

# Biosynthesis, Processing, and Control of Release of Melanotropic Peptides in the Neurointermediate Lobe of *Xenopus laevis*

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**ABSTRACT** The neurointermediate lobes of dark-adapted toads *Xenopus laevis* were incubated for 30 min in [<sup>3</sup>H]arginine and then "chased" for various time periods. By use of this pulse-chase paradigm there were detected 10 trichloroacetic acid (TCA)-precipitable peptides separated on acid-urea polyacrylamide gels and one TCA-soluble peptide separated by high-voltage electrophoresis (pH 4.9) with melanotropic activity. Each of these peptides had a different degree of melanocyte stimulating hormone (MSH) activity as revealed by the *Anolis* skin bioassay. Three of these TCA-precipitable peptides comigrated with ACTH,  $\beta$ -lipotrophin, and  $\alpha$ -MSH on acid-urea gels. Evidence suggesting a precursor-product mode of biosynthesis of the melanotropic peptides is presented. 7 of the 10 TCA-precipitable peptides and the one TCA-soluble peptide with melanotropic activity were released into the medium. The half-time of release of the TCA-precipitable peptides was about 2 h, whereas the half-time of TCA-soluble peptide release was about 30 min. The release of these peptides was inhibited by  $5 \times 10^{-5}$  M dopamine. Dopamine inhibition of release did not appear to affect the biosynthesis of the melanotropic peptides, but did appear to enhance the degradation of the newly synthesized TCA-soluble peptide in the tissue. White adaptation of the toads greatly decreased the biosynthesis of all of the TCA-precipitable melanotropic peptides.

## INTRODUCTION

Several types of melanotropic peptides have been found in the pars intermedia of lower vertebrates and mammals (Burgers, 1963; Preslock and Brinkley, 1970; Hopkins, 1972; Baker, 1973). These peptides, including  $\alpha$ - and  $\beta$ -melanocyte stimulating hormone (MSH), all contain a heptapeptide amino acid sequence, -Met-Glu-His-Phe-Arg-Trp-Gly-, which appears to be the "active core" essential for melanotropic activity (i.e. to cause the expansion of melanocytes) (Eberle and Schwyzer, 1975). Adrenocorticotrophic hormone (ACTH) and  $\beta$ -lipotrophin ( $\beta$ -LPH) also are found in the pars intermedia, possess melanotropic activity, and contain this heptapeptide sequence. Indeed, it has been proposed that ACTH and  $\beta$ -LPH are the precursors of  $\alpha$ - and  $\beta$ -MSH, respectively, in the pars intermedia (Scott et al., 1973; Lowry and Scott, 1975; Bradbury et al., 1976). Although the biosynthesis of both ACTH and  $\alpha$ -MSH has recently been demonstrated in the rat neurointermediate lobe (Scott et al., 1976), systematic pulse-

chase experiments showing the conversion of ACTH to  $\alpha$ -MSH in situ have not been done.

Another significant question relates to the mechanism of control of release and biosynthesis of the melanotropic peptides. Morphological studies have shown that two types of nerve fibers from the hypothalamus end on the secretory cells in the pars intermedia: one type contains electron-dense catecholaminergic vesicles, and the other contains large neurosecretory granules presumed to be peptidergic (Dawson, 1953; Etkin, 1962; Iturriza, 1964; Saland, 1968; Nakai and Gorbman, 1969; Hopkins, 1971). Although there have been proposals that hypothalamic peptides, i.e. melanocyte-stimulating hormone releasing factor (MSH-RH) and melanocyte inhibitory factor (MIF) may regulate MSH release (Taleisnik and Orias, 1965; Taleisnik and Tomatis, 1967; Kastin and Schally, 1966; Celis et al., 1971) there have also been extensive studies showing that MSH release is under hypothalamic inhibitory control by catecholamines (Iturriza and Kasal-Iturriza, 1972; Hadley et al., 1973, 1975; Bower et al., 1974; Olsson and Gorbman, 1973; Baker, 1976). A morphological-histochemical study by Terlou and his co-workers (Terlou and Van Straaten, 1973; Terlou and Van Kooten, 1974), concluded that the MSH-producing cells are under monoaminergic control. The catecholamine in *Xenopus* pars intermedia has been identified as dopamine (Terlou and Van Kooten, 1974). The results of various pharmacological studies (Tanzer and Thoenen, 1968; Smith, 1975; DeVolcanes and Weatherhead, 1976) have lent support to the hypothesis that the release of MSH is under inhibitory control by dopamine. The effect of dopamine to inhibit release raises the question of whether there is an inhibition of the biosynthesis of MSH by dopamine. Studies by Thornton (1974) suggested that there was no such effect of dopamine on phenylalanine incorporation into the toad neurointermediate lobe; however, direct evidence on the effect of dopamine on MSH synthesis is lacking.

The first part of this paper describes our studies on the heterogeneity and biosynthesis of melanotropic peptides in the neurointermediate lobe of the South African clawed toad *Xenopus laevis*. Some evidence is presented, from "pulse-chase" labeling studies, suggestive of a precursor-product mode of biosynthesis of the melanotropic peptides. The second part considers the mechanisms and time courses of release of the melanotropic peptides, their identification and correlation with the peptides synthesized in the neurointermediate lobe, and the regulation of the biosynthesis and release of these peptides by dopamine.

#### MATERIALS AND METHODS

##### *Animals*

Adult toads (*Xenopus laevis*) weighing 40–70 g were obtained from Amphibian Facilities, Ann Arbor, Mich., and maintained in plastic aquaria at 22°C. Before use in experiments, the animals were placed on either a white (white-adapted) or a black (dark-adapted) background for 7–15 days. Except where otherwise indicated, dark-adapted animals were used for the biosynthesis experiments and white-adapted animals were used for the bioassay experiments.

### *Incubation of Neurointermediate Lobes in [<sup>3</sup>H]Arginine*

Neurointermediate lobes were dissected from the toads and preincubated at 22°C in amphibian Ringer (NaCl, 112 mM; KCl, 2 mM; CaCl<sub>2</sub>, 2 mM; HEPES, 15 mM; glucose, 5 mg/ml; bovine serum albumin, 1 mg/ml; ascorbic acid, 1 mg/liter; pH 7.35) for 1 h to maximize melanotropic peptide biosynthesis *in vitro* (Hopkins, 1970). The neurointermediate lobes were then "pulse" incubated in the presence of 17.5 μM [<sup>3</sup>H]arginine (New England Nuclear, Boston, Mass., sp act = 28.7 Ci/mmol) for 30 min. After the "pulse," the neurointermediate lobes were either homogenized immediately in 0.1 N HCl or "chased" in amphibian Ringer in the presence of unlabeled arginine (1 mM) for varying time periods before homogenization in 0.1 N HCl. For the studies on the regulation of biosynthesis and the release of MSH, 5 × 10<sup>-5</sup> M dopamine (the concentration found to be effective in inhibiting MSH release [Bower et al., 1974]) was included in the pulse and/or chase medium. The chase medium was analyzed for melanotropic peptide released in the presence and absence of dopamine. After the incubation, HCl was added to the medium to a final concentration of 0.1 N.

### *Preparation of the Tissue Homogenate and Medium for Analysis*

The above homogenate (or medium sample) was heated at 100°C for 10 min, and 50 μg of bovine serum albumin was added to the solution as carrier. Proteins in the homogenates and media samples were then precipitated by cold 10% trichloroacetic acid (TCA). The TCA-precipitable fractions were separated from the TCA-soluble fractions by centrifugation at 12,000 *g* for 5 min in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). TCA was removed by extensive washing of both fractions with ether. The TCA-soluble peptides were then passed through a copper Sephadex column (Fazakerly and Best, 1965) to remove the unincorporated [<sup>3</sup>H]arginine. This was followed by gel filtration on a Sephadex G10 column (using 5% acetic acid as the eluant). The excluded volume (*V*<sub>E</sub>) and the included volume (*V*<sub>I</sub>, <700 daltons) of the Sephadex G10 were then collected for analysis.

### *Analysis of the TCA-Precipitable Peptides*

The TCA-precipitated pellet from tissue or medium was dissolved in 50 μl of 0.9 M acetic acid in 5 M urea. A 5-μl sample was then taken for radioactive counting. The remainder of the sample was analyzed by electrophoresis on 11% acid-urea, polyacrylamide (11 cm × 4 mm) cylindrical gels (Davis et al., 1972). 5 μg of cytochrome *c* was included in the sample as an internal standard marker. The run was terminated when the cytochrome *c* ran 5 cm. Gels were stained with Coomassie blue, destained, and sliced into 1.5-mm slices. Each slice was eluted in scintillation cocktail and counted for its radioactivity in a Beckman LS 330 scintillation counter. Details of the gel electrophoresis and counting procedures have been described elsewhere (Loh and Gainer, 1975). When the molecular weights of the TCA-precipitable peptides were to be determined, the labeled peptides on unstained acid-urea gel were cut out, and the peptides were eluted and re-electrophoresed on polyacrylamide gels in sodium dodecyl sulfate (SDS). Details regarding the SDS gel electrophoresis procedures have been previously reported (Gainer, 1971).

### *Analysis of TCA-Soluble Peptides*

A sample of the TCA-soluble peptides from the *V*<sub>E</sub> and *V*<sub>I</sub> of the G10 run was taken for radioactive counting and the remainder was lyophilized and resuspended in 20 μl of 5% acetic acid in methanol. This sample was then spotted on thin layer cellulose plates (250 μm) and separated by high-voltage electrophoresis with a pH 4.9 buffer (glacial acetic acid:pyridine:H<sub>2</sub>O; 45:50:900). Methyl green was used as a tracking dye during the

electrophoretic run. At the end of the run, the plates were air dried and scraped into 1-cm segments for analysis of their radioactivity.

#### *Bioassay of Melanotropic Peptides*

For the bioassay experiments, neurointermediate lobes from white-adapted toads known to store MSH granules (Hopkins, 1970) were used. The lobes were homogenized and treated as described above. TCA-precipitable peptides were eluted from 1.5-cm slices of the acid-urea gel after electrophoresis and bioassayed. TCA-soluble peptides were eluted from the cellulose plates (1-cm segments) after high-voltage electrophoresis. Peptides were eluted with *Anolis* medium and melanotropic activity was determined with the *Anolis* skin bioassay as described by Tilders et al. (1975). This method has been shown to be very sensitive and specific for MSH and related molecules. Specimens of *Anolis carolinensis* were obtained from Mogul-Ed Corp., Oshkosh, Wis.).

### RESULTS

#### *Incorporation of [<sup>3</sup>H]Arginine into Neurointermediate Lobe Peptides*

Comparison of the [<sup>3</sup>H]arginine incorporation into TCA-precipitable peptides in the neurointermediate lobe from white-adapted and dark-adapted toads is illustrated in Fig. 1. Dark-adapted lobes exhibited a linear incorporation of [<sup>3</sup>H]arginine with time, from 5 to 150 min of incubation (Fig. 1, filled circles). In contrast, lobes from white-adapted animals appeared to be nonlinear in their incorporation of [<sup>3</sup>H]arginine (Fig. 1, open circles). After 30 min of incubation in [<sup>3</sup>H]arginine, the TCA-precipitable radioactivity in dark-adapted lobes was five-fold greater than in white-adapted lobes. After 150 min of incubation, the ratio of radioactivity between the lobes fell to about 2.5. The increased incorporation rates in the lobes of the dark-adapted vs. white-adapted animals are consistent with the well-known metabolic responses of this gland to enhance MSH synthesis and release under dark-adapting conditions (Hopkins, 1970; Thornton, 1974). Neurointermediate lobes of dark-adapted animals were used in the pulse-chase experiments described below. The arginine chase data illustrated in Fig. 1 (filled squares, broken line) show that a chase in 1 mM unlabeled arginine after a 30 min incubation in [<sup>3</sup>H]arginine effectively prevented any further incorporation of [<sup>3</sup>H]arginine into TCA-precipitable peptides.

#### *Evidence for the Processing of Newly Synthesized Polypeptides in the Neurointermediate Lobe*

Fig. 2 illustrates the labeling patterns of TCA-precipitable peptides extracted from neurointermediate lobes which had been pulse incubated in [<sup>3</sup>H]arginine for 5, 15, and 30 min and separated by electrophoresis on acid-urea gels. In all three cases, the same labeled peak (designated *a*) dominates the profile, and no consistent qualitative differences in profile could be determined. Peak *i* is inconsistently seen in pulse experiments and therefore cannot be considered a significant difference between the 30-min and 5-min pulse profiles. Peak *a* also appears to be synthesized in white-adapted lobes (Fig. 2) although at much lower levels. Because of the similarities in profile between the 5-min and 30-min pulses, all subsequent pulse-chase experiments were done by use of 30-min pulse durations.

The fates of the peptides synthesized during a 30-min pulse were examined by chase incubations of the pulsed lobes for durations ranging from 15 min to 180 min. The results of these experiments are depicted in Fig. 3. After the 30-min pulse, peak *a* (Fig. 3, upper left panel) contained about 30% of the total

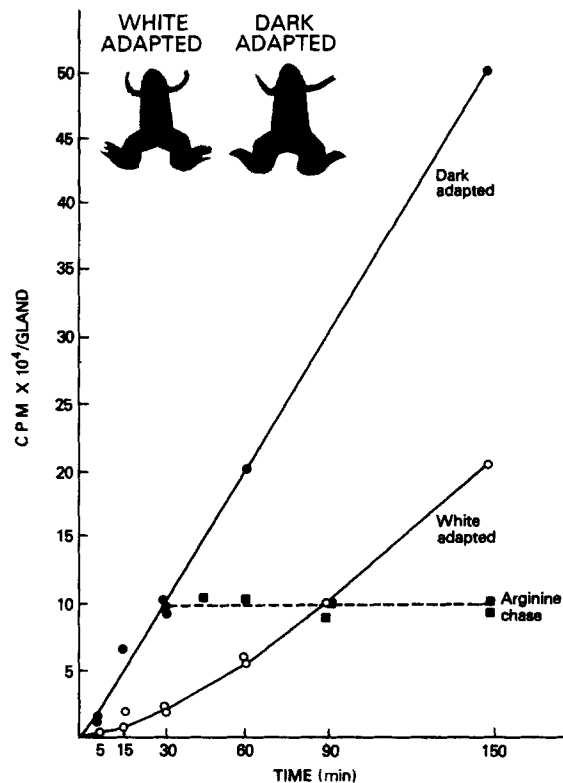


FIGURE 1. Time course of incorporation of [<sup>3</sup>H]arginine into TCA-precipitable peptides by neurointermediate lobes of dark-adapted and white-adapted toads. The ordinate shows the TCA-precipitable cpm per gland, and the abscissa the period (in minutes) of incubation. Note that the incorporation in dark-adapted animals (solid circles) is linear for 150 min and approximately five times greater than for white-adapted animals (open circles) in the first 30 min. The incorporation into the white-adapted animals appears to be nonlinear, with the slope increasing significantly after 60 min. When the lobes were chased in unlabeled arginine after a 30-min pulse in [<sup>3</sup>H]arginine, the further incorporation of [<sup>3</sup>H]arginine was completely inhibited, as shown by the dotted line (solid squares). The *inset* in the upper left corner shows the change in the animal's color after 2 wk of adaptation to a black or white background.

radioactivity on the acid-urea gel. In the subsequent chase periods (Fig. 3, 15–180 min) peak *a* progressively decreased in radioactivity concurrently with the emergence of several new labeled peaks (i.e. peaks *b–j*). After prolonged chase incubations (60–180 min), peak *a* was greatly decreased and peak *c* now dominated the profile. Significant radioactivity was also present in peak *e*. The

labeling profiles in Fig. 3 are suggestive of a processing of a larger precursor (peak *a*) to peptide products (peaks *b-j*) with time in the neurointermediate lobe. This suggestion is supported by the molecular weight analyses of the various peaks (Table I) discussed below. Since so many labeled peptides appear during the chase, it is not possible at present to describe the specific processing sequence.

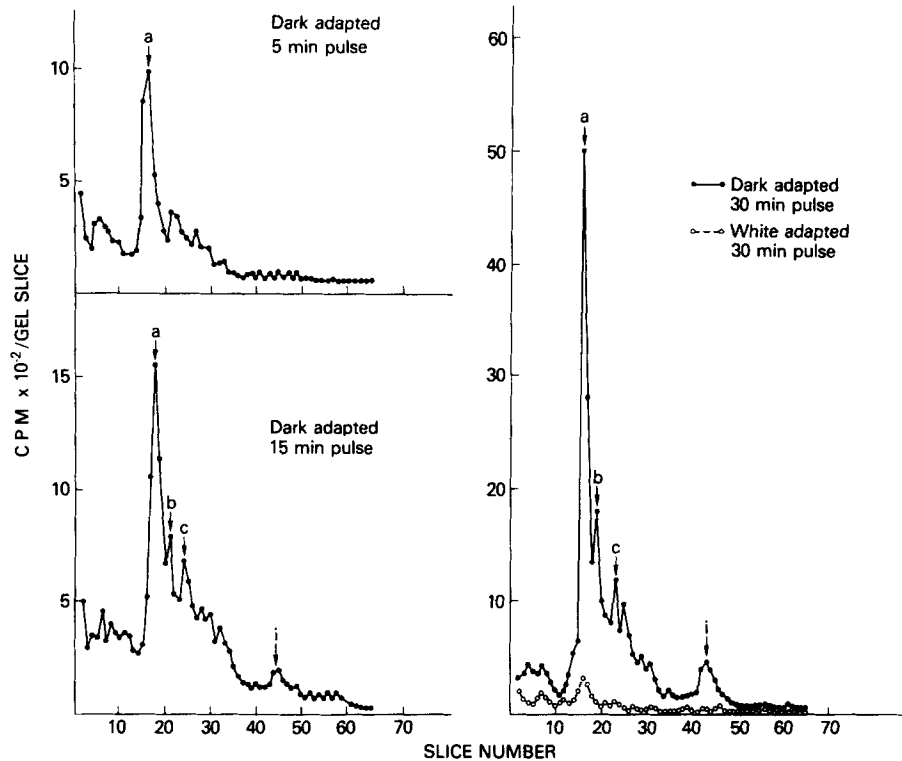


FIGURE 2. Acid-urea gel labeling profiles of TCA-precipitable peptides of neurointermediate lobes after 5-, 15-, and 30-min pulses in [ $^3\text{H}$ ]arginine using neurointermediate lobes from dark-adapted toads and a 30-min pulse of lobes from white-adapted animals. The ordinate shows the cpm/gel slice, and the abscissa the slice number. Peak *a* is the dominant peak in all the profiles.

This is further complicated by the appearances of peaks *k* and *l* after prolonged chases, since these peptides are clearly of higher molecular weight than peak *a*.

#### *Characterization and Identification of the Labeled Peaks*

Table I summarizes the  $R_f$  values of the various peaks seen in Fig. 3 ( $R_f$  equals the mobility relative to cytochrome *c* on acid-urea gels) and the molecular weights of some of these peaks as evaluated by electrophoresis on SDS gels. The molecular weight of peak *a* was 36,000 daltons. Since peaks *b-j* are clearly of lower molecular weight, these data are consistent with the notion of a precursor-product relationship between the polypeptide(s) represented by peak *a* and the

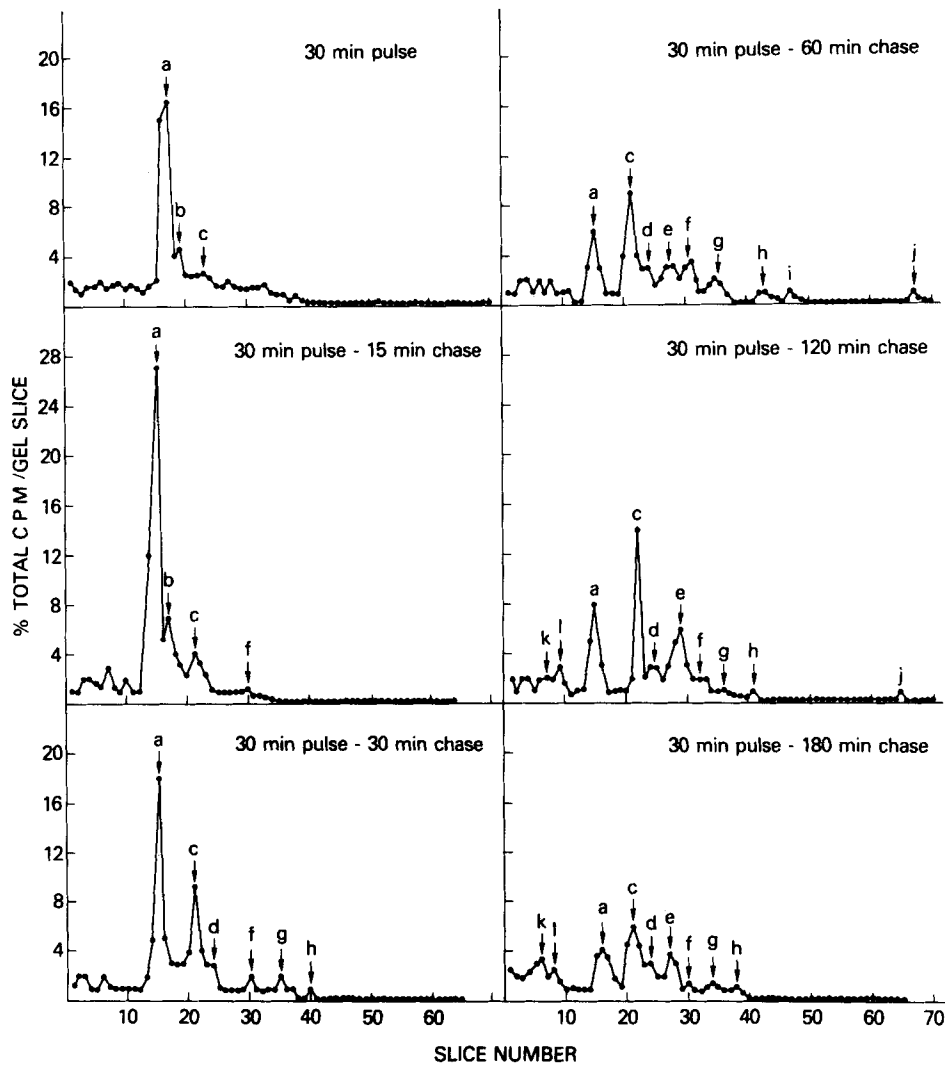


FIGURE 3. Acid-urea gel labeling profiles from pulse-chase experiments with neurointermediate lobes from dark-adapted animals. Each lobe was pulsed in [ $^3\text{H}$ ]arginine for 30 min and chased in unlabeled arginine for various times (ranging from 0 to 180 min). Only TCA-precipitable peptides were electrophoresed. The ordinate shows the percent total cpm on the gel/gel slice, and the abscissa shows the slice number (see text for details).

smaller peptides. Several of the labeled peaks comigrated on acid-urea gels with certain known melanotropic peptides. Purified  $\beta$ -LPH, ACTH,  $\beta$ -MSH, and  $\alpha$ -MSH had been found in preliminary experiments to be TCA precipitable and had  $R_f$  values equal to  $1.01 \pm 0.01$  (peak *g*),  $1.30 \pm 0.02$  (peak *i*),  $1.60 \pm 0.02$ , and  $1.72 \pm 0.02$  (peak *j*), respectively. In addition, the estimated molecular weights of peak *g* and peak *i* (Table I) compare favorably with the known molecular weights

of  $\beta$ -LPH and ACTH, respectively. On the basis of these correlations and the fact that all of these peaks are released by the neurointermediate lobe and have potent melanotropic activity, we suggest that the toad pars intermedia synthesizes, stores, and releases  $\beta$ -LPH, ACTH, and  $\alpha$ -MSH. Although there is some melanotropic activity on the gels with an  $R_f$  equal to 1.6 (i.e. corresponding to  $\beta$ -MSH), we have not detected a labeled peak in this region of the gel. In addition to these tentatively identifiable peaks, the toad neurointermediate lobe also synthesizes and releases at least four additional (unidentified) TCA-precipitable, melanotropic peptides (Table I).

TABLE I  
ANALYSIS OF TCA-PRECIPITABLE PEPTIDES SYNTHESIZED IN THE  
NEUROINTERMEDIATE LOBE OF THE TOAD\*

Labeled peak	$R_f$ (acid-urea gels)	Molecular weight	Released‡	Bioassayable§ melano- tropic activity
a	0.46±0.02 (23)	36,000±2,500 (3)	0	++
b	0.58±0.01 (12)	21,000±1,400 (2)	0	0
c	0.65±0.02 (23)	18,000 (1)	+	++
d	0.74±0.03 (11)	12,000±2,700 (3)	0	+
e	0.83±0.02 (13)	9,000 (1)	+	+
f	0.91±0.008 (12)	14,000±3,200 (3)	0	0
g	1.01±0.01 (12)	9,000±2,300 (2)	+	+++
h	1.2 ±0.01 (6)	—	0	0
i	1.31±0.036 (10)	4,300 (1)	+	+++
j	1.74±0.03 (5)	—	+	+++
k	0.16±0.006 (3)	—	+	+++
l	0.27±0.01 (3)	—	+	+

\* The labeled peaks are illustrated in Figs. 2-5.  $R_f$  represents the mobility of each peak on acid-urea gels relative to cytochrome *c*. Molecular weights were determined by elution of peaks from acid-urea gels, and subsequent electrophoresis on SDS gels (see Materials and Methods).  $R_f$  and molecular weight expressed as means ± SE. (The numbers in parenthesis represent the number of determinations). The  $R_f$  values on acid-urea gels for  $\beta$ -LPH, ACTH,  $\beta$ -MSH, and  $\alpha$ -MSH were 1.01 ± 0.01, 1.30 ± 0.02, 1.60 ± 0.02, and 1.72 ± 0.02, respectively.

‡ Peptides released into the medium denoted by +; not released, denoted by 0.

§ Bioassayable activity was determined by the *Anolis* assay (Tilders et al., 1975). 0 = no activity, + = 1 *Anolis* unit (AU), ++ = 10 AU, +++ = 100 AU. 1 AU is equivalent to the melanocyte response to 10<sup>-12</sup> M synthetic  $\alpha$ -MSH.

#### *Time Course and Dopamine Control of Release of TCA-Precipitable Peptides*

The pulse-chase experiments described in Fig. 3 showed that there was a redistribution of the radioactivity into different peaks with time. In addition, there was a slow decline of absolute TCA-precipitable radioactivity in the tissue simultaneous with an increase of TCA-precipitable radioactivity in the medium after long-term chases. These data are illustrated in Fig. 4. The release of the labeled peptides into the medium appeared to have a lag time. Release first became apparent 2 h after chase and was completed about 2 h later (i.e. after 3-4 h of chase). About 30% of the total TCA-precipitable radioactivity in the tissues after the pulse appeared in the medium after 3 h of chase. This lag time of 60 min for release of TCA-precipitable peptides is consistent with the observation



(in Fig. 3) that it takes about this long for the processed products (peaks *c-j*) to appear in the tissue.

The labeled peptides in the medium after 3 h of chase were analyzed by acid-urea gel electrophoresis. The labeling profile of the TCA-precipitable peptides in the medium is shown in Fig. 5. Seven peaks of radioactivity (peaks *c, e, g, i, j, k, and l*) were released (Fig. 5, filled circles) from the neurointermediate lobe, and each of these peaks ( $R_f$  values) contained melanotropic activity in the bioassay experiments on tissues from white-adapted animals (see Fig. 5 and

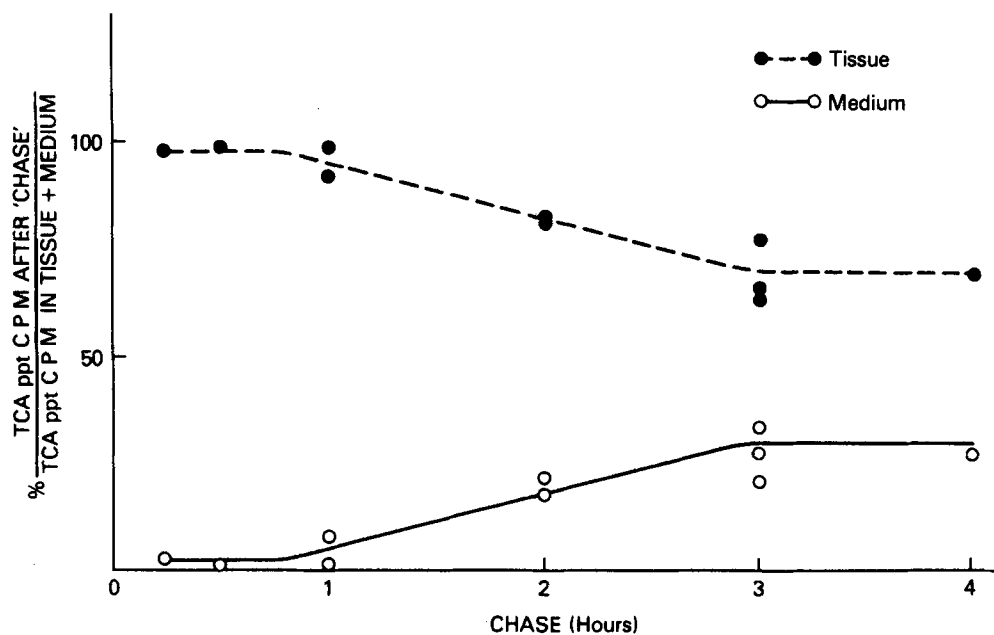


FIGURE 4. TCA-precipitable cpm in the tissue (filled circles) and medium (open circles), expressed as a percent of TCA-precipitable cpm in tissue plus medium with time of chase (abscissa). The labeled TCA-precipitable peptides in the tissue decrease after 1 h of chase to a steady value at 3 h. Labeled TCA-precipitable peptides first appear in the medium after 1 h of chase, and approach a steady value after 3 h.

Table I). Peaks *g, i, j, and k* appeared to have the most potent melanotropic activity. The apparent release of these peaks after synthesis may explain to some extent the variable presence of certain labeled peaks (such as peak *i*) in the tissue after pulse labeling. The release of these labeled peptides is completely inhibited if  $5 \times 10^{-5}$  M dopamine is included in the chase medium (Fig. 5, open circles). In the dopamine chase, only a small amount of the putative precursor, peak *a*, appears to have been released. Since dopamine does not appear to alter the biosynthesis and processing steps (see discussion of Fig. 7, below) in the lobes, it is unlikely that the release of peak *a* in dopamine chase medium was due to a pile-up of precursor in the cell.

Fig. 6 shows the quantitative data relating to the dopamine inhibition. In the

control tissue, which was chased for 3 h (open bar),  $72\% \pm 4\%$  of total incorporated radioactivity remained in the tissue and  $28\% \pm 4\%$  was detected in the medium. In the presence of dopamine  $89\% \pm 4\%$  of total incorporated radioactivity remained in the tissue and  $6.3\% \pm 0.8\%$  was recovered in the medium. Thus the  $5 \times 10^{-5}$  M dopamine treatment results in a  $78\% \pm 2.8\%$  inhibition of release of TCA-precipitable peptides.

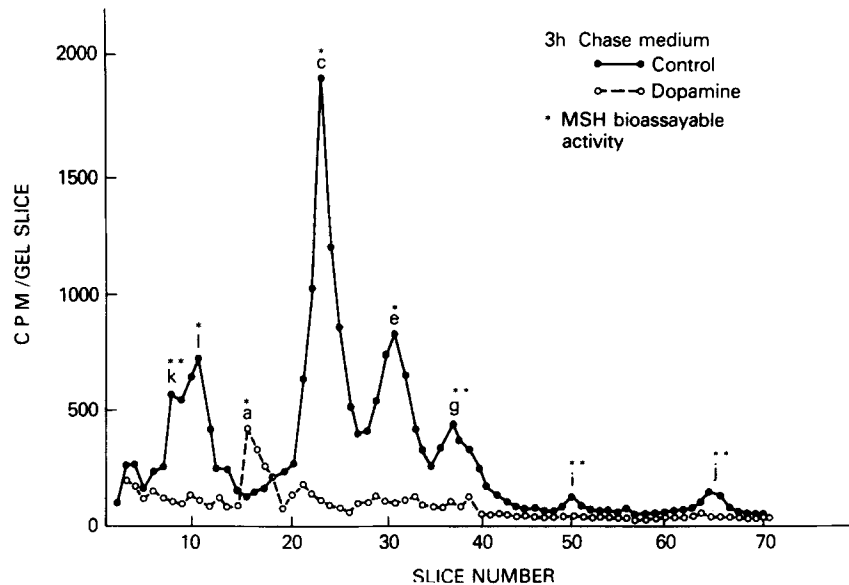


FIGURE 5. Acid-urea gel patterns of labeled TCA-precipitable peptides in the 3-h chase medium in the absence (solid circles) and presence of  $5 \times 10^{-5}$  M dopamine (open circles). The ordinate and abscissa are as described for Fig. 2. In the control (nondopamine treated),  $^3\text{H}$ -labeled peaks *k*, *l*, *c*, *e*, *g*, *i*, and *j* are released. In the medium from tissue which was pulsed and chased for 3 h in dopamine, there was inhibition of release except for a very small amount of peak *a*. Each of the released peaks comigrates with melanocyte-stimulating bioassayable activity. The extent of bioactivity is denoted by (\*), where (\*\*) > (\*). Bioassay data were obtained in experiments done on white-adapted animals.

#### *Lack of Effect of Dopamine on the Biosynthesis and Processing of TCA-Precipitable Peptides*

The above inhibition of release of the labeled peptides by dopamine raised the question of whether this was due to dopamine inhibition of the biosynthesis and/or processing mechanisms of the secretory cells. To test for these possibilities, lobes from dark-adapted animals were preincubated in  $5 \times 10^{-5}$  M dopamine and then pulsed (in [ $^3\text{H}$ ]arginine) for 30 min in the presence of  $5 \times 10^{-5}$  M dopamine (Fig. 7, filled circles). Another group of lobes were chased for 3 h in dopamine after the pulse (Fig. 7, open circles). The acid-urea gel labeling profile after a 30-min pulse in dopamine (Fig. 7, filled circles) was similar to that

observed in experiments in the absence of dopamine (see Fig. 2, and Fig. 3, upper left panel). Thus there appears to be no effect of dopamine on the incorporation of the [ $^3\text{H}$ ]arginine in these experiments. Similarly, the results of the chase incubation in dopamine (Fig. 7, open circles) show that there was no modification of the qualitative distribution of labeled peaks when compared to

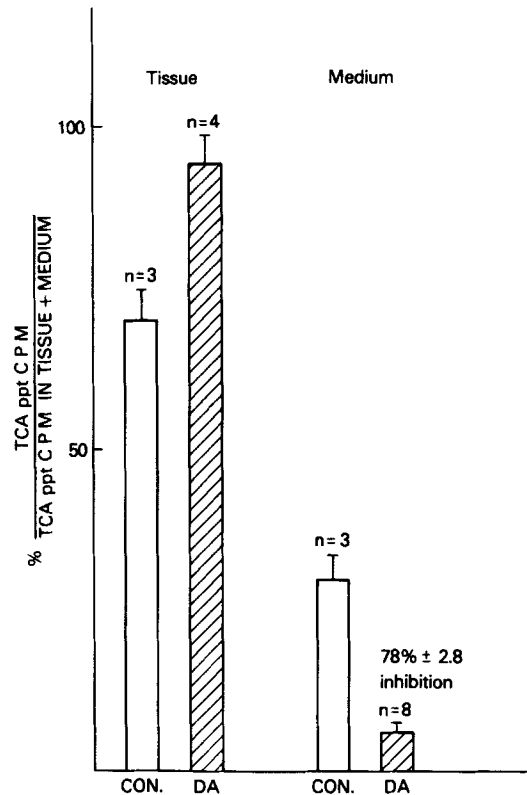


FIGURE 6. Bar graphs showing the effect of dopamine on the release of [ $^3\text{H}$ ]arginine-labeled TCA-precipitable peptides. In the control (open bar), 72%  $\pm$  4% of the radioactivity remained in the tissue, and 28%  $\pm$  4% was recovered in the medium after a 3-h chase. With  $5 \times 10^{-5}$  M dopamine in the medium during the 3 h chase (striped bars) 89%  $\pm$  4% of the radioactivity remained in the tissue and 6.3%  $\pm$  0.8% was recovered in the medium, representing a 78% inhibition of release by dopamine.

control chase experiments (see Fig. 3). The only difference is the apparent accumulation of the releasable peaks (seen in Fig. 5) in the dopamine-treated tissue (Fig. 7). This is consistent with the observed inhibition of release of these peaks by dopamine (Figs. 5, 6). Thus, in these relatively short-duration treatments of the lobes with dopamine, there appeared to be no effects on the biosynthesis or processing mechanisms in the tissue, and therefore, the dopamine appears to selectively inhibit the release mechanism.

*Time Course and Dopamine Control of Release of TCA-Soluble Peptides*

Fig. 8 shows that when neurointermediate lobes were pulsed for 30 min and then chased for various periods (1–4 h), there was a rapid decrease of TCA-soluble label in the tissue (solid circles) with time. The amount of labeled peptide recovered in the medium (open circles) reached a maximum after 1 h of chase.

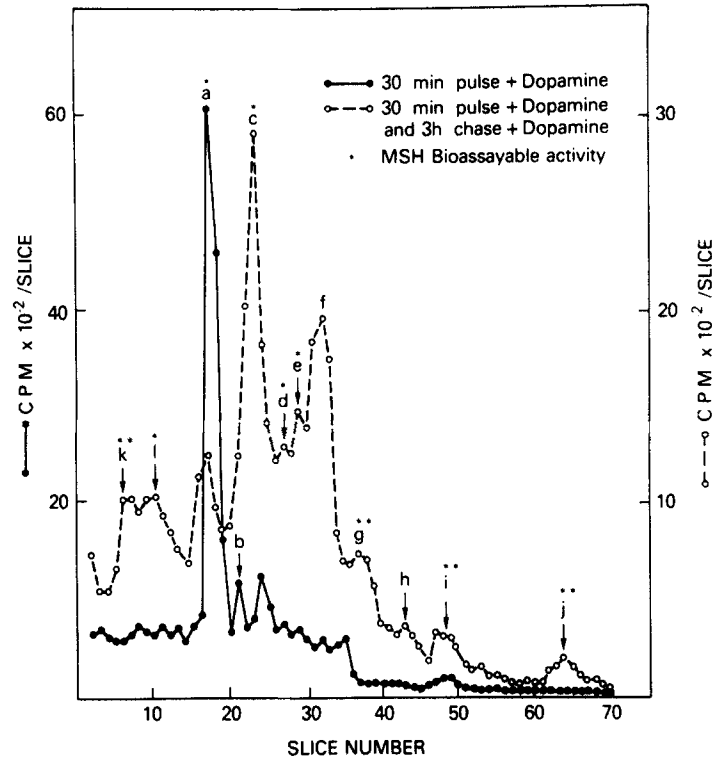


FIGURE 7. Acid-urea gel labeling patterns showing the effect of dopamine on the biosynthesis of melanocyte-stimulating peptides. The solid circles show the 30-min pulse profile after incubation in the presence of  $5 \times 10^{-5}$  M dopamine, while the open circles show the 30 min pulse-3 h chase profile in dopamine. The ordinates and abscissa are as described for Fig. 2. Neither the biosynthesis nor the processing mechanisms appear to be inhibited by dopamine. Note, however, that the products are accumulated in the tissue in the presence of dopamine. Each of the peaks marked by an asterisk (\*) comigrated with melanotropic bioactivity (\*\*>\*)).

Thus there appears to be a rapid release of TCA-soluble peptides from the tissue.

As with the TCA-precipitable peptides, the TCA-soluble peptide release mechanism was also inhibited by dopamine ( $5 \times 10^{-5}$  M). This is illustrated in Fig. 9. In the control tissue, which was chased for 3 h (open bars),  $9.5\% \pm 3.8\%$  of total TCA-soluble incorporated radioactivity remained in the tissue, whereas in the presence of dopamine there was virtually no release from the tissue.  $89.5\% \pm 4\%$  of the total TCA-soluble radioactivity was present in the control medium,

whereas in the dopamine-treated tissue  $2\% \pm 0.5\%$  of the TCA-soluble label was in the medium. Thus  $5 \times 10^{-5}$  M dopamine in the chase medium caused  $98\% \pm 0.5\%$  inhibition of the release of TCA-soluble peptides.

*Analysis of the Labeled TCA-Soluble Peptides Synthesized and Released by the Neurointermediate Lobe*

The labeled TCA-soluble peptides were fractionated into two groups by G10 Sephadex chromatography (see Materials and Methods). Bioassay for melanotropic activity of extracts of neurointermediate lobes from white-adapted toads

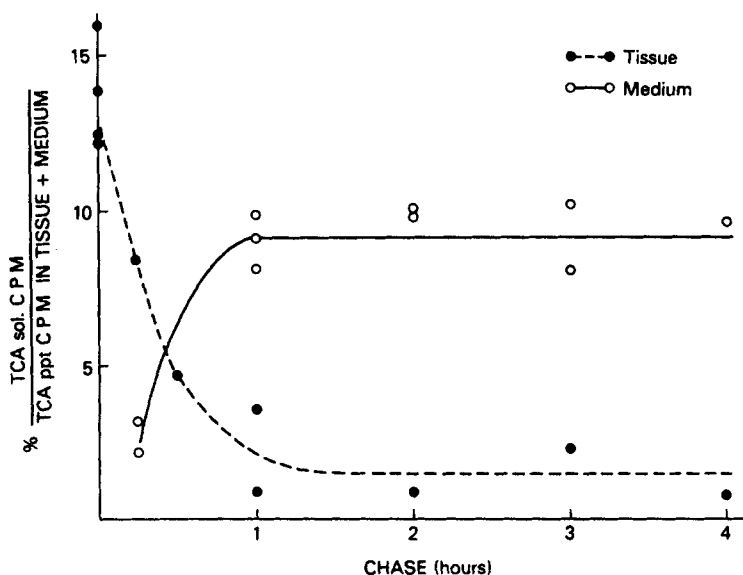


FIGURE 8. Release of labeled TCA-soluble peptides during the chase after a 30-min pulse labeling in [ $^3$ H]arginine. The ordinate shows the TCA-soluble cpm (in peptides, see Materials and Methods) as percent of the TCA-precipitable cpm in tissue plus medium. This was used as a method to normalize the data from different experiments. The labeled TCA-soluble peptides appear to be completely released by the tissue into the medium after about 1 h of chase.

showed that only the excluded volume ( $V_E$  fraction) was melanotropic. Therefore, only the  $V_E$  fraction (corresponding to molecular weights  $\geq 700$  daltons) of the labeled TCA-soluble peptides was separated by thin-layer high-voltage electrophoresis at pH 4.9. Fig. 10 shows the labeling patterns on the thin-layer plate after electrophoresis of the peptides found in lobes after a 30-min pulse in [ $^3$ H]arginine (filled circles) and in medium after a 30-min chase (open circles). In both samples there was a single *major* labeled peak (7 cm from the origin), which comigrated with the only bioassayable melanotropic peak found in TCA-soluble extracts of lobes from white-adapted animals. Two minor peaks of radioactivity (at the origin and 4 cm from the origin) which did not have melanotropic activity were also observed. Thus, the major labeled peak which has an  $R_f$  of 0.78 (relative to the mobility of methyl green on the plate) is both synthesized in the

tissue and released into the medium. This labeled peak does not correspond with either  $\alpha$ - or  $\beta$ -MSH which, in contrast, are TCA precipitable and have  $R_f$  values (on the cellulose plates) of 0.56 and 0.47, respectively.

*Effect of Dopamine on the Biosynthesis of TCA-Soluble Peptides*

Neurointermediate lobes were preincubated for 1 h in  $5 \times 10^{-5}$  M dopamine media and then pulsed in [ $^3$ H]arginine for 30 min in the presence of dopamine.

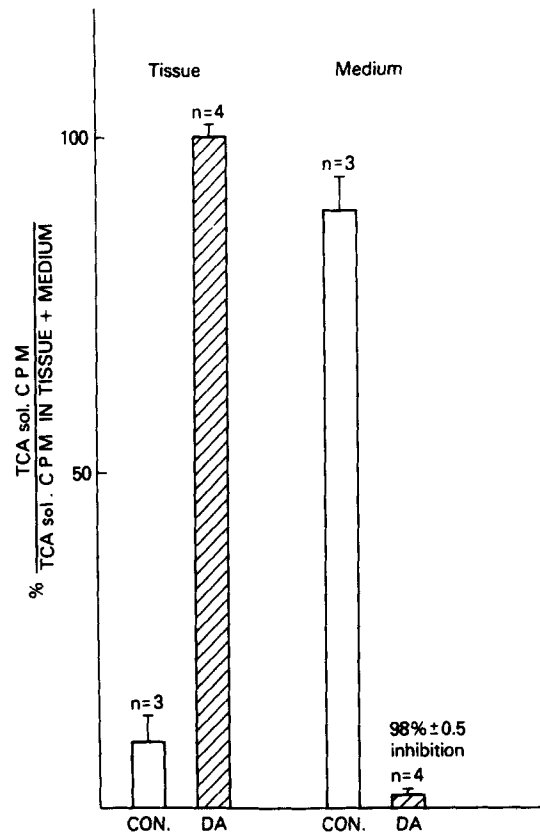


FIGURE 9. Bar graphs showing the effect of  $5 \times 10^{-5}$  M dopamine on the release of [ $^3$ H]arginine-labeled TCA-soluble peptides. The control (open bar) shows that  $9.5\% \pm 3.8\%$  of the total TCA-soluble radioactivity remained in the tissue after 3 h of chase and  $89.5\% \pm 4.5\%$  was released into the medium. With dopamine in the medium during the 3-h chase (hatched bars) only  $2\% \pm 0.5\%$  was released into the medium. This represents a  $98\% \pm 0.5\%$  inhibition of release of the TCA-soluble peptides by dopamine.

Comparison of the labeled TCA-soluble peptides (in the  $V_E$  fraction) synthesized in the presence and absence of dopamine is shown in Fig. 11. Separation of the control-pulsed peptides (Fig. 11, filled circles) revealed the typical major melanotropic peak ( $R_f = 0.78$ , seen also in Fig. 10 after a shorter electrophoretic separation). After dopamine treatment (Fig. 11, open circles) the labeling profile was quite different. The  $R_f$  0.78 peak was greatly reduced, whereas most of the

label appeared in a nonmelanotropic  $R_f$  0.26 peak. Therefore, unlike the case with the TCA-precipitable peptides, we cannot discount the possibility that the inhibition of release of the TCA-soluble peptides by dopamine was due to a modification of the biosynthesis mechanism (see Discussion).

## DISCUSSION

### *Biosynthesis of Melanotropic Peptides*

Studies on the incorporation of [ $^3\text{H}$ ]Arginine into TCA-precipitable peptides in

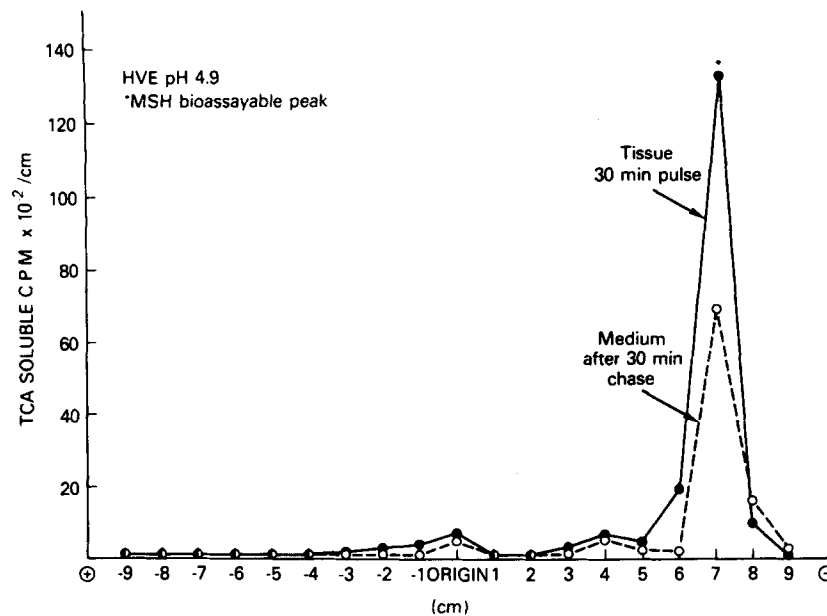


FIGURE 10. High-voltage electrophoresis (pH 4.9, cellulose thin-layer plates) of labeled TCA-soluble peptides. The ordinate shows the cpm/cm while the abscissa shows the distance the peptides migrated from the origin (in centimeters). After a 30-min pulse, the tissue contained a dominant  $^3\text{H}$ -labeled peak which migrated 7 cm towards the cathode from the origin. Minor peaks were also observed at the origin and 4 cm from the origin. After a 30-min chase, the medium showed a major labeled peak that also migrated 7 cm from the origin. Bioassay studies of white-adapted toads showed that this released peak contained melanocyte-stimulating activity (denoted by \*).

the neurointermediate lobes from dark- vs. white-adapted toads showed that lobes from dark-adapted animals had much higher incorporation rates (Fig. 1). Since it is well known that dark-adapting conditions cause profound changes in the morphology and increases in secretory activity of the neurointermediate lobe *in vivo* (Hopkins, 1970, 1972; Thornton, 1974; Terlou et al, 1974), it is perhaps not surprising that the biosynthetic activity of the lobe is changed as well. The shape of the incorporation curve for the lobe from white-adapted animals (see Fig. 1) is suggestive of an induction of biosynthetic activity with incubation time *in vitro*. Similar observations were made by Thornton (1974), although his initial

differences in incorporation (of [ $^3\text{H}$ ]leucine) into lobe peptides of white- and dark-adapted animals were not as dramatic as was found here (Fig. 1). This apparent induction *in vitro* may be related to the cessation of the hypothalamic inhibition of the lobe, normally occurring *in vivo*, when the neurointermediate lobe is dissected for incubation *in vitro*. We are currently investigating this possibility.

The nature of the newly synthesized TCA-precipitable peptides in lobes from

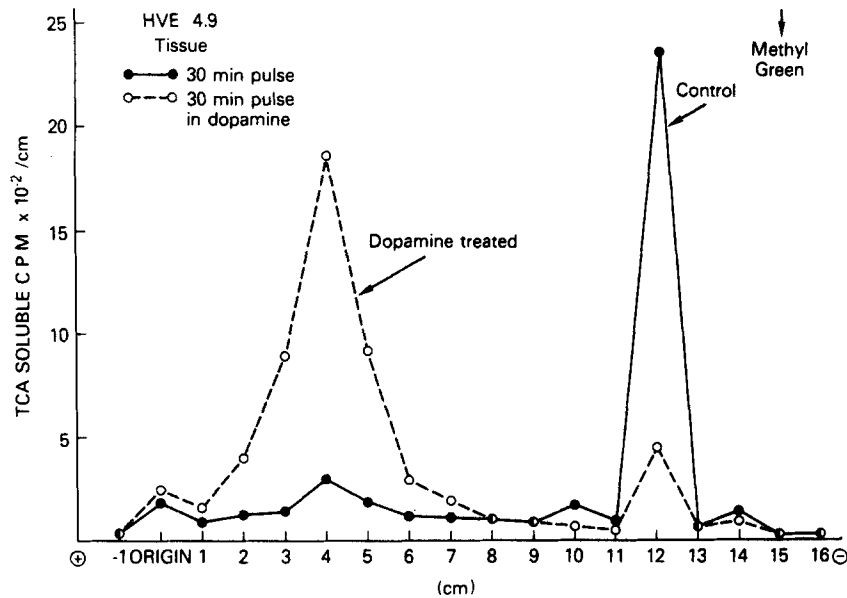


FIGURE 11. High-voltage electrophoresis (pH 4.9) of labeled TCA-soluble peptides from neurointermediate lobes incubated for 30 min in [ $^3\text{H}$ ]arginine in the absence (filled circles) and presence of  $5 \times 10^{-5}$  M dopamine (open circles). The ordinate and abscissa are as described in Fig. 10, except that in this case, in order to increase resolution, a longer migration towards the cathode was employed. Note that in the absence of dopamine the major labeled peptide in the lobe migrated 12 cm from the origin ( $R_f$  0.78, relative to methyl green), whereas in the dopamine-treated tissue the major labeled peptide peak migrated 4 cm ( $R_f$  0.26), with only a small peak at 12 cm.

dark-adapted animals was analyzed by acid-urea polyacrylamide gel electrophoresis. Pulse incubations ranging from 5 to 30 min (Fig. 2) revealed primarily one major labeled peak (peak *a*), with a molecular weight of 36,000 daltons (see Table I). Pulse-chase experiments showed that peak *a* decreased in time, as various new lower molecular weight peaks appeared (Fig. 3, Table I). Since there was no further incorporation of [ $^3\text{H}$ ]arginine during the chase (see Fig. 1, arginine chase curve), the progressive appearance of these lower molecular weight labeled peaks during the chase is probably due to the post-translational processing (cleavage) of larger labeled precursors. Although peak *a* appears to be a precursor of some of the processed peptides, it is difficult to identify the specific products derived from peak *a* due to the heterogeneity of the peaks seen.



Similarly, peak *b* which is seen during the pulse but rapidly disappears during the chase may also be a precursor or a transient intermediate form. In this regard, the recent report by Mains and Eipper (1976) on the biosynthesis of ACTH in mouse pituitary tumor cells is of interest. These authors propose that a 31,000 dalton ACTH is the biosynthetic precursor of the smaller forms of ACTH whose molecular weights are 23,000, 13,000, and 4,500. These molecular weights approximately correspond to the labeled peaks *a*, *b*, *d*, and *i* synthesized in the total neurointermediate lobe (Table I). Other studies using mRNA-directed cell free protein synthesis systems have shown that large immunoreactive forms of ACTH were synthesized with molecular weights comparable to peak *a* (i.e. 35,000 dalton molecular weight in bovine anterior pituitary, Nakanishi et al., 1976; 31,000 dalton molecular weight in mouse pituitary tumor cells, Jones et al., 1977). Peaks *k* and *l*, which become labeled after prolonged chases (Fig. 3) and appear to have significantly higher molecular weights than peak *a* (on the basis of their low mobility on acid-urea gels and of preliminary SDS gel analyses), also have significant melanotropic activity. Thus it is possible that they were derived from even larger labeled precursors which were not soluble in the 0.9 M acetic acid-5 M urea solution used to dissolve the TCA pellet in preparation for electrophoresis.

Comparisons of the  $R_f$  values on acid-urea gels, molecular weights, and melanotropic potencies of the released labeled peptides with standard purified (or synthetic) melanotropic peptides (Table I) suggest that peak *g* represents  $\beta$ -LPH, peak *i* is ACTH, and peak *j* is  $\alpha$ -MSH. Definitive identification of these labeled peaks must await the results of immunological experiments (in progress). Immunological evidence for the presence of "big ACTH" (Yalow and Berson, 1971) and ACTH<sub>1-39</sub> in the amphibian neurointermediate lobe has been reported (Estivariz and Iturriza, 1975). If, as is commonly believed, ACTH is derived from the cleavage of big ACTH, then immunoprecipitates of the homogenates of pulsed lobes (using the Kendall and West antibodies to ACTH) should reveal labeled peptide species comparable in molecular properties to big ACTH as well as ACTH and  $\alpha$ -MSH. In this regard it is interesting that we found a labeled peak (peak *g*) corresponding in  $R_f$  (1.01) to  $\beta$ -LPH, but no labeled peak corresponding to an  $R_f$  equal to 1.6 (i.e. the  $R_f$  of  $\beta$ -MSH). This finding is curious since  $\beta$ -MSH has been reported, on the basis of high-voltage electrophoresis separations and bioassay analyses (Burgers, 1963; Hopkins, 1972), to be in the amphibian neurointermediate lobe. We have found bioassayable activity on acid-urea gels in the migration position of  $\beta$ -MSH (unpublished data), but we could obtain no evidence at all for biosynthesis of this peptide. Since  $\beta$ -MSH is presumed to be derived from the precursor  $\beta$ -LPH (Scott et al., 1973) which appears to be amply labeled in our experiments, the question must be raised of whether this conversion is a physiologically significant one in vivo. Bertagna et al. (1974) also failed to observe a conversion of  $\beta$ -LPH to  $\beta$ -MSH in pulse-chase experiments on sheep pituitaries, and  $\beta$ -MSH could not be detected in human pituitary (Silman et al., 1976). Whether the  $\beta$ -MSH previously reported in amphibian neurointermediate lobes was the result of in situ biosynthesis mechanisms or of the degradation of the  $\beta$ -LPH during tissue preparation for analysis requires further investigation. It should be emphasized that the

dynamic state of the pars intermedia (i.e. the influence of the color adaptation of the animal) and the heterogeneity of melanotropic peptides synthesized and released makes analysis of the processing sequence in a pulse-chase paradigm very difficult. The use of specific antibodies (e.g., for  $\beta$ -LPH, ACTH, and  $\alpha$ -MSH) for immunoprecipitation of the labeled peptides after pulses and chases of various durations will be essential in future work.

Analysis of the TCA-soluble peptides showed that bioassayable melanotropic activity was found only in the excluded volume ( $V_E$ ) of the G10 Sephadex separation (i.e.  $V_E$  contains peptides with molecular weights  $\geq 700$  daltons). Peptides extracted from a 30-min pulsed intermediate lobe and found in the  $V_E$  were separated by high-voltage electrophoresis at pH 4.9. The synthesis of one dominant labeled peak which was released to the medium and has potent melanocyte-stimulating activity was detected (Fig. 10). This peak appears to be rapidly synthesized and released from the tissue with chase (Fig. 8). With a short pulse of 15 min, this TCA-soluble peptide is already found to be labeled (unpublished data). Whether its mode of synthesis is by cleavage of a precursor is unknown at present.

#### *Control of Melanotropic Peptide Release by Dopamine*

The relatively slow ( $t_{1/2} = 2$  h) release of the newly synthesized TCA-precipitable peptides is consistent with their relatively slow processing times in the tissue. The relatively rapid release ( $t_{1/2} = 30$  min) of the labeled TCA-soluble peptides is correlated with their rapid synthesis. Hopkins (1970) showed that maximum release of bioassayable melanotropic peptides from *Xenopus* lobes occurred between 2 and 6 h of incubation. This time course is comparable to that which we have found with the TCA-precipitable peptides. However, by using this biosynthetic paradigm we have found two independent release mechanisms: a rapid one for the small TCA-soluble peptide, and a slow one for the larger TCA-precipitable peptides.

The release of the TCA-precipitable and soluble peptides appears to be under inhibitory control by dopamine (Figs. 6 and 9). Dopamine caused  $72\% \pm 2.8\%$  inhibition of release of TCA-precipitable peptides and  $98\% \pm 0.5\%$  inhibition of TCA-soluble peptides. This dopamine effect was consistent with previous reports in the literature based on bioassay data (Hadley et al., 1973, 1975; Bower et al., 1974). These effects of dopamine to inhibit release raised the issue of whether dopamine acted via a feedback inhibition of the biosynthesis and/or processing of the peptides. We found that neither the synthesis nor the processing of the TCA-precipitable peptides was affected by dopamine after 1 h of pretreatment with dopamine in vitro (Fig. 7). Moreover, the inhibition of release allowed the accumulation of the releasable melanotropic peptides in the tissue (Fig. 7).

In contrast to the above lack of effect on the TCA-precipitable peptide biosynthesis by dopamine treatment of the lobe, the TCA-soluble peptide was profoundly affected (Fig. 11). Instead of the normal ( $R_t = 0.78$ ) peak of labeled melanotropic peptide in the tissue after a 30-min pulse, a highly labeled nonmelanotropic peak ( $R_t = 0.26$ ) was found. We interpret these data not as an effect of dopamine upon synthesis, but rather as an enhancement of the degradation

of the  $R_f$  0.78 peak to the  $R_f$  0.26 peak in the lobe evoked by dopamine. The reason for this interpretation is that we have observed a similar tendency for the labeled  $R_f$  0.78 peak to be converted to the labeled  $R_f$  0.26 peak in the media in long-term chase experiments in the absence of dopamine (unpublished data), presumably due to the degradation of the release peptide with time in the media. If this interpretation is correct, then dopamine inhibition of release of the TCA-soluble peptide may be coupled to an enhancement of degradative mechanisms in the tissue specifically directed at this peptide. Such a regulatory mechanism, if it indeed exists, would be particularly well suited to a biosynthesis and release system which requires a fast response to a specific input. That is, even after a long-term inhibition of release (as is the case in vivo in white-adapted animals), it would not require a long induction period for the biosynthesis of the released peptide (as appears to be the case for the TCA-precipitable peptides) upon removal of the inhibition. A rapid decrease in the post-translational degradative processes would then be the only requirement for a rapid response. That this system is in fact operating in the toad neurointermediate lobe is speculative at present and experiments are currently in progress to test this notion.

*Possible Physiological Significance of the Heterogeneous Forms of Melanotropic Peptides*

In this work we have demonstrated that the neurointermediate lobe of the toad synthesizes and releases eight distinct melanotropic peptides. Do these different forms have distinct functional roles? We can only speculate about this issue at this time. In the previous section we discussed the possibility that the presence of a rapidly synthesized and released melanotropic peptide (under degradative control) in combination with slowly synthesized and released melanotropic peptides may provide effective control mechanism for both short-term and long-term adaptive color changes. In addition, the various peptides may be inactivated at different rates in the blood and thus may be used at different times depending upon the specific dark adaptation strategy employed by the animal. We do not know whether all the melanotropic peptides are synthesized in and released by one cell type, or if each form is under independent control in a separate cell type. The latter possibility would, of course, be a more efficacious form of organization for the selective regulation mechanisms discussed above.

In recent years, attention has been focused on the extramelanotropic action of peptides containing the heptapeptide sequence essential for melanocyte expansion. For example, ACTH, ACTH fragments (e.g., ACTH<sub>4-10</sub>), and  $\alpha$ -MSH can profoundly affect neuronal activity and behavior (De Wied et al., 1972; Sandman et al., 1975; Krivoy and Guillemin, 1962). Whether some of the melanotropic peptide forms found in the neurointermediate lobe of the toad are involved in the control of the nervous system remains to be determined.

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