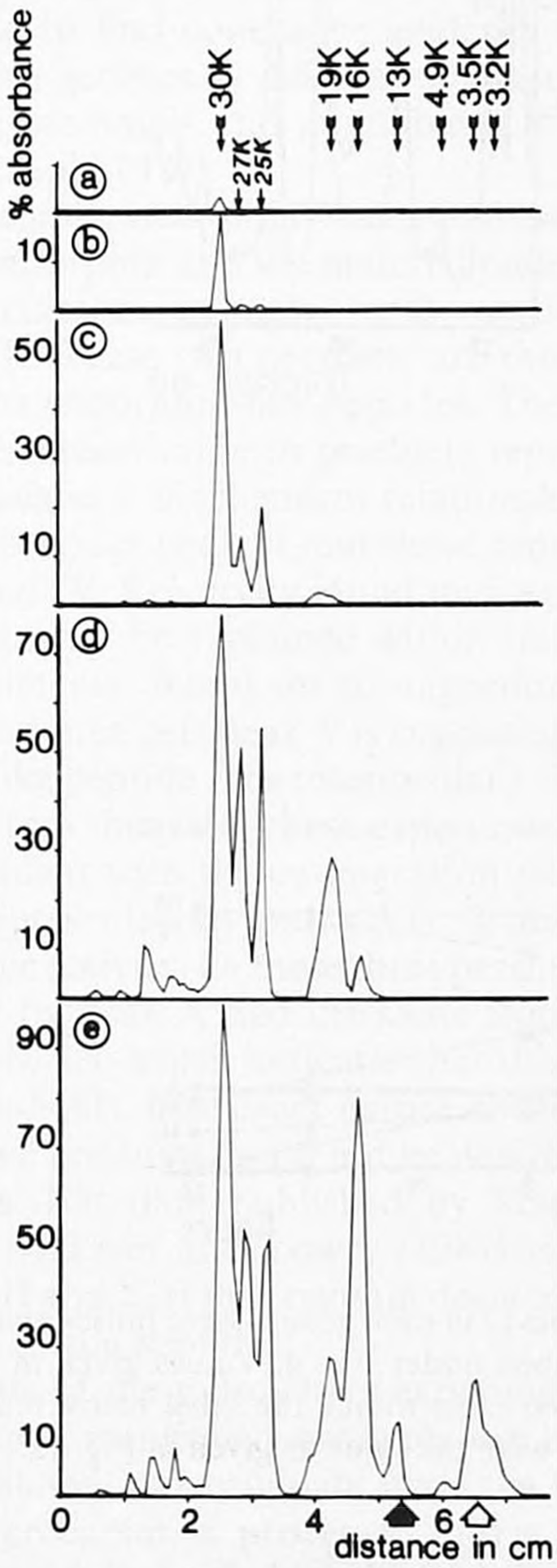

Fig. 3. Spectrophotometric scan of autoradiograph from SDS gel electrophoretic analysis of pars intermedia lobes (3 each lane) incubated for increasing periods of time in MDM containing 40 μCi ^{3}H-lysine. Incubation times were 5 min (a), 10 min (b), 60 min (c), 2 hr (d), and 4 hr (e). Black arrow: ACTH 1–39; white arrow: human-β-endorphin.

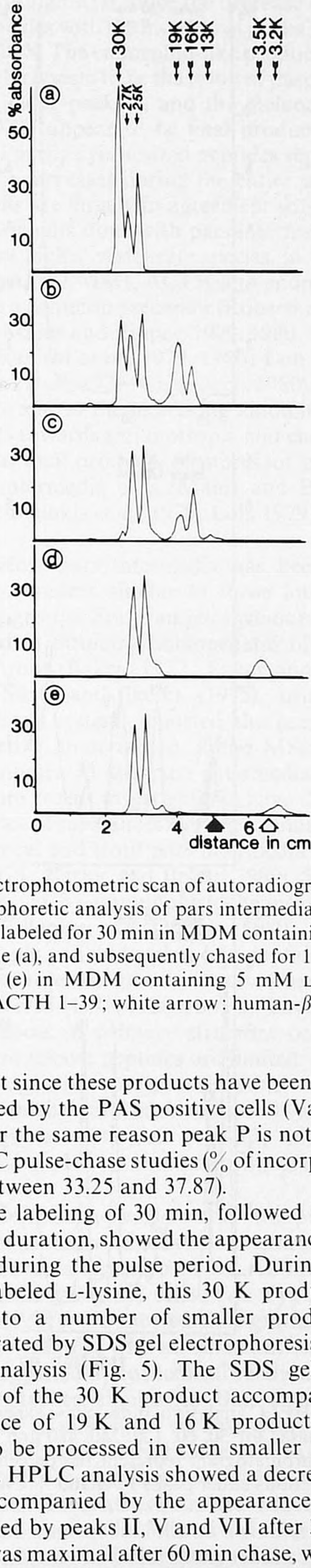

Fig. 4. Spectrophotometric scan of autoradiograph from SDS gel electrophoretic analysis of pars intermedia lobes (3 each lane) pulse labeled for 30 min in MDM containing 40 μCi/100 μl ^{3}H-lysine (a), and subsequently chased for 1 (b), 2 (c), 8 (d) and 24 hr (e) in MDM containing 5 mM L-lysine. Black arrow: ACTH 1–39; white arrow: human-β-endorphin.

in account since these products have been shown to be synthesized by the PAS positive cells (Van Eys *et al.*, 1983). For the same reason peak P is not discussed in the HPLC pulse-chase studies (% of incorporated label varied between 33.25 and 37.87).

A pulse labeling of 30 min, followed by chases of increased duration, showed the appearance of the 30 K product during the pulse period. During the chases with unlabeled L-lysine, this 30 K product was processed into a number of smaller products as was demonstrated by SDS gel electrophoresis (Fig. 4) and HPLC analysis (Fig. 5). The SDS gels showed a decrease of the 30 K product accompanied by the appearance of 19 K and 16 K products, which appeared to be processed in even smaller 13 and 3.2 K products. HPLC analysis showed a decrease of peak I to be accompanied by the appearance of products represented by peaks II, V and VII after 30 min chase. Peak II was maximal after 60 min chase, whereas peaks V and VII started to diminish after a 240 min chase period. The amount of newly synthesized products represented by peaks III, IV, XI, XII and XIII increased during the entire chase period. Label incorporation in products represented by peaks VI, VIII,

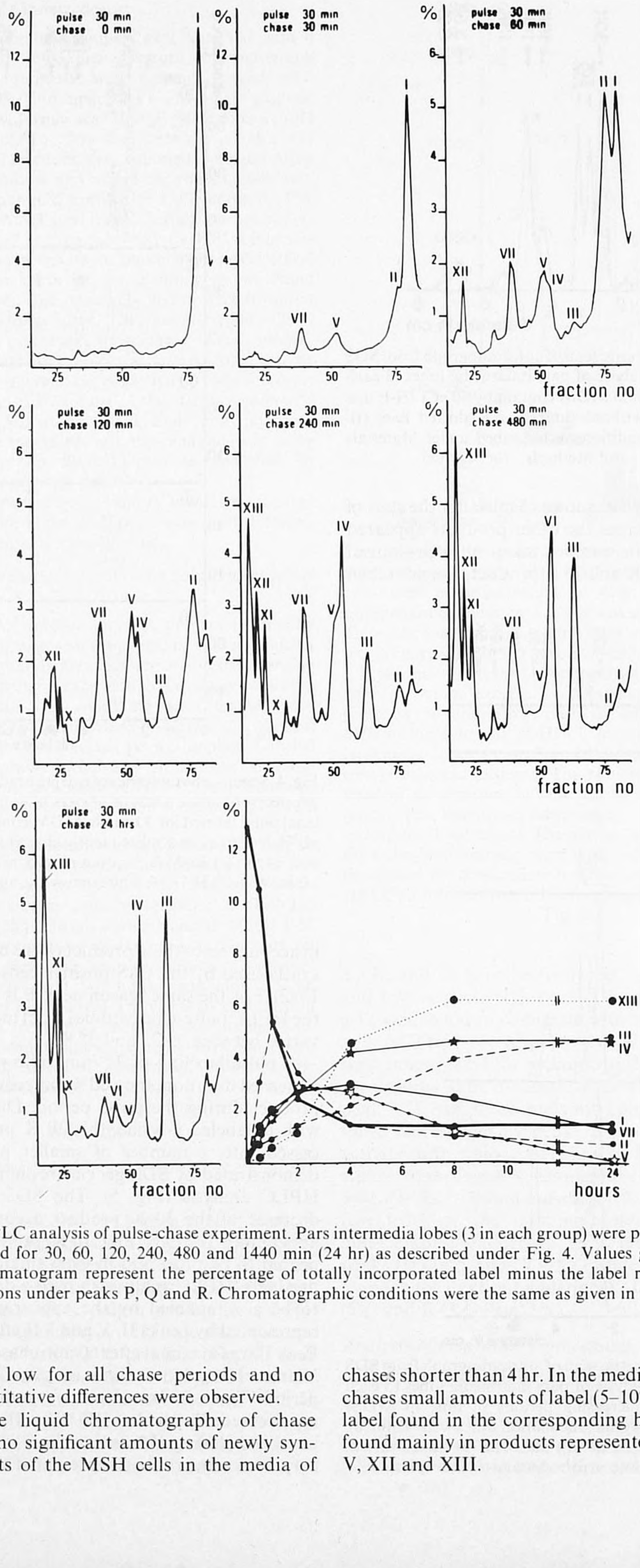

Fig. 5. HPLC analysis of pulse-chase experiment. Pars intermedia lobes (3 in each group) were pulse-labeled and chased for 30, 60, 120, 240, 480 and 1440 min (24 hr) as described under Fig. 4. Values given in the radiochromatogram represent the percentage of totally incorporated label minus the label recovered in fractions under peaks P, Q and R. Chromatographic conditions were the same as given in Fig. 1a.

IX and X was low for all chase periods and no noticeable quantitative differences were observed.

High pressure liquid chromatography of chase media revealed no significant amounts of newly synthesized products of the MSH cells in the media of chases shorter than 4 hr. In the media of the 8 and 24 hr chases small amounts of label (5–10% of the amount of label found in the corresponding homogenates) were found mainly in products represented by peaks III, IV, V, XII and XIII.

DISCUSSION

The results of autoradiography at the light microscopical level of the pars intermedia of *Sarotherodon mossambicus* (Van Eys *et al.*, 1983) as well as the results of the study on the incorporation of labeled lysine (Van Eys, 1981) show that the biosynthetic activity of the MSH cells continues after transfer of the pars intermedia lobes into the incubation medium.

A number of products of the MSH cells may be tentatively identified. Peak I, the 30 K product, was the first to appear during pulse labeling. It was apparently processed during chase incubations as it disappeared simultaneously with the appearance of smaller labeled products in the homogenates. Further, no significant quantities of the 30 K product were found in the chase media. Therefore, the 30 K product is likely to function as a precursor. The 30 K is somewhat smaller than the precursors reported for mammals and amphibians (Crine *et al.*, 1981; Roberts and Herbert, 1977a; Martens *et al.*, 1982). This may be accounted for by the lack of glycosylation, since neither incorporation of glucosamine nor changes in molecular weight as a result of tunicamycin treatment were observed. This conclusion seems to be in agreement with the observed minimal stainability of the MSH cells with periodic acid–Schiff (see also: McLean and Lowry, 1981). Notwithstanding some indications, so far we have been unable to find conclusive evidence for the existence of two genetically different precursor forms, as reported in mammals and amphibians (Crine *et al.*, 1980, 1981; Loh, 1979).

Peak IV comigrated on SDS and acid urea gels with human-β-endorphin and the material under peaks III and IV reacted strongly with anti-salmon-β-endorphin II. These two products are therefore considered to be endorphin-like peptides. The crossreactivity of the antiserum with products represented by peak II indicates a biochemical relationship between the products under peak II and those represented by peaks III and IV. Reactivity found under peaks R, IX and VIII can not be explained within the context of these experiments. Based on co-migration found on SDS and acid urea gels peak V is suggested to contain an ACTH-like peptide. The intermediary character, as concluded from the pulse-chase experiments, seems to be in agreement with the co-migration patterns. The products represented by peaks VII, X and XIII had melanotropic activity. Of these three products, the one represented by peak X had the same elution time as synthetic α-MSH, which indicates that this product is acetylated α-MSH. The exact nature of the other two melanotropic products could not be derived from our experiments. But data published by Martens *et al.* (1983) and McLean and Lowry (1981) indicate that peaks XI, XII and XIII may contain des-acetyl-α-MSH and a γ-MSH-like peptide.

The results of the pulse-chase experiments suggest that the above mentioned products are biosynthetically interrelated and originate from the MSH cells. The 30 K precursor is processed into a number of intermediate products of which the slightly endorphin-crossreactive product under peak II and the ACTH-like and melanotropic products represented by peaks V and VII respectively are the most prominent ones. The HPLC data indicate that the latter two may function as intermediates for products represented by peaks X through XIII, since the decrease of those two peaks coincides with the increase of peaks XIII, XII, XI and slightly X. The endorphin-like products (peaks III and IV), which seem to be the result of the processing of products under peak II, and the melanotropic products X–XIII appear to be final products, since the amount of newly synthesized peptides represented by these peaks increases during the entire chase period. Such results are largely in agreement with those of *in vitro* experiments done with pars intermedia lobes or cell lines of higher vertebrate species, in which it has been shown that MSH, ACTH and endorphins originate from a common precursor (Roberts and Herbert, 1977a, b; Mains and Eipper, 1979, 1980; Mains *et al.*, 1977; Nakanishi *et al.*, 1979, 1980; Loh and Gainer, 1977; Jenks *et al.*, 1979; Crine *et al.*, 1980). In addition, our results add to the increasing amount of evidence that points towards melanotropic and endorphin-like peptides as final products of precursor processing in the pars intermedia cells (Mains and Eipper, 1979, 1980; Gianoulakis *et al.*, 1979; Loh, 1979; Crine *et al.*, 1980).

The teleost pars intermedia has been shown to contain substances similar to those found in other vertebrate groups. For example, melanotropic activity was found in pituitary homogenates of the eel and rainbow trout (Baker, 1972; Baker and Ball, 1975), whereas Scott and Baker (1975), using a radioimmunoassay system, reported the presence of immunoreactive material to anti-α-MSH and anti-ACTH antisera in the pars intermedia of rainbow trout. More recent investigations have demonstrated the presence of substances with endorphin(opiate)-like activity in 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). In addition, melanotropic and endorphin-like peptides have been isolated from teleost pituitaries and characterized by Kawauchi *et al.* (1979, 1980a, b, c, d). They showed that the differences in primary structure between mammalian and teleost peptides are limited. Although all these findings point to a great similarity with respect to processes in the pars intermedia cells among higher vertebrates, no data are available concerning the biosynthesis and the interrelationship of these peptides in fish.

In *Sarotherodon mossambicus* we previously reported the presence of substances immunoreactive to antisera directed against α-MSH, ACTH 1-24 and β-endorphin in the MSH cells (Van Eys and Van den Oetelaar, 1981). We showed that granules contained substances reacting with all three antisera and therefore we suggested a biochemical relationship between these substances similar to that found for other vertebrates. In this report we have demonstrated such a relationship and defined some of the characteristics of a number of the products of the MSH cells. Our data suggest that biosynthesis in the MSH cells of the teleost pars intermedia follows a similar pattern as in other vertebrates and ultimately results in similar final products. However, further investigations may reveal that in addition minor differences in primary structure of the peptides synthesized by teleost MSH cells, there may be differences in processing, as indicated by the lack of glycosylation of the precursor.

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