

A CELLULAR BASIS FOR THE DIFFERENCES IN REGULATION OF SYNTHESIS AND SECRETION OF ACTH/ENDORPHIN PEPTIDES IN ANTERIOR AND INTERMEDIATE LOBES OF THE PITUITARY

BY PATRICIA A. ROSA, PAUL POLICASTRO AND EDWARD HERBERT

Department of Chemistry, University of Oregon, Eugene, Oregon 97403

SUMMARY

The focus of research in our laboratory over the past few years has been the regulation of synthesis, processing and release of the ACTH/LPH family of peptides. These peptides are derived from a common precursor protein that is found in both the anterior and intermediate lobes of the pituitary (Roberts *et al.* 1978) and in the hypothalamus (Liotta *et al.* 1979). In the anterior lobe this protein gives rise to $\alpha(1-39)$ ACTH, β -lipotropin and an N-terminal fragment of undefined function. In addition, a variety of intermediate lobe pituitary peptides can be derived from the precursor by further processing of ACTH and β -LPH. In this paper we compare the structure of the precursor in the anterior and intermediate lobes of mouse and rat pituitary. Processing of the precursor to its constituent hormones is then contrasted in primary cultures of anterior and intermediate lobe cells using pulse label and pulse chase techniques with radioactive amino acids and sugars. Finally, we discuss the difference in behaviour of anterior and intermediate lobe cells in culture with regard to their rates of secretion and intracellular turnover of hormones and regulation of these processes by hypothalamic factors, glucocorticoids and catecholamines.

INTRODUCTION

Cells that produce the ACTH/LPH family of peptides are found predominantly in the anterior and intermediate lobes of the pituitary and in smaller numbers in the brain, gut and placenta (Kraicer, Gosbee & Bencosme, 1973; Pelletier & Racadot, 1971; Scott *et al.* 1974; Orwell & Kendall, 1979; Liotta *et al.* 1977, 1979). The anterior lobe has at least six different cell types as defined by characteristic secretory products, while the intermediate lobe has only one (Moriarity, 1973; Martin, Weber & Voigt, 1979). Although the cells of the intermediate lobe are distinct from those of the anterior lobe by morphology and staining characteristics (Kurosumi, Matsuzawa & Shibasaki, 1961), they are related on a biochemical basis in that two major peptides found in the intermediate lobe cells, α -MSH and CLIP, are identical in

This work was supported by National Institutes of Health Grant AM 16879 to E.H., National Institutes of Health Grant GMO7759 to the Institute of Molecular Biology of which P.R. is a PHS Fellow and by BRSG Grant S07RR07080 awarded by the Bio-Medical Research Support Grant Programs, Division of Research Resources, National Institutes of Health.

sequence to $\alpha(1-13)$ ACTH and $\alpha(18-39)$ ACTH respectively (Scott *et al.* 1974). It is postulated that α -MSH arises along with CLIP by cleavage of ACTH followed by acetylation of the N-terminus and amidation of the C-terminus of $\alpha(1-39)$ ACTH.

The relationship between anterior lobe corticotrophs and intermediate lobe cells acquired a new dimension with the discovery that ACTH itself is a cleavage product of a much larger protein which also contains the structure of β -LPH, another anterior lobe hormone (Roberts & Herbert, 1977*a, b*; Mains, Eipper & Ling, 1977). β -LPH in turn contains the opiate peptide, β -endorphin (Bradbury, Smyth & Snell, 1976*a*, Bradbury *et al.* 1976*b*; Li & Chung, 1976*a, b*).

ABBREVIATIONS

SDS, sodium dodecyl sulphate

TCA, trichloroacetic acid

β -LPH, lipotropic hormone

β -endorphin, β -(61-91) sequence of β -lipotropic hormone

γ -endorphin, β -(1-58) sequence of β -lipotropic hormone

ACTH, adrenocorticotrophic hormone

MSH, melanocyte stimulating hormone

CLIP, corticotropin-like intermediate lobe peptide [the $\alpha(18-39)$ ACTH sequence]

CNS, central nervous system

DMEM, Dulbecco's Modified Eagle's Medium

The molecular weight forms of ACTH, β -LPH and β -endorphin are referred to by K values (20K ACTH, for example, is the 20000 molecular weight form of ACTH).

IDENTIFICATION OF THE PRECURSOR

The initial demonstration that ACTH and β -LPH are derived from the same precursor protein was done with mouse pituitary tumour cells (AtT-20/D_{16v} cells) because these cells secrete large quantities of ACTH and β -LPH and can be grown easily in culture.

Tumour cells were labelled with radioactive amino acids, extracted, and immunoprecipitated with antiserum to either ACTH or β -LPH. Fractionation of the immunoprecipitates by SDS gel electrophoresis showed that a glycoprotein of molecular weight 30000 contained both ACTH and β -LPH determinants (Mains *et al.* 1977). Furthermore, RNA from the tumour cells directed the synthesis of an unglycosylated protein of molecular weight 28500 that contained both ACTH and β -LPH determinants (Roberts & Herbert, 1977*a, b*).

Peptide mapping studies revealed the structure of the precursor as represented in Fig. 1 (Roberts & Herbert, 1977*a, b*). The structure has been confirmed by determination of the amino acid sequence of the precursor in bovine pituitary by recombinant DNA technology (Nakanishi *et al.* 1979). ACTH and β -LPH occupy the C-terminal half of the molecule leaving a stretch of about 110 amino acids (N-terminal half) without a defined function. The precursor forms present in tumour cells are glycosylated at two or possibly three sites (Herbert *et al.* 1979; Phillips, Budarf &

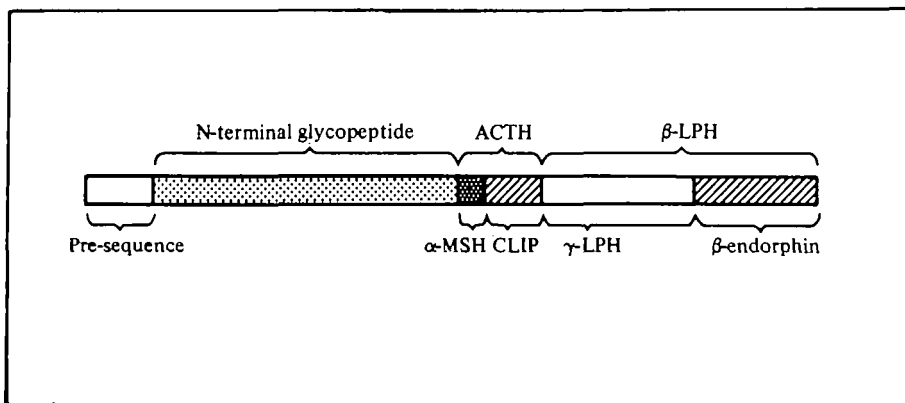
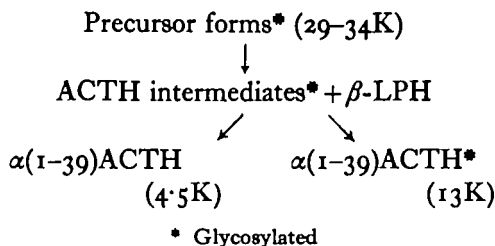


Fig. 1. Structure of the ACTH/LPH precursor.

Herbert, 1980). The oligosaccharides are of the complex type linked to an asparagine residue in the protein via an *N*-acetyl-glucosamine residue (Eipper & Mains, 1977). The following simple processing scheme was also worked out in the tumour cells (Roberts *et al.* 1978).



β -LPH is cleaved very slowly in tumour cells to β -endorphin-like material $\beta(61-91)$ -LPH and possibly γ -LPH [$\beta(1-58)$ LPH] (the latter component has not been identified as yet). These studies were done with a tumour cell line because it was a convenient source of material. However, the precursor could also be identified in both the anterior and intermediate lobes of the pituitary, and was, therefore, not merely an artifact of the tumour cell line (Roberts *et al.* 1978).

ACTH/LPH-RELATED MOLECULES PRESENT IN THE PITUITARY

A more detailed comparison of the distribution of ACTH/LPH peptides in the anterior and intermediate lobes of mouse pituitary was made by fractionating proteins from each lobe by SDS polyacrylamide tube gel electrophoresis. The tube gels were sliced and eluted and radioimmunoassay of eluates from gel slices was performed with an N-terminal ACTH antiserum (MSH region) and a C-terminal LPH antiserum (β -endorphin region). In this manner a profile of the different sizes of molecules bearing

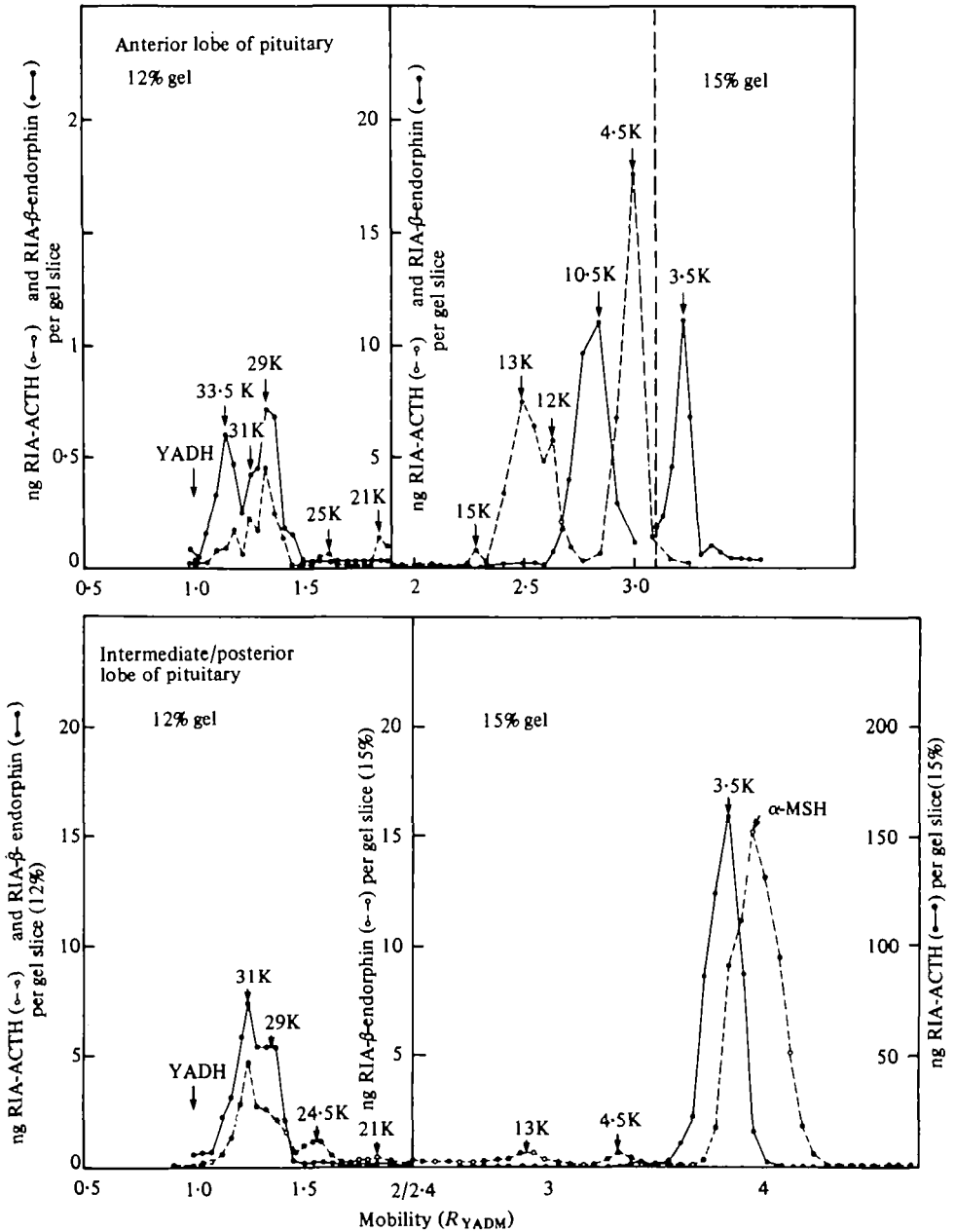


Fig. 2. Analyses of immunoreactive forms of ACTH and endorphin in the anterior and intermediate-posterior lobes of mouse pituitaries. Anterior and intermediate-posterior lobes were extracted with acetic acid (Roberts *et al.* 1978). Each extract was fractionated by electrophoresis on two SDS gel systems to achieve maximum resolution of all of the forms of the hormones. Twelve per cent gels were used to resolve molecules in the range of 15 000–30 000 in molecular weight and 15 % gels were used to resolve the lower molecular weight forms. The figure is a composite of the two gel runs. The gels were sliced and eluates of the gel slices were analysed by radioimmunoassay with β -endorphin and ACTH antisera..

neither ACTH or LPH determinants from both lobes was obtained, as shown in Fig. 2 (Roberts *et al.* 1978).

From this analysis it appeared that similar forms of the precursor and ACTH intermediates were present in each lobe but that the end products of processing were different. We will discuss the processing of the precursor and the structure of the intermediates in greater detail later in the paper.

As previously mentioned, ACTH undergoes cleavage in the intermediate lobe to generate an α -MSH-size molecule. However, CLIP was not detected in the above studies because the antibody used did not react with the C-terminal region of ACTH. An interesting fact uncovered by this analysis was that β -LPH was not present in a significant amount in the intermediate lobe. Instead, a further cleavage product of LPH was detected. This molecule was similar in size and antigenic characteristics to β -endorphin, a potent opiate whose sequence represents the 31 C-terminal amino acids of β -LPH (Li & Chung, 1976b). As with CLIP, the remainder of the β -LPH molecule (γ -LPH) was not detected in this assay because the antibody used did not cross react with this portion of the molecule.

COMPARISON OF THE STRUCTURE OF THE PRECURSOR FROM ANTERIOR AND INTERMEDIATE LOBES OF RAT AND MOUSE PITUITARIES

The question arises as to whether differences in processing of the precursor can be explained by structural differences in the precursor from the two lobes. An easy structural difference to observe would be a size difference as monitored by mobility on SDS-polyacrylamide gels. Primary cultures were made from the anterior and intermediate lobes of both mouse and rat pituitaries. These cultures were labelled with [35 S]methionine, immunoprecipitated with an ACTH antibody and analysed by SDS-polyacrylamide slab gel electrophoresis. We hoped that slab gel electrophoresis would give better resolution of precursors and intermediates than tube gels used previously. We compared the precursor forms present in anterior and intermediate lobes of mouse and rat pituitary. The results in Fig. 3, lanes B, C, E, and F show that the precursors from the anterior and intermediate lobes are the same size within a species. However, precursors from rat pituitary migrate more slowly in this gel system than do precursors from mouse pituitary. There are at least two possible explanations for the slower migration of rat precursors than mouse precursors: (1) the primary amino acid sequence of the mouse precursor is shorter than that of the rat precursor or (2) rat precursors are more heavily glycosylated than mouse precursors.

STRUCTURE OF THE ACTH/LPH PRECURSOR MADE IN A CELL-FREE PROTEIN SYNTHESIZING SYSTEM

Glycoproteins do not migrate according to molecular weight on SDS gels (Weber, Pringle & Osborn, 1972; Segrest & Jackson, 1972). Therefore, it is difficult to compare molecular weights of glycosylated proteins by this procedure. One way to avoid this problem is to compare the mobilities of proteins synthesized in the reticulocyte cell-free system under the direction of extracted mRNA because glycosylation of proteins

does not occur in this system. We thought it would be useful to take advantage of this feature of the cell-free system to distinguish among the possibilities presented above. A shorter amino acid sequence of the mouse ACTH/LPH precursor than the rat precursor would be apparent in a comparison of the cell-free products. A species difference in glycosylation or processing of the precursor would be obvious when the intact cell and cell-free products from each species are compared.

We analysed the cell-free products made under the direction of RNA extracted from the rat anterior and intermediate pituitary and the mouse tumor cell line. The RNA was translated in the presence of [35 S]methionine, immunoprecipitated with ACTH specific antisera and separated by SDS slab gel electrophoresis. An autoradiogram of the slab gel is shown in Fig. 3, lanes A, G, and H. Three important points are apparent from these results: (1) the cell-free precursors from the anterior and intermediate lobes of the same species migrate with the same mobility, (2) rat cell-free precursors migrate more slowly than mouse cell-free precursors and (3) in addition to one major band, several minor bands exist for rat cell-free precursors. The existence of several bands for the rat cell-free product could indicate the presence of more than one gene product for the ACTH/LPH precursor or a single translation product present in several partially denatured states (or a combination of both of these explanations). The second possibility seemed quite likely because of the large number of cysteine residues present in the N-terminal region of the bovine precursor as shown by the sequencing work of Nakanishi *et al.* (1979). Although the gels are run in the presence of SDS and mercaptoethanol, there is a chance for disulfide bonds to reform between cysteine residues as the proteins concentrate at the interface between the stacking gel and the separation gel.

To test this idea we analysed the mobilities of the precursors on 8 M urea gels (Swank & Munkres, 1971) in which the proteins are completely denatured and disulfide bonds have less opportunity to reform (Budarf & Rosa, unpublished observation, data not shown). Under these conditions, only one band exists for the cell-free product for each species. This indicates that the multiple banding pattern we previously observed was probably due to different denatured states of the same molecule. Equally important is the finding that the mouse cell-free precursor migrates with the same mobility as the rat cell-free precursor in the urea gel. This indicates that the differences in mobilities of rat and mouse cell-free precursors on SDS slab gels are probably due to differences in amino acid sequence rather than differences in length of polypeptide chains as has been shown to be the case for a histidine transport protein (Noel, Nikaido & Ferro-Luzzi Ames, 1979).

TRYPTIC PEPTIDE MAPS OF RAT PITUITARY CELL-FREE PRODUCTS

From the above analysis it appears that the peptide backbone of the precursors from both lobes are the same size. In order to detect differences in sequence we used tryptic peptide mapping. RNA from each lobe was translated separately in the presence of radioactive leucine (Leu) or phenylalanine (Phe) and immunoprecipitated with ACTH-specific antisera. The immunoprecipitated protein was digested with trypsin and the peptides in the digest were analysed by paper electrophoresis. The maps are shown in Fig. 4.

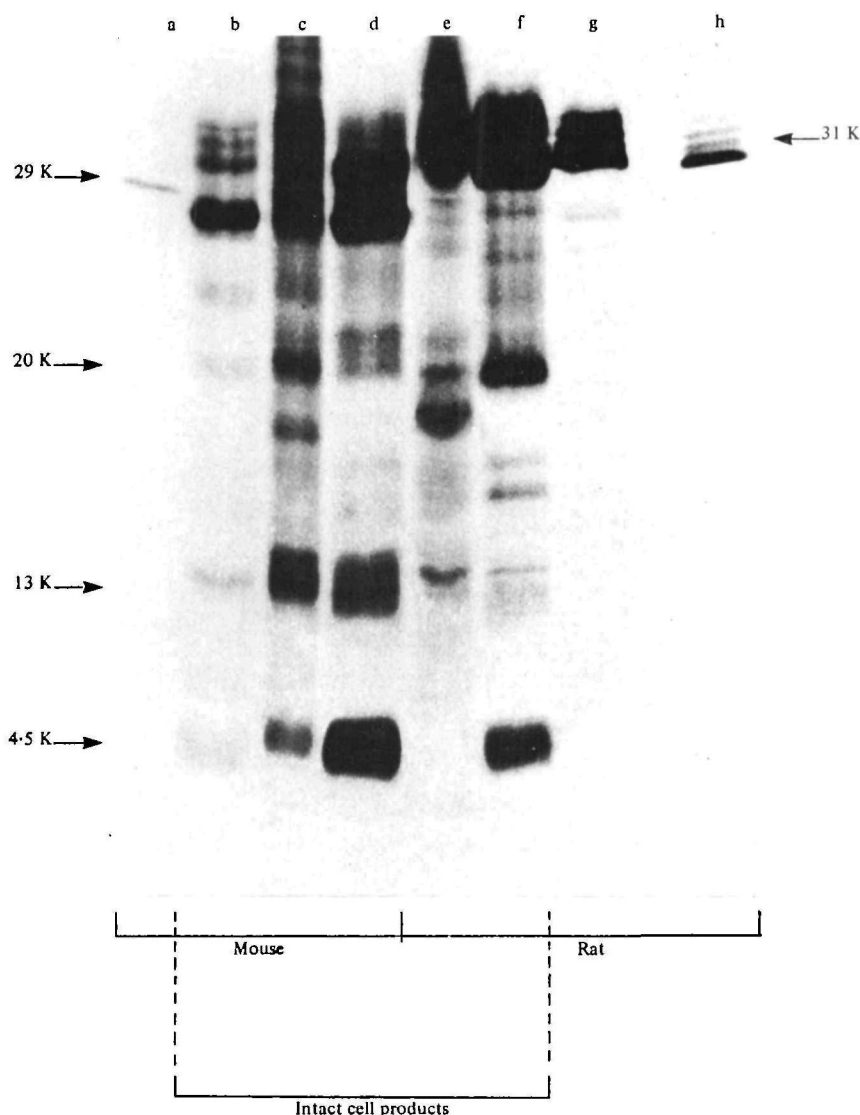


Fig. 3. Relative mobilities of [^{35}S]-methionine labelled rat and mouse intact cell and cell-free, ACTH/LPH precursors. Monolayer cultures of mouse pituitary tumour cells, mouse anterior lobe, mouse intermediate lobe, rat anterior lobe, and rat intermediate lobe pituitary were incubated in microwells with 200 μCi of [^{35}S] methionine for 4 h in 200 μl of methionine-deficient medium (DMEM). Cellular protein was extracted and immunoprecipitated with ACTH antiserum (Roberts *et al.* 1978). RNA was extracted from AtT-20 cells and from rat anterior and intermediate lobe cells (Chirgwin *et al.* 1979) and translated in a reticulocyte cell-free system in the presence of 50 μCi of [^{35}S]methionine (Roberts & Herbert, 1977a). Cell-free translation products were immunoprecipitated with ACTH antiserum. All immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis using a 12.5 % acrylamide slab gel. An autoradiograph was made of the dried gel. (a) AtT-20 cell-free product. (b) Mouse intermediate lobe primary culture cell products. (c) Mouse anterior lobe primary culture cell products. (d) AtT-20 culture cell products. (e) Rat anterior lobe primary culture cell products. (f) Rat intermediate lobe primary culture cell products. (g) Rat intermediate lobe cell-free product. (h) Rat anterior lobe cell-free product.

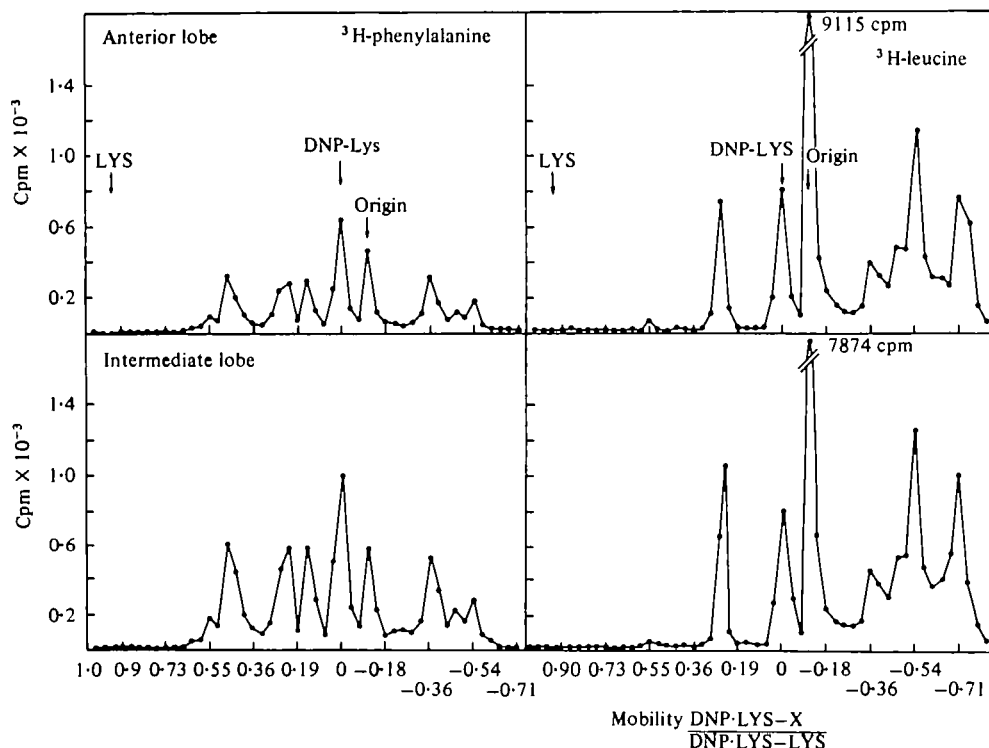


Fig. 4. Tryptic peptide maps of [^3H]leucine and [^3H]phenylalanine-labelled ACTH/LPH precursor: comparison of rat anterior and intermediate lobe pituitary cell-free products. RNA was extracted from rat anterior and intermediate lobes (Chirgwin *et al.* 1979) and translated separately in the reticulocyte cell-free protein synthesizing system. Cell-free products were immunoprecipitated with ACTH antiserum and separated by SDS gel electrophoresis on 12 % tube gels (Roberts *et al.* 1978). Gels were cut into 1 mm slices, eluted, and a fraction of each eluate was counted in liquid scintillation fluor, in order to determine the exact position of the ACTH/LPH precursor in the gel. Tryptic peptides of the isolated precursors were prepared and analysed by paper electrophoresis at pH 6.5 (Roberts & Herbert, 1977a).

The tryptic peptides from the precursor containing radioactive Leu or Phe amino acids appear to be identical for both lobes. Furthermore, the tryptic peptide maps of the cell-free products of the rat are similar when compared to those of the mouse tumour cell-free product but the mobilities of the peptides are slightly different (data not shown).

We conclude that there are no easily detectable differences in the ACTH/LPH precursors from the anterior and intermediate lobes of the pituitary. The primary sequences of the cell-free precursors from each lobe appear to be identical on the basis of tryptic peptide mapping and mobility on SDS-polyacrylamide gels. The translational modifications of the precursors from both lobes (glycosylation and cleavage of the N-terminal signal sequence) also appear to be the same based upon the finding that the forms of the precursor in the anterior lobe migrate with the same mobility as forms of the precursor in the intermediate lobe (relative to the mobilities of the cell-free products). There are detectable differences in the mobilities of the precursor from rat and mouse pituitary. This may be due to different amino acid

Table 1. *Comparison of precursor mRNA content of anterior and intermediate-posterior lobes of rat pituitary*

(A) RNA and proportion of corticotrophs in lobes of the pituitary				
	Total weight	'Corticotrophs'	Total RNA	
	(mg)	(%)	(μg)	
Anterior lobe	5.45	3.5	13	
Intermediate/ posterior lobe	1.04	27	1.05	
(B) Cell-free translation				
	Total TCA Precipitable Radioactivity		Radioactivity in ACTH immunoppt.	cpm ACTH immunoppt. (cpm in TCA ppt. × 100)
	Labelled Precursor	(cpm/μg/RNA)	(cpm/μg/RNA)	
Anterior lobe	³⁵ S-met	23 000	840	3.7 %
	³ H-phe	61 000	1 900	3.1 %
	³ H-leu	177 000	6 700	3.7 %
				Average 3.5 %
Intermediate/ posterior lobe	³⁵ S-met	2 700	1 400	50 %
	³ H-phe	17 000	8 400	50 %
	³ H-leu	86 000	37 000	43 %
				Average 48 %

TCA, trichloroacetic acid.

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The lobes were separated and blotted and weighed. RNA was extracted as described in the legend of Fig. 4 by the method of Chirgwin *et al.* (1979). The RNA was translated in a reticulocyte lysate cell-free system using either ³⁵S-met, ³H-phe or ³H-leu as the labelled amino acid (Roberts & Herbert, 1977). A portion of the incubation mixture was treated with trichloroacetic acid (TCA) to a final concentration of 5%. The TCA insoluble material was counted as described by Roberts *et al.* to give the total TCA insoluble radioactivity – a measure of total protein synthesizing capacity of the system (total mRNA activity). The remainder of the reticulocyte lysate was immunoprecipitated with antiserum to ACTH and the immunoprecipitate was counted in scintillation fluor (Roberts & Herbert, 1977). The numbers for weights of the two lobes represent an average of weights of 99 male rats, weighing 175–225 gm (50–60 days of age). The last column in Part (B) of the table is a measure of the capacity of the cell-free system to translate precursor mRNA relative to its capacity to translate total mRNA.

sequences in the precursor from the two species (in particular a difference in the number or distribution of cysteine residues in the precursors) (Noel *et al.* 1979). This cannot be proven, however, until the complete amino acid sequence of the precursor from each species is determined.

RELATIVE AMOUNT OF ACTH mRNA IN THE LOBES OF THE PITUITARY

In addition to providing information about the structure of the precursor in the the anterior and intermediate lobes of the pituitary, the translation assay reveals how much of the total mRNA in the two lobes is ACTH/LPH precursor mRNA. The amount of ACTH/LPH mRNA present is measured by translating the RNA in the cell-free system and determining the amount of radioactive amino acid incorporated into ACTH immunoprecipitable material relative to the amount incorporated in

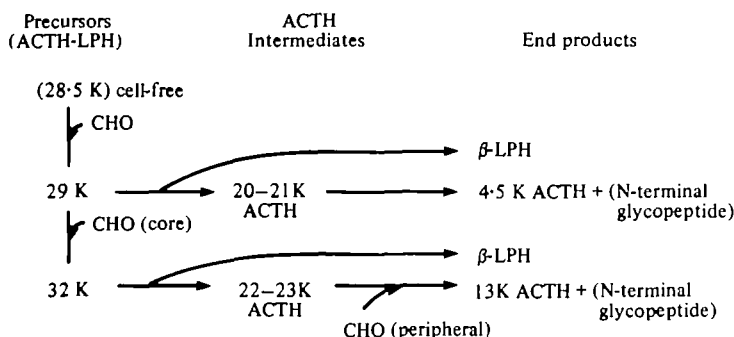


Fig. 5. Model of processing of ACTH/LPH precursors in the anterior lobe of the pituitary.

trichloroacetic acid precipitable material (a measure of total translating capacity of the RNA or total protein synthesis). These data are summarized in Table 1, along with data on the proportion of cells in the two lobes which synthesize ACTH/LPH molecules [corticotrophic cells as measured by the immunostaining technique (Moriarty, 1973; Legait & Legait, 1964)].

When cell-free translation is directed by RNA extracted from the anterior lobe of the rat, the percentage of total translating capacity attributable to ACTH/LPH mRNA in the above assay is about 3.5%; the percentage of corticotrophs in the anterior lobe is 3.7% (Moriarty, 1973). When the same assay is performed with RNA extracted from the intermediate/posterior lobe (of which approximately 27% of the cells synthesize ACTH/LPH related molecules) (Legait & Legait, 1964), 48% of the RNA translating capacity is attributable to ACTH/LPH mRNA.

These results indicate that there is a correlation between the percentage of cells identified as corticotrophs (by immunostaining) and the capacity of the lobes to synthesize precursor mRNA.

ACTH/LPH PRECURSOR PROCESSING IN THE INTERMEDIATE LOBE

The details of processing of the ACTH/LPH precursor were originally worked out using the tumour cell line and then confirmed in mouse anterior lobe primary cultures (Hinman & Herbert, 1980). The detailed model diagrammed in Fig. 5 is an elaboration of the scheme presented earlier. The biosynthetic relationships of the different forms were established by continuous and pulse chase labelling experiments with radioactive amino acids and sugars, and the forms were identified by tryptic peptide mapping. The tumour cells appear to be a fairly good model system for the anterior lobe corticotrophs on the basis of similarities in the processing pathway and regulation of secretion of ACTH/LPH peptides.

To briefly summarize the processing scheme in the anterior lobe, β -LPH is the first molecule to be cleaved out of the precursor leaving intermediate forms of ACTH of approximately 20K molecular weight. The ACTH intermediates are then cleaved to form $\alpha(1-39)$ ACTH and N-terminal fragments. ACTH processing ceases at this point in the anterior lobe and in the tumour cell line. As shown in Fig. 2, both the

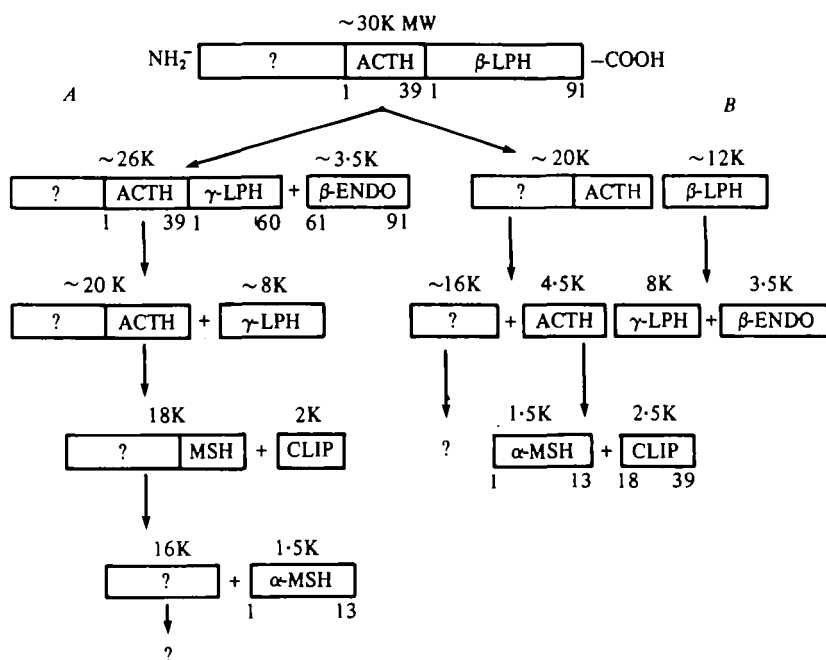
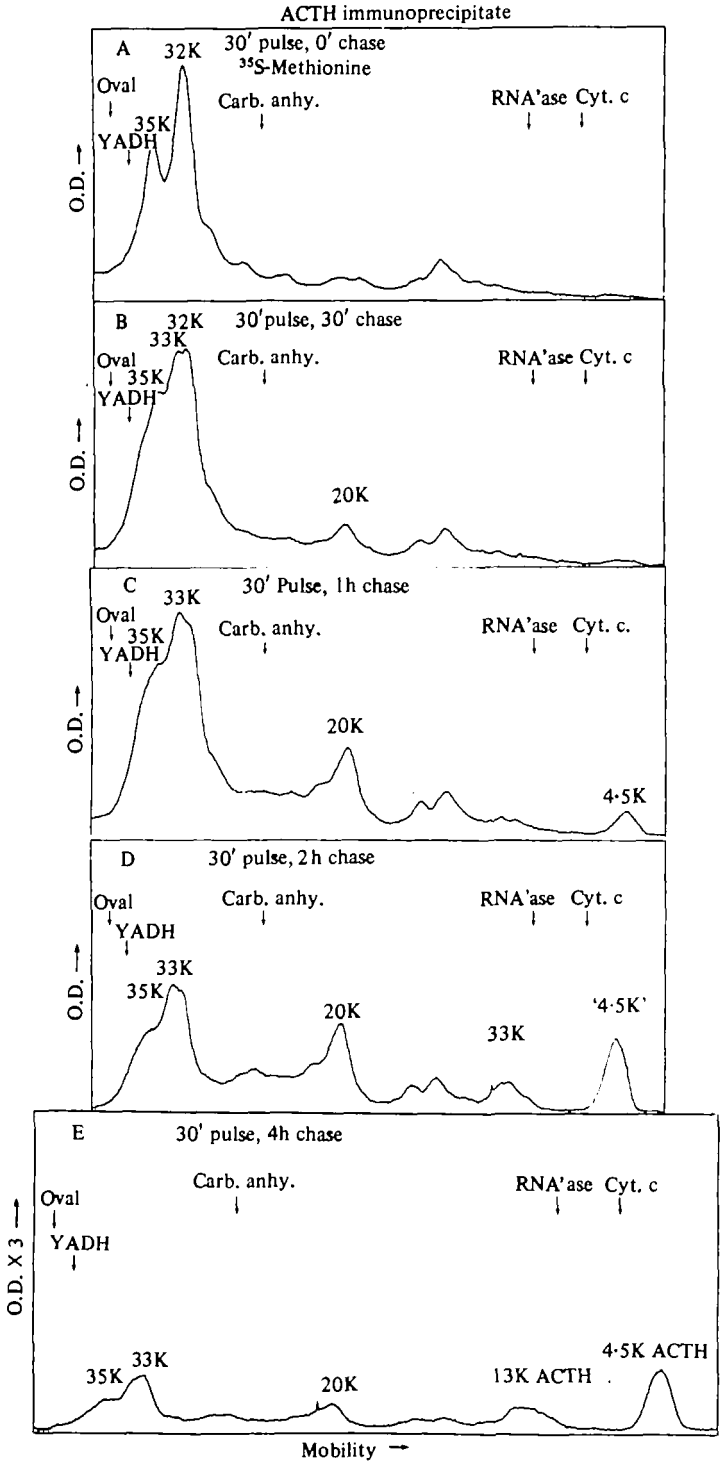


Fig. 6. Possible ACTH/LPH precursor processing pathways in the intermediate lobe of the pituitary.

precursors and intermediate forms of ACTH are present in the intermediate lobe of the pituitary, but in contrast to the anterior lobe, neither ACTH nor β -LPH is present in significant amounts. Instead, α -MSH and endorphin, fragments of ACTH and β -LPH respectively, are detected. There are at least two ways in which these products could arise, as shown in Fig. 6.

In pathway A, molecules are cleaved out sequentially from the carboxy terminal of the precursor, in the form in which they are found in the pituitary. β -endorphin is the first molecule to be cleaved out. This is followed rapidly by the cleavage of γ -LPH [$\beta(1-58)$ LPH] from the precursor, generating a 20K ACTH intermediate. This intermediate has been found in both lobes. A similar sequence of cleavage events would occur with 20K intermediate, resulting in the release of CLIP, and then α -MSH. In summary, this pathway would result in formation of an ACTH intermediate larger than the 20K form, bearing γ -LPH but not endorphin, and a smaller molecule with N-terminal precursor and α -MSH but not CLIP. The latter forms

Fig. 7. Pulse chase study of precursor processing in rat intermediate pituitary with [35 S]-methionine; analysis of ACTH immunoprecipitates. Five microwell cultures were incubated for 30 min (pulse period) with 200 μ Ci of [35 S]methionine in methionine deficient medium (DMEM), and then incubated for (a) 0, (b) 30, (c) 60, (d) 120 or (e) 240 min in complete, unlabelled DMEM containing methionine (chase periods). Cellular protein was extracted and immunoprecipitated with ACTH antiserum (Roberts *et al.* 1978). Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis, using 12.5% slab gels. Autoradiographs were made of the dried gels, and the films were scanned with a microdensitometer.



have not been detected in the lobes of the pituitary by radioimmunoassay methods. This does not mean they are not present – they could have been missed because the time course of the cleavages is very rapid and because the molar reactivities of these forms with the ACTH antisera are not as high as with other forms. In the alternate scheme (B), the original cleavage events are identical to those of the anterior lobe, but additional cleavages of ACTH and β -LPH take place quite rapidly to generate α -MSH and CLIP, and γ -LPH and β -endorphin.

PULSE CHASE LABELLING WITH THE INTERMEDIATE LOBE CULTURES

In order to distinguish between the possibilities presented above, we used the same experimental design that had been employed to study processing in the anterior lobe. Intermediate lobes were cultured for 5 days and then pulse labelled with ^{35}S -met for a relatively short time period to ensure that only the precursor forms were radioactive. A chase with non-radioactive amino acid was then performed for varying periods of time.

The cells were extracted and half of each extract was immunoprecipitated with an ACTH antibody and the other half with an endorphin antibody. These immunoprecipitates were separated by slab or tube gel electrophoresis and the radioactivity in the gels was quantitated either by performing densitometry of autoradiograms of the gels or by slicing the gels and counting eluates of the slices. The results of these experiments are shown in Figs. 7 and 8. The use of ^{35}S -met in these experiments makes it easy to quantitate the amount of N-terminal fragment, ACTH and β -LPH or β -endorphin because each of these fragments of the precursor has a single Met residue.

The first molecules to be labelled with the pulse of [^{35}S]methionine are the precursors. In the ACTH immunoprecipitation, label is chased from the precursors ($\sim 30\text{K}$ mol. wt) into an intermediate ($\sim 20\text{K}$ mol. wt) class of molecules. This 20K class of molecules does not appear in the endorphin immunoprecipitate of the sample from the same time period; instead β -LPH size material is present. This indicates that the first cleavage to occur liberates a complete β -LPH-like molecule and leaves the N-terminal fragment and ACTH intact. With time, label chases from 20K material into 13K and 4.5K ACTH immunoprecipitable material and then disappears from these forms. β -LPH-like material chases to endorphin size material.

These experiments demonstrate quite clearly that β -LPH and ACTH-like material are cleaved out of the precursor as intact molecules and then undergo further processing to yield α -MSH and CLIP, γ -LPH and β -endorphin. The presumed conversion of ACTH to α -MSH is not apparent because the antibody used in the immunoprecipitation does not cross react with α -MSH. However, the conversion of LPH to a β -endorphin-like molecule is quite apparent. Gianoulakis *et al.* (1979) and Mains & Eipper (1979) have demonstrated a similar pathway of precursor processing in intermediate lobe cultures. This processing pathway is similar to that of Fig. 8B, in which the initial cleavages are identical to those of the anterior lobe, but ACTH and β -LPH each undergo further processing. It has not been determined whether the N-terminal portion of the molecule also undergoes further processing in the intermediate lobe.

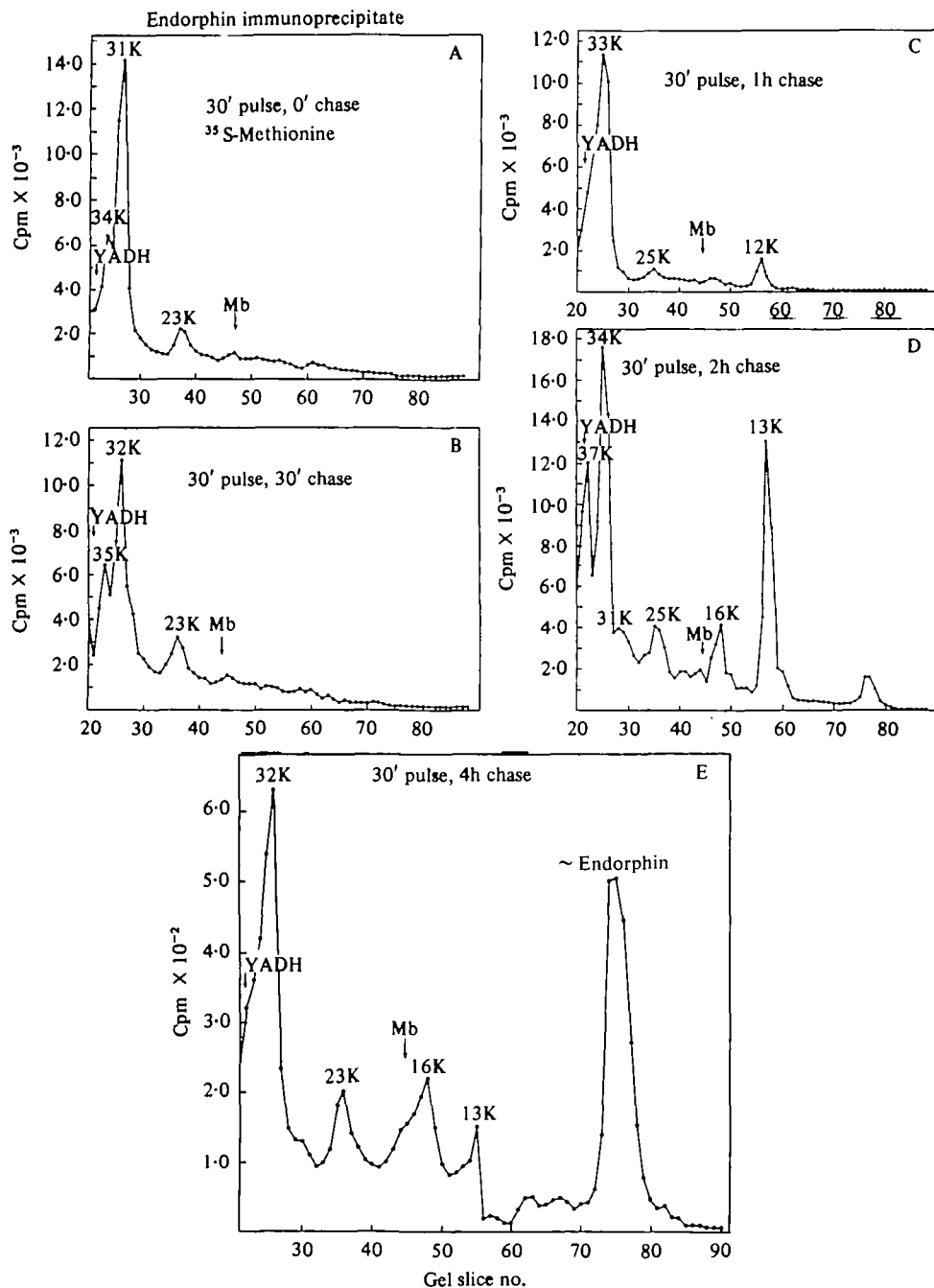


Fig. 8. Pulse chase study of precursor processing in rat intermediate lobe: analysis of endorphin immunoprecipitates. Five microwell cultures were incubated for 30 min with 200 μ Ci of [35 S]-methionine in methionine deficient medium (DMEM) and then incubated for (a) 0, (b) 30, (c) 60, (d) 120 or (e) 240 min in complete unlabelled DMEM. Cellular protein was extracted and immunoprecipitated with endorphin antiserum (Roberts *et al.* 1978). Immunoprecipitates were analysed using 15 % acrylamide tube gels. Gels were cut into 1 mm slices, eluted, and counted in liquid scintillation fluor.

CHARACTERISTICS OF INTERMEDIATE LOBE CELLS IN CULTURE

Several rather surprising results were observed when rat intermediate lobe cultures were subjected to long term labelling (3 days with phenylalanine) in order to obtain a picture of the steady-state distribution of labelled hormones within the cells. SDS gel profiles of sequential ACTH and endorphin immunoprecipitations of both cells and medium are shown in Fig. 9. Three major points arise from these results:

(1) There is a substantial amount of ACTH-size material present in both the cells and the medium.

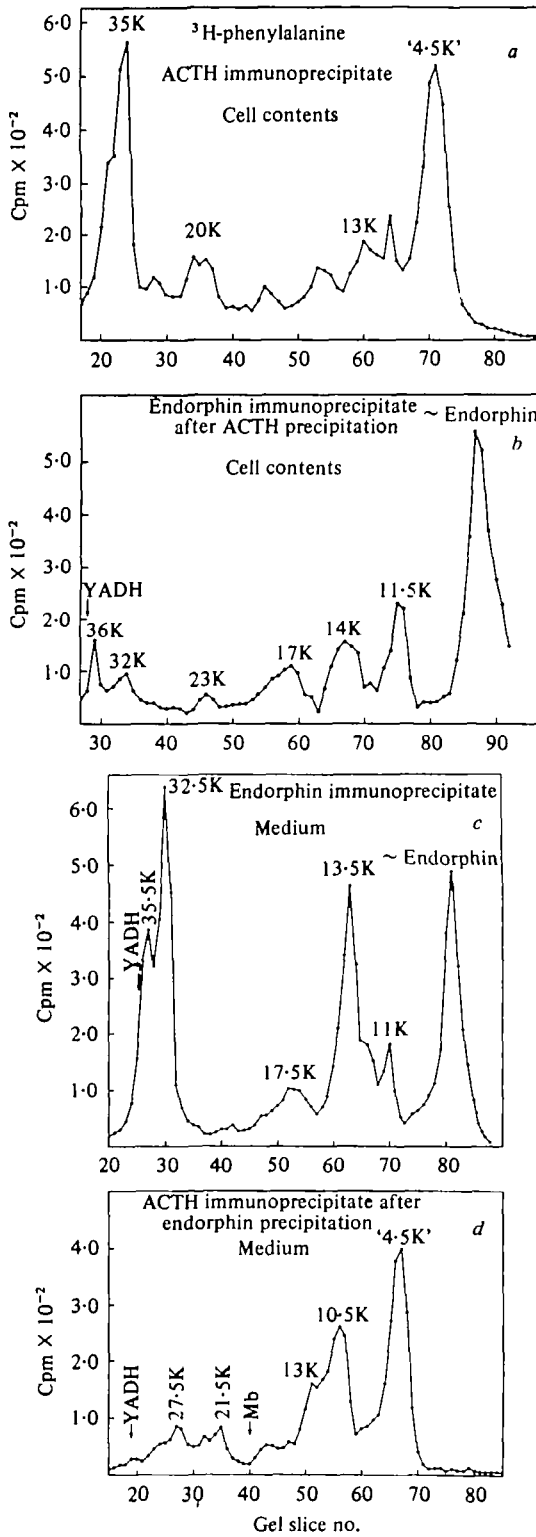
(2) The ratio of precursor to end products in these cultures is much higher than in mouse anterior lobe primary cultures, or in the intermediate lobe extracts, although one can't make a direct comparison to the molar amounts of precursor found in the pituitary as measured by RIA, since the immunoreactivity of the precursor with the antibodies is not known. When cpm in the labelled forms are normalized to Phe content (to give relative molar amounts), there is about four times as much end product as precursor in intermediate lobe primary culture cells maintained for 5 days as opposed to about 20 times as much in fresh intermediate lobe extracts. This difference is unlikely to be explained on the basis of antibody reactivity alone, since in the anterior lobe the ratio of forms extracted from the pituitary looks quite similar to that of labelled cultures (after 5 days in culture).

(3) Intermediate lobe cultures secrete at least 25 % of their ACTH/LPH contents/day. By contrast, anterior lobe cultures appear to secrete only 5–10 % of their ACTH/LPH contents per day in basal state (Hinman & Herbert, 1980). The 25 % figure for intermediate lobe cells is undoubtedly a low estimate, since under the conditions in which this experiment was done, a substantial amount of the material secreted was very likely degraded.

This number also does not take into account the period of time during which the cells were secreting, but the molecules had not reached steady-state labelling. In making this estimation we have extrapolated to account for the processed portions of the precursor molecules which are not recovered in immunoprecipitations because of the antibody specificities (N-terminal, α -MSH, CLIP, γ -LPH). Without this extrapolation, the immunoprecipitable counts in the medium after 3 days are 96 % of the immunoprecipitable counts within the cells (or 32 % secreted per day).

By radioimmunoassay, 5-day-old rat intermediate lobe primary cultures appear to secrete 8 % of their ACTH/LPH contents in 4 h (approximately 100 % in 24 h) (unpublished observation). It is difficult to interpret these data, however, since the

Fig. 9. Steady-state labelling with [3 H]phenylalanine: analysis of sequential ACTH and endorphin immunoprecipitates. One microwell was incubated with 200 μ Ci of [3 H]phenylalanine for 3 days in complete medium (DMEM). The medium was collected and frozen until immunoprecipitated with endorphin antiserum. Cellular protein was extracted and immunoprecipitated with ACTH antiserum (Roberts *et al.* 1978). A second immunoprecipitation was then done on the supernatants resulting from the first immunoprecipitation with either the ACTH (culture medium) or endorphin antiserum (cell extract). Immunoprecipitates were analysed by SDS gel electrophoresis using 12 % and 15 % polyacrylamide tube gels. Gels were cut into 1 mm slices, eluted, and counted in liquid scintillation fluor. (a) Cell extract; ACTH immunoprecipitation. (b) Cell-extract; endorphin immunoprecipitation of supernatant from ACTH immunoprecipitation. (c) Culture medium; endorphin immunoprecipitation. (d) Culture medium; ACTH immunoprecipitation of supernatant.



forms of the molecules secreted were not determined and if secretion were preferential for the smaller, more immunoreactive forms of ACTH and LPH, a high estimation would result. This would not be reflected in the gel profiles of the immunoprecipitated medium from the long-term label experiment since these smaller forms are more susceptible to proteolytic degradation.

In summary, intermediate lobe primary cultures appear to secrete ACTH/LPH related molecules at a much higher rate than anterior lobe cultures. The pattern of forms present in 5-day intermediate lobe cultures is different from that seen in extracts of intermediate lobe prior to culture, in that substantial amounts of ACTH can be isolated, and the ratio of end products to precursors is much lower. This is in contrast to the anterior lobe, in which no striking differences are noticed between pituitary extracts and five day old primary cultures. We interpret this to reflect the difference in regulation of each lobe in the animal, and thus the different response of each lobe to being removed from the animal and cultured. These results could be explained if it is postulated that secretion by intermediate lobe cells is under constant inhibition in the animal, whereas the anterior lobe corticotrophs in the basal state are inactive and need a positive stimulus in order to secrete. When intermediate lobe cells are put in culture (and removed from the inhibitory agent) they secrete constitutively at a very high rate (faster than the time necessary to process). This could account for the lower ratio of end product to precursor found in intermediate lobe cultures compared to fresh extracts, if the cells preferentially store and secrete the smaller forms, or if the period of time spent within the cell dictates the amount of processing which can take place. Thus the higher level of ACTH detected in these cultured cells could be a function of faster turnover and less time spent in the cell to be processed to α -MSH. It could also reflect the absence of an extrapituitary signal required for processing. Unlike the cessation of conversion of ACTH to α -MSH in culture, β -LPH continues to be cleaved quite completely to endorphin-like material. This could be due to the fact that β -LPH is the first molecule to be cleaved from the precursor and is, perhaps, accessible to processing enzymes within the cell for a longer period of time.

Anterior lobe corticotrophs in culture need a positive stimulus to secrete and thus appear to behave quite similarly to corticotrophs in the pituitary of intact animals. Studies by Scott & Baker (1975) on cultures of anterior and intermediate lobes of the pituitary of the Rainbow Trout are in agreement with our observations. Although Scott & Baker (1975) use grossly different culture techniques, they find that anterior lobe cultures secrete low but constant amounts of ACTH, while intermediate lobe cultures secrete up to 60 times their original ACTH contents by 8 days in culture. In addition, they noticed a shift in the distribution of immunoreactive forms in the intermediate lobe from α -MSH to ACTH, both in the cells and in the culture medium with time in culture. When fractionated by gel filtration a substantial amount of this immunoreactivity is eluted as 'large form' (precursor?) ACTH, in addition to bioactive $\alpha(1-39)$ ACTH.

REGULATION OF PROCESSING AND SECRETION OF ACTH/LPH MOLECULES
IN THE PITUITARY

The above studies show that the peptide backbone of the precursor is very similar in anterior and intermediate lobes of the pituitary but that the end products of processing of the precursor are different in the two lobes. These studies also show that the initial steps in processing of the precursor are very similar in the two lobes. The major difference appears to be that intermediate lobe cells have the capability of converting ACTH to α -MSH and CLIP and β -LPH to β -endorphin. These observations provide a basis for asking meaningful questions about regulation of synthesis, processing and secretion of ACTH/LPH peptides in anterior and intermediate lobe corticotrophs. We have designed experiments to test whether differences in regulation of secretion of these peptides reported in *in vivo* studies can be explained by the actions of specific regulators (CRF, glucocorticoids and catecholamines) in cell culture systems.

There are many indications in the rat that anterior lobe corticotrophs and intermediate lobe cells respond to different stimuli *in vivo*. When examined immunohistochemically, different *in vivo* stimuli lead to the depletion of ACTH-related material from their secretory granules (Moriarty, Halmi & Moriarty 1975). Anterior lobe corticotrophs respond to 'classical stresses' such as heat, cold, electrical shock and ether, while intermediate lobe cells respond to neurogenic stresses such as flashing lights or high pitched sounds (Moriarty *et al.* 1975). Adrenalectomy, which leads to hypertrophy of the anterior lobe, and an increase in plasma ACTH, alters neither the morphology nor the cytological response of the intermediate lobe (Moriarty *et al.* 1975). Lesions of the paraventricular nucleus of the hypothalamus, however, lead to an increase in both plasma and intermediate lobe MSH levels (Thody, 1974). In some species the intermediate lobe is either vestigial or absent, while corticotrophs are still present in the anterior lobe.

Secretion from anterior lobe corticotrophs both *in vitro* and *in vivo* appears to be under positive control by CRH and under negative control by glucocorticoids secreted by the adrenals (Guillemin & Rosenberg, 1955). The effects of hypothalamic extract and glucocorticoids on the corticotrophs of the anterior lobe of the pituitary are well established; CRH itself has been partially purified but its structure is unknown. In contrast to this, various hypothalamic factors which are putative intermediate lobe regulators have been isolated and their structures completely determined. Pro-leu-gly-amide and tocinoic acid (fragments of oxytocin), are reported to inhibit release of MSH (Bower, Hadley & Hruby, 1971; Celis, Taleisnik & Walter, 1971; Hruby *et al.* 1972). However, the effects of these factors on the secretion of MSH from intermediate lobe cells vary greatly depending upon the investigator and frequently they have no effect at all (Grant, Clark & Rosanoff, 1973; Fischer & Moriarty, 1977). We have never seen an effect by these factors on cultures of intermediate lobe cells.

Of obvious interest when trying to understand the regulation of a gland, are anatomical structures which could elucidate control mechanisms. It is relevant, therefore, that the anterior lobe is directly connected to the hypothalamus by portal vessels which have extensive capillary beds in the anterior lobe; while the inter-

mediate lobe is very poorly vascularized, being supplied indirectly by the capillary bed of the adjacent posterior lobe (Green, 1951). There are, however, dopaminergic nerve endings in the intermediate lobe (Kurosumi *et al.* 1961; Dawson, 1953), and the posterior lobe is richly innervated, while the anterior lobe contains only a few nerve endings which terminate predominantly (if not exclusively) on prolactin-secreting cells (Calabro & MacLeod, 1978). This type of observation nicely supports control of anterior lobe corticotrophs by blood-born factors and regulation of intermediate lobe cells by direct innervation or by posterior lobe factors.

The question arises as to whether differences in responsiveness of anterior and intermediate lobe cells observed in animals are due to differences in the cells or to other factors. Different modes of regulation could reflect merely the anatomical differences between the lobes or accessibility to blood-born factors versus neurotransmitters. In order to answer this question, it is necessary to test the effects of regulators on secretion of hormones from cells in culture.

We began by looking at the effects of known regulators of secretion of ACTH/LPH peptides on cultures of anterior lobe corticotrophs (glucocorticoids and CRH) and intermediate lobe cells (dopamine). The results of these experiments are shown in Fig. 10. Apomorphine, a dopamine agonist, was used because it is much more stable than dopamine. As shown in Fig. 10 apomorphine inhibits secretion of both ACTH and endorphin in intermediate lobe cultures, but has no effect on anterior lobe corticotrophs. In contrast to this, dexamethasone (a stable fluorinated derivative of hydrocortisone), a known inhibitor of ACTH secretion from the anterior lobe (Yates & Maran, 1974), has no effect on the secretion of ACTH and endorphin by intermediate lobe cells. We also tested the effect of CRH on intermediate lobe cultures. The same CRH preparation which gave a sixfold stimulation of ACTH and endorphin secretion from anterior lobe cells in culture had no effect on basal secretion of ACTH or endorphin by intermediate lobe cells.

We thought it possible that basal secretion of ACTH and endorphin by intermediate lobe cells was already so high in culture that further stimulation might be difficult to detect. For this reason, we pretreated intermediate lobe cultures with apomorphine to inhibit secretion, and then administered CRH in the presence of apomorphine. Even under these conditions CRH is unable to stimulate secretion of ACTH and endorphin by the intermediate lobe cells in culture. However, CRH in the presence of apomorphine is capable of stimulating ACTH and LPH secretion by anterior lobe cells and dexamethasone can inhibit this stimulation.

In summary, anterior lobe cultures are stimulated to secrete ACTH and LPH by a partially purified hypothalamic factor(s) termed CRH. This stimulation can be inhibited by glucocorticoids. Apomorphine, a dopamine agonist, has no effect on either the basal or stimulated secretion of ACTH and LPH in anterior lobe cultures. The regulation of secretion by tumour cells is similar to that of the anterior lobe. In contrast, secretion of ACTH and endorphin from intermediate lobe cells in culture is strongly inhibited by apomorphine, while dexamethasone has no effect. CRH does not stimulate ACTH or endorphin secretion in intermediate lobe cells in the inhibited state (apomorphine treated) or basal state. Preliminary experiments (data not shown) suggest an increase of the proportion of smaller forms of ACTH and LPH in intermediate lobe cells inhibited by apomorphine.

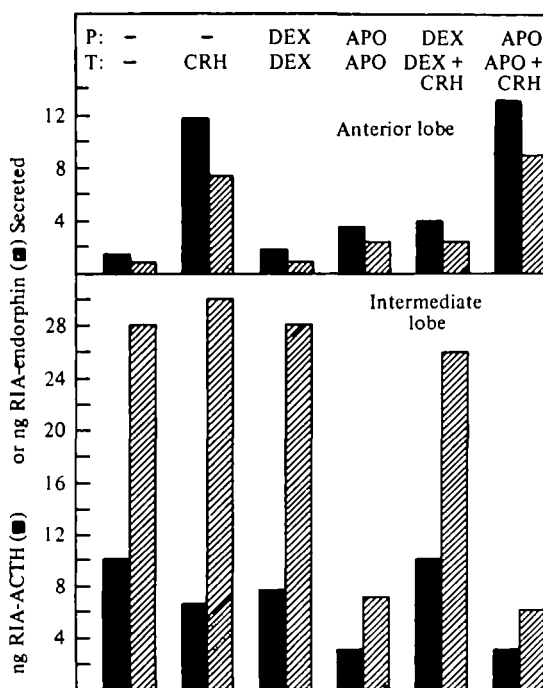


Fig. 10. Regulation of secretion of hormones from anterior and intermediate lobe pituitary cells in culture by dexamethasone, apomorphine and corticotropin releasing hormone(s) (CRH). Rat anterior and intermediate lobe cells were dissociated by a combination of enzymatic and mechanical dispersion techniques and plated at a density of 1 intermediate lobe and 1/5 anterior lobe equivalent per microwell. On the 4th day in culture a preincubation (designated P at top of upper chart) was begun with medium containing 5×10^{-6} M apomorphine, dexamethasone or nothing. After 24 h, the medium was changed and a treatment (T) was started with medium containing 5×10^{-6} M dexamethasone, apomorphine, dexamethasone + corticotropin-releasing hormone (CRH), apomorphine + CRH, CRH, or nothing. After 3 h, the medium was collected, frozen and subsequently analysed by radioimmunoassay with both ACTH and endorphin antisera. CRH was prepared from rabbit hypothalami by acid extraction followed by gel filtration of the extract on G-25 sephadex columns (Allen *et al.* 1978).

These experiments indicate quite clearly a difference in responsiveness of anterior and intermediate lobe corticotrophs in culture. The *in vivo* indications of different modes of regulation do not merely reflect the anatomical differences between the lobes or accessibility to blood-born factors or neurotransmitters. It appears that the ACTH/LPH producing cells of the cells of the anterior and intermediate lobes of the pituitary in addition to exhibiting processing differences *in vivo*, also respond to different regulatory agents *in vitro*.

This type of analysis is important in assessing the role of each lobe of the pituitary in the animal. When anterior lobe corticotrophs are cultured, they are removed from the source of positive regulation (CRH from the hypothalamus) and show very low levels of ACTH/LPH secretion. When intermediate lobe cells are cultured they are removed from the source of negative regulation (dopamine from the hypothalamus) and secrete ACTH (α -MSH) and endorphin at a high constitutive rate. It will be interesting to determine if a positive regulator of secretion exists for the intermediate

lobe, which can override the dopamine inhibition, or if a physiological stimulus for intermediate lobe α -MSH/endorphin secretion is mediated merely by a reduction of dopamine levels and, thus, release of inhibition.

We are currently attempting to determine if apomorphine inhibition of secretion of intermediate lobe cells in culture is sufficient to ensure the cleavage of ACTH to α -MSH. It has recently been reported that β -endorphin [β -(61-91)LPH] is rapidly converted to β (61-87)LPH (C' fragment) in the intermediate lobe by removal of four carboxy terminal amino acids (Zakarian & Smyth, 1979). The C' fragment can also be acetylated (Smyth & Zakarian, 1979). We would like to investigate the processing of β -endorphin to C' fragment and the acetylation of ACTH (or α -MSH) and endorphin (or C') in order to determine if the capacity to make these modifications decreases with time in culture.

SPECULATION ABOUT TARGETS OF HORMONES

Along with different mechanisms of regulation of secretion, one must consider the targets of the secretory products, the routes by which they reach their targets, and the effect they exert upon their targets. The only unambiguous case in which the target is known is that of plasma ACTH. Upon reaching the adrenals via the blood, ACTH stimulates secretion of glucocorticoids (Li, Kalman & Evans, 1949). The role of β -LPH and β -endorphin in the periphery is not as well understood, nor is it clear that all the anterior lobe products are directed to the blood stream (and not the central nervous system). It is difficult to imagine an efficient entrance of intermediate lobe products into the blood stream, since this lobe is so poorly vascularized. It is attractive to postulate retrograde axonal flow or some such mechanism whereby the target for the intermediate lobe products would be in the central nervous system. The role of α -MSH in higher vertebrates is very poorly understood. In lower vertebrates it is involved in pigment changes for light adaptation, but only in pathological cases (and Agouti mice) does it alter pigmentation in higher vertebrates (Levitan, Gomez Dumm & Iturriza, 1979). Although by no means definitive, there are numerous implications of MSH effects on learning, behaviour and displacement activities in higher vertebrates (Veith *et al.* 1978; Delius, 1970). β -endorphin, another molecule derived from the ACTH/LPH precursor, has a very potent opiate activity in the brain (Li & Chung, 1976a); however, the main forms of endorphin found in the intermediate lobe – *n*-acetylendorphin, C'(61-91)LPH and *n*-acetyl-C' – have very weak opiate activity (Geisow *et al.* 1977). Therefore, the role of intermediate lobe endorphins is not understood at this time. The only reported systemic role of any intermediate lobe product in higher vertebrates is that for CLIP. This peptide stimulates insulin secretion in a mutant strain of mice which has unusually high pituitary and plasma levels of α -MSH and CLIP (Beevor *et al.* 1978; Beloff-Chain, Edwardson & Hawthorn, 1977).

Although tempting, it is premature to assign a systemic role to anterior lobe corticotrophs and CNS role of intermediate lobe cells. A portion of this paper represents work we have done on anterior and intermediate lobe primary cultures with respect to the effects of hypothalamic factors, neurotransmitters and glucocorticoids.

on the secretion of ACTH/LPH related molecules. It is our hope that by understanding the differences in regulation of secretion at a cellular level it will be easier to assess the roles of the anterior and intermediate lobes of the pituitary in the animal.

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