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CHARACTERIZATION OF POMC-DERIVED PEPTIDES FROM GUINEA-PIG AND HUMAN PITUITARIES

by

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ABSTRACT

The biochemical characterization of the pro-opiomelanocortin (POMC) products, isolated and purified from a human anterior lobe pituitary adenoma associated with Cushing's Syndrome, illustrates the processing of this prohormone. In the tumor, 87% of the acidic joining peptide (AJP) is found as a dimer rather than as a monomer, the prevalent form found in normal pituitary tissue. The corticotropin (ACTH) purified and characterized from the tumor was found not to be phosphorylated. In contrast, human ACTH of normal pituitary origin is found to be 30% phosphorylated at the serine residue at position 31. ß-lipotropin (ß-LPH) is partially processed into γ-LPH and a single form of ß-endorphin as seen in normal anterior pituitary tissue. Further HPLC and amino acid analysis did not reveal any modifications in the purified ß-LPH such as the formation of an amino-terminal pyroglutamate. The identities of these POMC derivatives was confirmed by determination of their masses by ion-spray mass spectrometry (IS-MS). Finally the amino-terminal fragment of POMC was found to be subject to both N- and O-linked glycosylation. No evidence of cleavage at residues 49-50 to produce γ_3-MSH and the 1-49 fragment was found. The elution pattern of the peptides closely corresponds to what was expected for anterior pituitary processing. However, the biosynthetic machinery of the tumor causes an exaggerated production of the peptides and certain biochemical modifications of some of the peptides, especially those at the N-terminus.

ACTH was purified from extracts of guinea-pig anterior pituitaries and characterized in terms of its amino acid composition and molecular weight using IS-MS and HPLC. The unusual structural characteristics of guinea-pig ACTH, namely having an alanine for proline substitution at position 24 and the lack of phosphorylation, were confirmed. The dose-response characteristics of guinea-pig ACTH were compared with those of a synthetic human ACTH(1-39) standard in both isolated rat fasciculata-reticularis and glomerulosa cell bioassays. Guinea-pig ACTH was found to have a similar activity to that of human ACTH with respect to the maximal steroid output of corticosterone and aldosterone. However, it proved to be slightly more potent in

terms of the concentration which elicited half-maximal steroid secretion. Under the assay conditions used, guinea-pig ACTH does not seem to a superagonist as suggested by a previous study. Combining amino acid compositions, mass spectrometric data, and the recent determination of the cDNA sequence for guinea-pig ACTH, the identification of various purified biosynthetic derivatives of guinea-pig POMC was facilitated.

Joining peptide, a major product of POMC processing, was found in extracts of both anterior and neurointermediate lobes. The purified peptide corresponded exactly in amino acid composition and mass to the predicted structure established by the cDNA sequence. Also, by using ion-spray mass spectrometry, post-translational modifications of other products of intermediate lobe proceesing were observed. N- and O-acetylation of α-melanotrophin, partial O-phosphorylation of corticotrophin-like intermediate lobe peptide and carboxyl-terminal amidation of ß-melanotrophin were identified.

ABREGE

En utilisant la chromatographie liquide à haute pression en phase inverse, la purification, l'isolation, et la caractérisation biochimique des produits de la POMC ont pu être faites et permettre d'illustrer le processing cellulaire d'une prohormone provenant d'un adénome du lobe antérieur hypophysaire associé à la maladie de Cushing. 87% de l'AJP (acidic joining peptide) isolé de la tumeur était sous forme dimérique ceçi étant différent de ce que l'on retrouve dans les tissus normaux soit la forme monomérique. La corticotropine (ACTH), purifiée et caractérisée de la tumeur, n'est pas phosphorylée contrairement à ce que l'on aurait pu s'attendre du fait qu'il y a présence d'un site de phosphorylation (ser_{31}) dans le peptide. La ß-lipotropine (ß-LPH) est partiellement clivée en ß-endorphine et γ-LPH comme retrouvé dans l'adénohypophyse normale. De plus, l'HPLC et leur analyse en acides aminés n'a pas révelé de modifications post-translationnelles telle que la formation d'un pyro-glutamate en position N-terminale chez la ß-LPH. Le fragment N-terminal de la POMC a aussi été isolé et purifié. Nous avons pu démontrer que ce peptide a subit une double glycosylation de type N et O, et qu'il n'y avait aucune évidence de clivage entre les acides aminés 49-50 pour produire le fragment 1-49 et la γ-MSH. Le tracé chromatographique de l'élution des différents fragments de la POMC correspond de très près à celui que l'on retrouve pour ce précurseur hormonal. Cependant, des altérations biochimiques sont présentes sur certains des peptides produits, indiquant probablement l'existance de modification au niveau de l'appareillage enzymatique des cellules tumorales.

Le deuxième volet de cette étude concerne la POMC de cochon d'inde. En utilisant une technique de spectrométrie de masse et l'HPLC en phase inverse, l'ACTH de cochon d'inde, provenant d'extraits d'adénohypophyses, a pu être purifiée et caractérisée en fonction de sa compostion en acides aminés et de poids moléculaire. La structure de cette hormone, qui contient des différences fondamentales avec l'ACTH humaine telles que la présence d'alanine au lieu de proline en position 24 et une absence de phosphorylation, a été confirmé. L'activité biologique de l'ACTH de cochon d'inde a ensuite été comparé à un standard synthétique d'ACTH (1-39) humain avec l'aide de deux bioessais.

Les bioessais consistent en un système in vitro de cellules de surrénales de rat dispersées, provenant soit des zones fasciculée et réticulée productrices de gluco-corticoïdes ou soit de la zone glomérulée productrice de minéralo-corticoïdes. La production maximale de stéroïdes (corticostérone et aldostérone) était similaire entre l'ACTH de cochon d'inde et l'ACTH humaine. Cependant, l'ACTH de cochon d'inde est plus puissante que l'ACTH humaine car elle nécéssite une concentration de 2 à 3 fois moindre pour atteindre 50% de la dose maximale de stéroïde produite. Aussi, d'après les conditions des bioessais utilisés, l'ACTH de cochon d'inde ne semblerait pas un superagoniste comme suggéré par une autre étude. Grâce à la spectrométrie de masse, la composition d'acides aminés, et la séquence d'ADN pour la POMC de cochon d'inde récemment établie, l'identification de peptides originant de la POMC a été rendu plus facile. L'AJP, un des produits de la biosynthèse de la POMC, a été isolé autant dans l'extrait de neurohypophyse que celui d'adénohypophyse. Le peptide purifié correspond exactement en composition d'acide aminés et en masse à la séquence d'ADN prédit par clonage moléculaire. Aussi, en utilisant la spectrométrie de masse, des modifications post-translationnelles de certains peptides purifiés de la neurohypophyse de cochon d'inde ont pu être identifiés. L'acétylation N et O de l'α-mélanotrophine, la O-phosphorylation partielle du peptide CLIP, et l'amidation de la ß-mélanotrophine ont été observés.

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ABBREVIATIONS

MEASURES

g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
kDA	kilodalton
N	normal
mM	millimolar
L	liter
ml	milliliter
µl	microliter
nmoles	nanomoles
pmoles	picomoles
CPM	counts per minute
V/V	volume per volume
W/V	weight per volume
^0C	degrees Centigrade
mm	millimeter
µm	micrometer
K_m	Michaelis-Menton constant

MATERIALS and METHODS

HPLC	- high performance liquid chromatography
RP-HPLC	- reversed-phase high perfomance liquid chromatography
GP-HPLC	- gel permeation high performance liquid chromatography
ODS-silica	- octadecylsilyl-silica
MECN	- acetonitrile
TFA	- trifluoroacetic
HFBA	- heptafluorobutyric acid
TRIS	- hydroxymethyl aminomethane hydrochloride
RIA	- radioimmunoassay
MRI	- magnetic resonance imaging

BIOLOGICAL MOLECULES

ACTH	Adrenocorticotropin
AJP	Acidic joining peptide
AP	Aminopeptidase ß-like enzyme
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CLIP	Corticotropin-like intermediate peptide
CMO	Corticosterone methyl oxidase
CPB	Carboxypeptidase ß-like enzyme
CRF	Corticotropin releasing factor
DBH	Dopamine ß-hydroxylase
DHEA	Dehydroepiandrosterone
DOC	11-deoxycorticosterone
ER	Endoplasmic reticulum
FSH	Follicle stimulating hormone
Fuc	Fucose
GABA	Gamma-aminobutyric acid
Gal	Galactose
Glc	Glucosamine
GlcNAc	N-acetyl glucosamine
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
3ß-HSD	3ß-hydroxysteroid dehydrogenase
LDL	Low density lipoprotein
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
ß-LPH	ß-lipotropin
4MA	4 NN-diethyl-4-methyl-3-oxo-4-aza-androstan-17-ß-carboxamide
Man	Mannose
MSH	Melanotropin
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
P45011ß	11ß-hydroxylase cytochrome P_{450}
PAM	Peptidyl-glycine α-amidating mono-oxygenase
PBR	Peripheral benzodiazepine receptor
PCE(PC)	Prohormone converting enzyme
POMC	Pro-opiomelanocortin
RER	Rough endoplasmic reticulum
SA	Sialic acid
SCP-2	Sterol carrier protein 2
SRP	Signal recognition particle
TSH	Thyroid stimulating hormone
16K	Amino-terminal fragment

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CHAPTER 1: INTRODUCTION

1.1. Steroidogenesis in the Adrenal Cortex

The synthesis of steroid hormones occurs in many tissues- the adrenal cortex, gonads, adipose tissue in postmenopausal women, the fetus, the placenta of pregnant women and certain peripheral tissues. These hormones, such as aldosterone, cortisol, and estrogen are part of regulatory mechanisms which are of vital importance for the maintenance of homeostasis and bodily functions. Because there are many sites for steroidogenesis, attention will be focused on what happens at the level of the adrenal cortex.

Cholesterol is the biosynthetic precursor needed for the production of all steroid hormones. In most species, it can be derived de-novo from acetate (1) or from plasma low density lipoproteins (2,3,4,5) which can be taken up at the cell surface by a high affinity LDL receptor (6). In the rat, the main source of cholesterol comes from high density lipoproteins (HDL) (7,8). The key enzyme in cholesterol biosynthesis is 3-hydroxy 3-methylglutaryl coenzyme A (HMG CoA) reductase and in the human adrenal cells it can be suppressed by elevated concentrations of LDL (9). LDL cholesterol esters are taken up by receptor-mediated endocytosis (10) and are

either stored in lipid droplets or converted to free cholesterol. However, a lack of either LDL (abetalipoproteinaemia) or the LDL receptor (hypercholesteraemia) can lead to a decrease of steroid hormone production, following chronic ACTH stimulation (3,11,12,13,14).

Cholesterol storage in lipid droplets is controlled by two enyzmes -cholesterol ester hydrolase and cholesterol ester synthetase. The liberation of free cholesterol is called the acute response. This response is found primarily in the adrenal cortex. Thus a hormone such as ACTH binds to its specific cell surface receptor, activates adenylate cyclase which in turn increases intracellular cAMP levels. This in turn brings about the stimulation of cholesterol ester hydrolase, liberating free cholesterol for steroid hormone biosynthesis, while the cholesterol ester synthetase is inhibited (15,16,17).

Free cholesterol is insoluble in the cytosol and its rapid mobilization to the mitochondria may involve a Sterol Carrier Protein 2 (SCP-2) (18,19,20). At the level of the mitochondria, cholesterol is first incorporated into the outer mitochondrial membrane (21) where P-450_{scc} (side chain cleavage) converts it into pregnenolone (22,23,24). Work by several groups has shown that intramitochondrial transport of cholesterol to the inner membrane can be blocked by cyclohexamide (22), and several proteins have been postulated to participate in this process (25,26,27). However, recent work indicates that a mechanism, coupled to peripheral type benzodiazepine receptors(PBR), mediates the translocation of free cholesterol from the outer to the inner mitochondrial membrane in adrenocortical cells (28). This supports the detailed studies that demonstrate that the rate limiting step in steroid hormone synthesis is not the reaction

involving P-450$_{scc}$ but rather the transport of the free cholesterol to the mitochondrial membranes (22,29,30).

Common to all steroidogenic pathways, the conversion of cholesterol to pregnenolone in the mitochondria involves a cytochrome P450 system that includes three distinct enzymes (adrenodoxin reductase, adrenodoxin, and P450$_{scc}$) (31) which together form an electron transport chain (32,33,34). Adrenodoxin reductase, a flavoprotein loosely associated with the inner mitochondrial membrane, accepts electrons from NADPH (35,36,37) and then transfers them to an iron-sulfur protein, adrenodoxin, found in the mitochondrial matrix (36,37,38,39). Adrenodoxin transfers the electrons to P450$_{scc}$, located on the inner aspect of the inner mitochondrial membrane (40,41) and acting as a terminal oxidase. The reaction uses three molecules of NADPH and three molecules of oxygen, which are needed for 20α-hydroxylation, 22-hydroxylation, and cleavage of the cholesterol side-chain at the bond between carbon atoms 20 and 22 to give pregnenolone and isocaproic acid. It is also noteworthy to mention that adrenodoxin and adrenodoxin reductase both serve as electron transport proteins for all the mitochondrial P450s, even those in non-steroidogenic tissues (42,43,44).

Pregnenolone produced by the P450$_{scc}$ system leaves the mitochondria and migrates to the endoplasmic reticulum. There, it can undergo two further transformations. One of these involves the action of the 3ß-hydroxysteroid dehydrogenase / $\delta^5 \rightarrow \delta^4$ isomerase enyzme (3ß-HSD). It has recently been shown using purified 3ß-HSD that 4MA (N,N-diethyl-4-methyl-3-oxo-4-aza-5α-androstan-17-ß-carboxamide), a known inhibitor of 5α-reductase, is also a potent inhibitor of 3ß-

HSD activities while Trilostane, a well known inhibitor of 3ß-HSD, can inhibit both the dehydrogenase and isomerase activity of the purified enzyme (45). Thus, these data seem to suggest that both the dehydrogenase and isomerase activities of the 3ß-HSD are on separate sites within the 3ß-HSD protein.

The other transformation that pregnenolone may undergo involves the 17α-hydroxylase cytochrome P-450 enzyme and leads to the formation of 17α-hydroxypregnenolone. This steroid may then undergo cleavage of the C-17,20 carbon bond to give rise to the precursor of the sex steroids, dehydroepiandrosterone (DHEA). Progesterone can also be converted by the 17α-hydroxylase enzyme to give 17α-hydroxyprogesterone and androstenedione when there is cleavage at the C-17,20 bond. This P-450 enzyme is located on the smooth endoplasmic reticulum, where it accepts electrons from P450 reductase, a flavoprotein, which has been shown to be immunologically distinct from the one used in the mitochondria and does not use an iron/sulfur-like adrenodoxin (46,47). The presence of cytochrome b_5 and certain phospholipids at the level of the endoplasmic reticulum also facilitates the transfer of electrons to P450-17α (48,49,50). The distinction between the 17α-hydroxylase and 17,20 lyase activities seems more functional than genetic or structural, and it appears that the factors regulating electron transport to P450-17α play an important role in this differentiation. In steroid hormone synthesis, the P450-17α enzyme represents a key branching point between the mineralocorticoid and glucocorticoid pathways in the adrenal cortex, and also its 17-20 lyase activity allows it to catalyse the formation of the major sex steroids. However, adrenals of guinea pig, hamster, rabbit, and rat do not contain the P450-17α enzyme and these species rely strictly on the 3ß-

HSD enzyme to convert pregnenolone into progesterone.

Once progesterone and 17α-hydroprogesterone are synthesized, they undergo a third hydroxylation at position 21 by a unique enzyme, 21-hydroxylase P450 ($P450_{c21}$), to form 11-deoxycorticosterone (DOC) and 11-deoxycortisol (S). This enzyme is found in the smooth endoplasmic reticulum and uses the same flavoprotein, P450 reductase , as the P450-17α to transport the electrons from NADPH. One of the interesting aspects of this enzyme is the fact that it is found to be deficient in about 1 in every 7000 persons, and inherited in an autosomal recessive fashion. Some of the clinical symptons of this disease, called congenital adrenal hyperplasia (CAH), include decreased cortisol and aldosterone levels which often lead to sodium depletion, hypertension, cardiovascular collapse, and even death (within the first month after birth) (25). Because of the decreased synthesis of cortisol, the negative feedback loop with the anterior pituitary is impaired causing overproduction of ACTH. Thus in utero, there will be an increase in androgen production since the 17-hydroxyprogesterone cannot be converted by the $P450_{c21}$ (51,52).

The final enzyme involved in the production of glucocorticoids in fasciculata and reticularis cells of the adrenal cortex is 11ß-hydroxylase cytochrome P450 ($P450_{11ß}$). Once DOC and S are produced, they leave the endoplasmic reticulum to re-enter the mitochondria where the $P450_{11ß}$ enzyme is located in the inner mitochondrial membrane (53). This enzyme uses the same electron transport chain (adrenodoxin reductase, and adrenodoxin) as described for $P450_{scc}$ (54). Thus, the 11ß-hydroxylation of DOC leads to the synthesis of corticosterone (B), the major

glucocorticoid in rodents, while S is converted into cortisol (F), the main glucocorticoid in man.

Aldosterone synthesis takes place exclusively in the zona glomerulosa of the adrenal cortex. It has recently been shown in rats that a second form of the $P450_{11\beta}$ enzyme of lower molecular weight (49K) than the one found in the zona fasciculata and reticularis exists (55), and both these forms have been cloned and sequenced (56,57). In the zona glomerulosa, both forms of the enzyme have been found, and under proper stimulus, the gene encoding for the 49K form is favored (58,59). Results indicate that the function of the 51K form of the $P450_{11\beta}$ enzyme would be to convert DOC into corticosterone (B) (60,61), using its 11ß-hydroxylase activity to insert an oxygen atom at the C-18 position of B, followed by a loss of water. Then, the 49K form of the enzyme would be responsible for the final two steps of aldosterone biosynthesis (55,62). This first step involves the transformation of B to 18-hydroxycorticosterone by the enzyme's 18-hydroxylase activity (63,64). 18-hydroxycorticosterone is then converted into aldosterone by corticosterone methyl oxidation (CMO 1 and 2) (55). Thus, the two forms of the $P450_{11\beta}$ enzyme may explain the zonal specificity of aldosterone biosynthesis.

1.2. Cushing's Disease

The study of Cushing's disease encompasses the physiology of the whole hypothalamic-pituitary-adrenal axis, since ultimately these three endocrine glands are affected by the syndrome. The two key elements are ACTH, from the processing of proopiomelanocortin

(POMC) (Figure 1.01), and cortisol, a glucocorticoid produced in excessive amounts. Its is through a deregulation of the these hormones that this pathological condition takes place.

Cushing's disease is persistent, inappropriate hypercortisolism, resulting most often from pituitary ACTH hypersecretion. Although cases resembling Cushing's Syndrome appeared at the turn of the twentieth century (65), it was not until Harvey Cushing's report (66), describing cases of "pituitary basophilism" that the disorder was clinically defined. Cushing's view was that the disease was of pituitary origin, however, others also emphasized the role of the adrenal cortex in the pathogenesis of the syndrome. This disease is primarily a disorder of women of childbearing age, accounting for approximately 70% of adult patients with Cushing's Syndrome according to current studies (67).

1.2.1. Pathology of Cushing's Disease

Although early studies seemed to indicate that many patients with Cushing's disease did not have tumors, many surgical series in the 1970s showed that more than 90% of patients have demonstrable tumors at surgery even though they were not necessarily visible on roentgenograms (68,69). These tumors can be classified into two distinct categories: tumors of primary origin and those of secondary origin.

In cases of tumors of primary origin, which represent approximately 30% of the causes

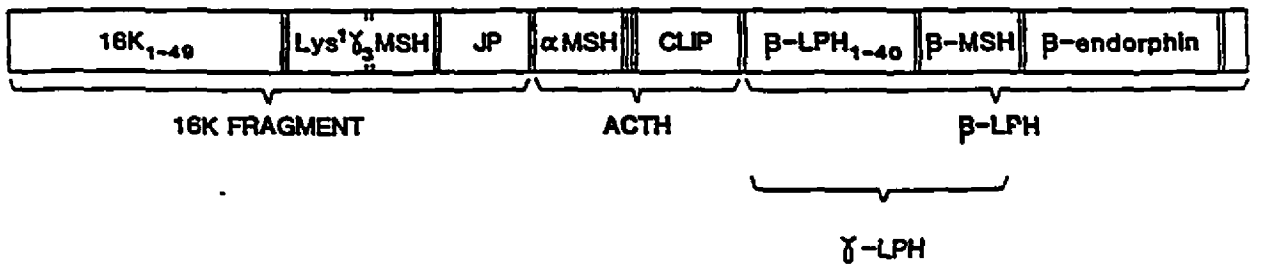


Figure 1.01 Schematic representation of POMC processing in the pituitary.

of the disease, they are considered to be ACTH-independent since pituitary secretion is suppressed by the excessive amount of cortisol that is produced either by adrenocortical tumors, autonomous hyperplastic nodular glands, or rare cases of ectopic tumors (70). Thus the negative feedback loop of the adrenal with respect to the pituitary is always "on". However, it is not totally clear whether Cushing's disease caused by hyperplastic adrenal glands is truly of primary origin. It has been suggested that the pituitary originally regulates cortisol secretion while the adrenal eventually becomes autonomous (71), and that, in the process, there may be an increased adrenocortical cell sensitvity to ACTH (72). Also, when the secretion of ACTH by these normal pituitary glands ceases, the ACTH-dependent portion of the adrenals become atrophic. The administration of large doses of dexamethasone, a synthetic glucocorticoid that can suppress ACTH secretion, is without effect on plasma cortisol or urinary 17-ketosteroids because the cortisol produced originates from adrenocortical tumor tissue and does not depend upon pituitary ACTH release which is already suppressed.

Confirming Cushing's original description of the disease, approximately 80% of the patients with Cushing's disease have pituitary adenomas of secondary origin or ectopic ACTH tumors. 80 to 90% of these are microadenomas of a diameter of less than 10mm, some of which have been found to be less than 2mm (73, 74). The majority of these small tumors are located in the periphery of the gland and are confined to the osteoaponeural sheath iof the sella turcica. These pituitary lesions can usually be seen by imaging techniques such as MRI (magnetic resonance imaging) and CT scans. Petrosal sinus sampling with or without CRH stimulation can also be helpful in localizing these lesions (75, 76, 77). Large ACTH secreting macroadenomas (> 10mm)

which account for the rest of the pituitary tumors often cause sellar enlargement and tend to be locally invasive with possible supra sellar expansion (78).

Although the true nature, origin, and biochemical characteristics of microadenomas involved in Cushing's disease are not always clear (79,80), they nonetheless have the following characteristics of being basophilic, unencapsulated, and located within the anterior lobe of the pituitary (69,74). The macroadenomas causing the disease tend to be chromophobic (81). Usually, these corticotroph adenomas are composed of a homogeneous, well-granulated cell type forming compact sheets with sinusoidal arrangement. They also exhibit Crooke's changes, a zone of perinuclear hyalinization, resulting from the hypercortisolism itself (74). Immunohistochemical staining generally shows the presence of ACTH and ß-lipotropin in these tumor cells while other POMC-derived products may or may not be seen using this technique (82). Moreover, the processing of POMC would seem normal, in contrast to ectopic ACTH-secreting adenomas (83,84), but heterogeneity does clearly exist at the level of the secretory granules where their size and number can vary considerably from cell to cell.

1.2.2. Physiology of Cushing's Disease

In a normal individual, hypothalamic CRF stimulates ACTH secretion in a pulsatile fashion (85,86), and other factors such as angiotensin II and arginine vasopressin help to modulate this response. Regulation of the hypothalamic-pituitary-adrenal axis depends on diurnal rhythms,

stress, and negative feedback due to the rate of change and concentration of cortisol. Because of the increased secretion of ACTH involved in an ectopic or pituitary adenoma of Cushing's disease (87), there is a loss of this normal negative feedback of ACTH by cortisol even though an augmentation of the total cortisol secreted by the adrenal glands is evident (88). There is also a loss of normal circadian rhythm in Cushing's disease of secondary origin. This absence of rhythmicity of ACTH and cortisol magnifies the differences between normal plasma levels of these hormones found in the afternoon and evening.

Another result of Cushing's disease is hypersecretion of adrenal androgens which is proportional to that of ACTH and cortisol. Characteristically, patients will respond to metyrapone, an 11-ß-hydroxylase inhibitor commonly used in the differential diagnosis of Cushing's disease, and suppression with high doses of dexamethasone, although up to 40% will exhibit resistance to these drugs (89).

Ectopic ACTH-dependent adenomas are not rare and have been recognized for a long time as a potential cause of Cushing's disease (90,91). These cases have been coined ectopic ACTH syndrome by Liddle et al (92). The most frequent neoplasms associated with ectopic ACTH production are the carcinoma of the lung, predominantly oat cell or small round cell type, thymic carcinoma, and pancreatic carcinoma and they account for approximately 70% of all the tumors involved in this disease (78). In this situation, the negative feedback of cortisol on the pituitary is fully functional, indicating that most of the ACTH found in the circulation originates from the ectopic tumor and that pituitary ACTH content is low. This is why patients fail to

respond to dexamethasone or metyrapone.

Recently, ectopic CRH production as the direct cause of Cushing's disease has been identified (93). Tumors that can secrete CRH include bronchial carcinoids, medullary thyroid carcinoma, and prostatic carcinoma. Morphologically, pituitary corticotroph hyperplasia is a direct cause of the increased CRH stimulation. However, this type of ectopic ACTH syndrome may respond to negative feedback (94).

1.2.3. Clinical Expression of Cushing's Disease

Cushing's disease is usually a disorder of women between the ages of 20 to 40 years with a female to male ratio of 8:1. The clinical manifestations of this disease reflects the biological state due to corticosteroid excess. Although some of the symptoms of the disease, for instance hypertension, diabetes, androgen-type hirsutism, and acne, can be found in patients without cortisol excess, several other clinical signs can be used to help diagnose Cushing's disease. They include the following: 1) muscle weakness due to protein catabolism, 2) osteoporosis (95), 3) obesity, especially of the neck, face, abdomen, and trunk, found with a high incidence, and often caused by a redistribution of fat since no weight gain may be seen (96), 4) skin problems, such as facial plethora, purple striae, and hirsutism, in cases of grossly increased concentrations of adrenal androgens (96), 5) hypogonadism where hypercortisolemia and hyperandrogenism suppress the hypothalamic-pituitary-gonadal axis (97), 6) hyperpigmentation due to excess ACTH

secretion, and 7) neuropsychiatric manifestations (98,99).

1.2.4. Treatment of Cushing's Disease

Treatment of Cushing's disease depends on its origin. Four approaches are presently used in the management of this pathological condition when it is classified as ACTH-dependent of pituitary origin: adrenal surgery, drug therapy, pituitary irradiation, and pituitary surgery (100,101,102). Of these, the treatment of choice is microsurgical removal of the pituitary adenomas using a trans-sphenoidal operation which has an efficacy of 80 to 90% (68,73). In cases of an ectopic ACTH syndrome, treatment involves the surgical resection of the primary tumor followed by radiation therapy or chemotherapy. Adrenocortical adenomas should be surgically removed (103) and followed by replacement therapy with physiologic doses of cortisol since adrenal insufficiency occurs post-operatively.

1.3. PEPTIDE BIOSYNTHESIS AND PROCESSING

1.3.1. Introduction

1.3.1.1. Regulation of Secretion

In the pituitary gland, corticotrophs, which synthesize and process POMC, represent 2% to 10% of pituitary cell content (104). Histologically, this gland is composed of two or three distinct zones. In the human, the pituitary is divided in two zones, the anterior lobe and the posterior lobe; however cells of a third zone, the intermediate lobe, absent from most primates, have been shown to be dispersed throughout the anterior lobe of the adult human pituitary (105) while in the rodent family, the cells are found in a very well defined intermediate lobe. Also, virtually all cells of the intermediate lobe have the ability to produce and secrete peptides derived from POMC.

The mechanisms for the regulation of POMC release are different in the anterior and intermediate lobes. In the anterior lobe, many substances have been implicated in the release of POMC-derived peptides, such as serotonin (106), adrenalin (107,108), and angiotensin II (109,110,111). However the main stimulator of this release is the corticotropin releasing factor (CRF) (112). This peptide, originating from the hypothalamus, is active in vivo and causes a rapid accumulation of cAMP in anterior pituitary cells (113). The release of POMC-derived

peptides, most notably ACTH, may then be elicited by activation of protein kinase C (114) and appears to be Ca^{2+} dependant (112). It should be noted that a variety of stessful or painful stimuli can also stimulate the release of POMC-derived peptides from the anterior pituitary (115,116). The inhibitory control of peptides of POMC origin is primarily achieved by direct glucocorticoid negative feedback from the adrenal gland (117). Glucocorticoids, via a feedback loop, can also cause a rapid decrease in CRF content of the median eminence (118), demonstrating that anterior pituitary POMC derivatives can be regulated centrally. Moreover, it has been suggested that the inhibitory control may be mediated via hypothalamic gamma-aminobutyric acid (GABA) (119,120).

In contrast to the anterior lobe, the intermediate lobe of the pituitary in rodents is directly innervated by neurons from the brain, and its regulation is thought to be primarily under negative dopaminergic control (121,122,123). Results indicate that activation of D2-dopaminergic and alpha-adrenergic receptors caused an inhibition of alpha-MSH secretion from this lobe, while activation of beta-adrenergic receptors stimulated release (121). Other factors such as GABA (124) and CRF (125) can also play a regulatory role in POMC-derived peptides from the intermediate lobe. However conflicting results concerning GABA-mediated inhibition exist (119,124). Finally, the intermediate lobe may be regulated by the stress of pain but the mechanisms of control would be different (126).

1.3.1.2. General Overview of Peptide Biosynthesis

The events that lead to the biosynthesis and secretion of hormonal peptides and proteins occur at the cellular level. Since the cell is highly compartmentalized, organelles involved in hormone synthesis have a very specific function in the biosynthesis and maturation of peptides and these events take place in a sequential manner.

The biosynthesis of hormonal peptides and proteins first begins at the level of the ribosomes within the cytoplasm which translate mRNA into protein. This translation is arrested after the formation of the pre-sequence and continues only when the ribosomes are associated with the membrane of the rough endoplasmic reticulum (RER). The nascent protein then enters into the lumen of the RER. During entry, early biosynthetic co-translational processes take place: cleavage of the pre-sequence, disulfide bond formation, and the initation of N-linked glycosylation. Once this has occurred, the precursor is then transported to the Golgi apparatus. It is at this level that N-linked glycosylation is completed and that other post-translational processes also occur, namely, O-linked glycosylation, sulfation and phosphorylation-the latter two events follow glycosylation. Hydroxylation of lysine can also take place in the Golgi fraction.

Finally, it is in the trans face of the Golgi fraction and in the secretory granules that the last modifications of the precursor leading to the production of the mature and secreted form of the peptide. This first involves the action of cleavage enzymes or convertases which first cleave the precursor at pairs of basic residues and then specific exopeptidases which remove the amino and carboxyl terminal basic amino acids that are revealed. Once this has occurred, other

enzymes come into play which further modify the peptides that have just been generated. Amidation of the C-terminal, acetylation of the N-terminus and the formation of pyrolidone carboxylic acid from glutamine at the N-terminus can take place on various peptides before they are secreted by the cell.

1.3.2. Events in the Rough Endoplasmic Reticulum

As the mRNA leaves the nucleus via nuclear pores, it contains the message that encodes for the peptide that will be produced by the cell. Once in the cytoplasm, in the vicinity of the endoplasmic reticulum (ER), it associates with ribosomes and translation begins. The first part of the mRNA that is translated corresponds to a stretch of 20 to 30 amino acids, which are mainly hydrophobic in nature and constitute the pre-sequence or the signal sequence. It is this pre-sequence that will interact with the signal recognition particle (SRP), located in the cytoplasm (127).

The SRP, a ribonucleoprotein (128), attaches itself to the large sub-unit of the ribosome and functions as an adapter between the elongation mechanism in the cytoplasm and the translocation process that occurs at the level of the ER membrane. As the nascent peptide chain emerges during translation, the affinity of the SRP for the ribosome increases because of the presence of the pre-sequence (129). This SRP molecule is composed of 6 non-identical polypeptide chains and divided into 2 specific domains (130): The elongation arrest domain, made up of sequences that are homologous to the repetitive ALU DNA sequence family and the signal recognition domain.

The affinity between the pre-sequence and the SRP at the level of the signal recognition domain (54kDa) (131) increases as the pre-sequence gets more and more elongated and evidence for this interaction has been provided by cross-linking experiments (132). It has been shown that in the case of preprolactin, labelled with a photoactivable cross-linking reagent on the amino terminus of the pre-sequence, direct contact existed. This is very important in the biosynthetic

process because once the nascent polypeptide reaches approximately 70 amino acids long (including the pre-sequence) (133), elongation stops and this prevents continued synthesis of the polypeptide chain in the cytoplasm. The arrest may have a regulatory role in the sense that it could provide the cell with an on/off switch or function as a more graded rate-controlling mechanism but this remains to be established (134). Recently, results using an in vivo system (Xenopus oocytes) have demonstrated that elongation arrest may not always take place (135) and thus this may challenge the results obtained with in vitro systems (most of these experiments involved the use of a wheat germ cell-free translation system).

While the interaction between the SRP and the growing chain takes place, the ribosome migrates towards the membrane of the endoplasmic reticulum. Once the ribosome is at the level of the membrane, the elongation arrest is terminated. This follows the binding of SRP to the SRP-receptor, located on the cytosolic surface of the ER membrane, and thus releases the nascent protein since, under these conditions, the SRP does not retain its high affinity for the pre-sequence. The receptor (72kDa) is composed of 2 components, one being in the cytoplasm (60kDa) which binds to the SRP located on the ribosome, and the other of approximately 12kDa which is implanted in the membrane of the ER (136). Since both the SRP and its receptor seem to be present in sub-stoichiometric amounts with respect to the membrane-bound ribosome, their interaction may be a transient one and once the targeting of the ribosome has taken place, it would appear that the SRP and the SRP receptor are both free to be recycled (137).

At this point, the ribosome involved in the translocation process seems to interact with a 180-kDa ER membrane protein and a 34-kDa trypsin-sensitive protein (136,138). However, since protein translocation can occur post-translationally, the purpose of these receptors has been

questioned (139). The process culminates in the translocation of the peptide across the ER membrane as it is released from the arrest brought about by SRP. The translocation process would seem to involve the hydrophobic pre-sequence of the nascent protein. This allows the entry of the translated protein chain into the lumen of the ER either directly through the lipid membrane of the ER or through the translocon, a hydrophobic proteinaceous pore (139). Evidence now supports the presence of the translocon (139). This pore is composed of the signal peptidase complex which has 6 polypeptides present in stoichiometric amounts and found in almost equivalent quantities to the amount of bound ribosomes (140). One component of this complex is the signal peptidase. This enzyme is responsible for the cleavage of the pre-sequence of the nascent prohormone or peptide precursor. Since no strict linear sequence homology can be found between the primary structure of the various pre-sequences, it appears that both the secondary and tertiary structures of the peptide would come into play to direct the cleavage (141). In fact, it has been shown that the pre-sequences are composed of hydrophobic α-helices (or ß-pleated sheets) followed by a ß-turn and this possibly functions to direct cleavage of the pre-sequence by the signal peptidase. However, evidence from the bovine opsin system (142) and from molecular modeling studies (143) of the conformation of certain integral membrane proteins has shown that not all the pre-sequences are cleaved during translocation.

After the cleavage of the pre-sequence by the signal peptidase, the first post-translational event (although recent evidence has pointed to a co-translation process in most cases) that takes place in peptide biosynthesis is N-linked glycosylation on the lumenal side of the RER membrane. This mechanism seems to be shared by all eukaryotic organisms and the fact that oligosaccharide chains are covalently bound to the peptide backbone adds to the uniqueness of

glycoproteins and also explains some of the chemical and physical properties that they possess.

The N-linked oligosaccharides of glycoproteins are very diverse in structure but they nonetheless can be divided into 2 broad categories: the complex oligosaccharides and the simple or high mannose oligosaccharides. Both of these classes are composed of an identical inner core of mannose $_3$ N-acetylglucosamine $_2$ at the reducing terminal which is linked to the asparagine residue. Their differences are at the level of the sugars that they contain outside the inner core (144). Complex oligosaccharides can have as external sugars acetyl-glucosamine (GlcNac), galactose (Gal), sialic acid (SA) and fucose (Fuc). High mannose oligosaccharides, as their name implies, contain additional mannose (Man) residues outside the core structure.

This process occurs co-translationally or shortly after translocation and is mediated by the enzyme oligosaccharide transferase. Recent studies, using canine oligosaccharide transferase, have indicated that two of its subunits are ribophorin I and ribophorin II (145). These glycoproteins can be cross-linked to ribosomes and antibodies raised against ribophorin I inhibit translocation into the ER (146). Thus, this enzyme may play a dual role both in protein translocation and in protein glycosylation.

Research involving a variety of glycoproteins of viral, plasma, membrane, and secretory origin have shown that both complex and simple oligosaccharides have a common biosynthetic pathway that starts with a high-mannose, dolichol-linked precursor oligosaccharide which has a composition of Glc_3 Man_9 $GlcNAc_2$. The synthesis of the carbohydrate moities requires linkage to the lipid carrier (namely dolichol phosphate) via N-acetylglucosamine. It is only when the synthesis is complete that the small sugar polymers are then transferred "en bloc" to the protein by the enzyme oligosaccharide transferase. Also, it should be pointed out that evidence

from studies with inhibitors (144) and mutant cell lines (147) has demonstrated that N-linked glycosylation absolutely requires the presence of lipid-linked oligosaccharides.

As mentioned earlier, the transfer of the oligosaccharide to the protein involved a membrane-bound oligosaccharide enzyme called oligosaccharide transferase which has a possible function in pore formation for the translocation process. For this transfer to take place, two factors must be considered: first, the nascent protein must get extruded through the ER membrane, and then it must have the proper stucture in order to become glycosylated. This requires the presence of a specific three amino acid sequence --asn-xaa-ser/thr-- where xaa can be any amino acid except possibly proline. Moreover, evidence from topological studies (148) supports the idea that the domain of the oligosaccharide transferase that recognizes the asn-xaa-ser/thr acceptor sequence is lumenally orientated. There are however other determinants involved since not all the asn-xaa-ser/thr sequences get glycosylated. One of these seems to be the presence of beta-turns in the vicinity of the site of glycosylation, emphasizing the importance of there being accessible sites for this process to take place (149). Another determinant is the role that serine and threonine residues in the tri-peptide sequence play in the initiation of glycosylation. Bause and Legler (150) proposed a mechanism to explain how the ser/thr participates in the transfer of the oligosaccharide from the dolichol to the asn in the protein or peptide. The hydroxy amino acid side-chain would act as a hydrogen bond acceptor, while the amide group of the asn would be a hydrogen bond donor. This would increase the nucleophilicity of the amide electron pair, giving rise to a higher reactivity towards the glycosyl donor, and lead to the attachment of the inner GlcNAc to the amide nitrogen of the asn, thus resulting in the core glycosylation of the protein.

Once the transfer of the oligosaccharide has taken place, the removal of the glycosyl residues that are located on the carbohydrate moities occur through the action of a series of three glycosidase which are integral membrane proteins and appear to be located on the lumenal surface of the RER (151). The localization supports the assumption that they participate in the processing of glycoproteins, present in the lumen of the RER. This removal of these glucose residues seems to be a necessary event in the biosynthesis of glycoproteins because very few molecules enter the Golgi apparatus possessing glucose residues and it also allows the Golgi α-1,2 mannosidase to remove the Man residues for further oligosaccharide modifications. Examples of glycoprotein hormones include thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH) and the adrenocorticotropin precursor, POMC. In looking at the rat ACTH precursor, two possible sites for N-linked glycosylation are apparent. While the site within the γ_3-MSH, located at asn_{65}, gets fully glycosylated, the other one present on asn_{29} of ACTH only gets partially glycosylated (152).

The formation of disulfide bridges is also an event that takes place either co-translationally or immediately post-translationally in the lumen of the ER. From the findings of Peters and his collaborators (153) on the biosynthesis of rat serum albumin, disulfide bridge formation begins while the nascent polypeptide chain is still attached to the ribosome and as it enters the RER. The final disulfide bridging would apparently be completed shortly after the protein is released into the lumen of the ER. This would most likely be the route taken by POMC which is known to have disulfide bridging between cys_2-cys_{24} and cys_8-cys_{20} (154). The enzyme involved in such a process is called protein disulfide isomerase (PDI). It is considered as the in vivo catalyst of the reaction and participates in co-translational pre-protein processing

(155).

Evidence from the study of glycosylation (156) and disulfide bridging (157) show that there is no protein folding on the cytoplasmic side prior to translocation (this is because of the arrest of translation by the binding of SRP to the pre-sequence) and that the nascent polypeptide chain must enter the ER membrane in an extended fashion. Then PDI, which is located in the vicinity of the translocation apparatus, maintains the growing polypeptide chain in a reduced state, as suggested from a study on prolactin biosynthesis (158). This ensures that the nascent chain would attain its native conformation in stages or in domains following a defined biosynthetic pathway, without any limitations imposed by incorrect disulfide pairing. The recent determination of the PDI sequence, using the cDNA for the rat liver enzyme, has shown that it contains two active domains. This would permit corroboration of the results obtained with prolactin in the following way: since both domains can react with sulfhydryl groups, correct disulfide bond formation might be due to the intrinsic folding energy of the nascent polypeptide, bringing the two active sites opposite to each other, thus facilitating disulfide bond exchange and the formation of the most stable tertiary structures for the protein. Thus, because of conformational changes and steric hindrance, disulfide bridging could in part explain why not all the asn-xaa-ser/thr sites are available for glycosylation in the RER.

1.3.3. Events in the Golgi Apparatus

The final processing of N-linked glycosylation takes place in the Golgi apparatus after the transport of the pro-peptide from the RER. The removal of the glucose residues in the RER is most likely necessary for efficient movement through the secretory pathway. The reason for this could be that either the deglucosylated oligosaccharides form part of a recognition site for a receptor necessary for the transport of certain secretory proteins or that the removal of the glucose allows for the maturation of the glycoprotein to a correct functional conformation.

The capping of the glycoproteins with terminal sugars occurs in the last two compartments of the Golgi (the medial and the trans), while in the cis Golgi, the preparation for this capping takes place. In the cis Golgi, the trimming process of the high mannose oligosaccharides of secretory glycoproteins by α-1,2 mannosidase yields a $man_5GlcNAc_2$ structure that allows for the addition, in the other compartments, of the sugars that will form complex oligosaccharides. The production of complex carbohydrate moities starts in the medial cisterna of the Golgi. This involves the addition of Fuc and GlcNAc by specific enzymes to the $Man_5GlcNAc_2$ core. Once this is done, the final step in N-linked glycosylation occurs in the trans Golgi and may lead to the addition of Gal followed by SA in the cases where these sugars are needed to form particular complex oligosaccharaides. In terms of the role of glycosylated products, it seems that they would play a major part in the recognition of cellular elements at the level of the cell surface (159).

O-linked glycosylation is another type of post-translational processing. This occurs entirely within the Golgi compartment as shown by Roth (160) by studies on the biosynthesis of glycoproteins within intestinal goblet cells. O-linked glycosylation involves the attachment of sugar moities onto the hydroxyl groups of serine or threonine residues. It has been shown that these serine and threonine residues must be accessible to the biosynthetic machinery and are usually in thc vicinity of multiple proline residues (161). Another distinctive feature of O-linked glycosylation is that generally no mannose residues are present and linkage to ser or thr is via N-acetyl-galactosamine NH_2. There is no requirement for a lipid intermediate or oligosaccharide pre-assembly for O-linked glycosylation.

One of the best examples of O-linked glycosylated hormones is human chorionic gonadotropin (hCG) (159). It is glycosylated at four serine residues located towards the carboxyl-terminal region of the ß- subunit. By removing the appropriate sugars (SA,Gal,and GlcNAc) from the O-linked serine of the hCG, its binding to receptors was not greatly influenced but the amount of cAMP accumulation was diminished. Also, as suggested by Bennett, O-linked glycosylation found at thr_{45} of POMC may have a role to play in regulating the extent of processing of the 16kDa amino-terminal fragment (162,163) of this multihormone precursor.

It has been shown that the Golgi membranes are enriched for sulfotransferase activity and that sulfation can occur both on sugar moieties and tyrosine residues of secretory proteins. Sulfation of tyrosine is mediated by the enzyme tyrosyl-protein sulfotransferase (also called tyrosine sulfotransferase) (164). It is a tissue specific enzyme since the sulfotransferases of the pituitary, placenta, and liver differ in their range of acceptable substrates. Recent work on the

biosynthesis of immunoglobin M by mouse hybridoma cells has shown that the modification of tyrosine takes place in the same compartment as terminal glycosylation events, such as sialylation and galactosylation (165). Thus, the next step in the biosynthesis of peptides and proteins is the covalent attachment of a negatively charged sulfate onto tyrosine residues which takes place in the trans-Golgi compartment (Figure 1.02).

Sulfation of tyrosine residues appears to be a very specific and highly selective process. However, recent studies by Huttner (164) indicate that this post-translational modification is relatively common for secretory proteins. The preference of the sulfation process for specific tyrosine residues relies on the distribution of several amino acids aroung the sulfation site and possible candidates can be identified using the following criteria (166, 167):

1) presence of an acidic amino acid at position -1 or -2
2) presence of at least 3 acidic residues within positions -5 to +5 of the tyrosine
3) no more than 1 basic residue within 5 residues of the tyrosine
4) no more than 3 hydrophobic amino acids within 5 residues of the tyrosine
5) absence of cysteine or glycosylation sites within 15 residues of the tyrosine

Also the secondary structure of the peptide aroung the sulfation site is important. The general conclusions are that the sulfation sites are bordered by α-helices and that they occur within short segments which can be identified as ß-turns or random coils. Examples of secreted proteins that contain sulfated tyrosine residues include gastrin II, CCK, caerulein, and [LEU]

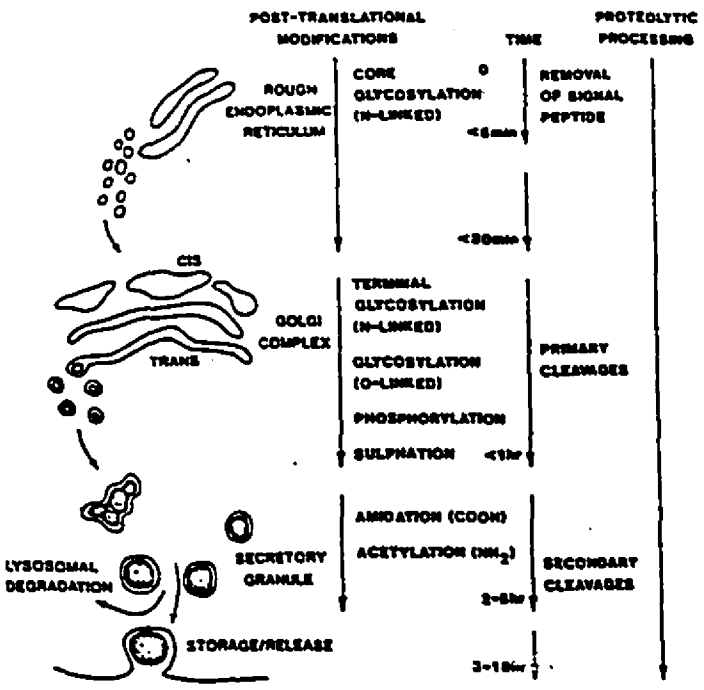
enkephalin.

Recently, evidence from studies using fast atom bombardment-mass spectrometry revealed that the tyr_{28} residue of the beta-lipotropin portion of bovine POMC is partially sulfated (168).

Although the physiological role of tyrosine sulfation has not yet been established, it nonetheless must be concluded that it has some significance in peptide biosynthesis since it is such a common modification. Some of the roles that have been postulated for its action involve the sorting, processing, and function of secretory proteins (169).

Phosphorylation of peptides and proteins is a protein kinase-mediated process that occurs in the Golgi and seems to be species specific since in the case of rat, mouse and human ACTH and CLIP, phosphorylation takes place to different degrees while in bovine ACTH, phosphorylation does not occur (170). In human and rat ACTH, phosphorylation occurs at the serine residue within the sequence ser_{31}-ala_{32}-glu_{33} . The sequence ser-X-acidic AA conforms to the sequence requirements of physiological casein kinase. It has been suggested that phosphorylation should follow glycosylation in POMC since a free serine hydroxyl is necessary for efficient glycosylation of $asparagine_{29}$ of ACTH (170). Phosphorylation does not seem to have any effect on the activity of ACTH (170).

ACTH is not the only peptide that can undergo this modification. It has been shown that ovine growth hormone is phosphorylated and that sites on pig pro-gastrin, rat pro-calcitonin, bovine pro-enkephalin, and also pro-parathyroid hormone have the potential to be modified in this way (171).



(From Bennett, H.P.J. In Recent Progress in Cancer Research (1985) 99, 34-45)

Figure 1.02

1.3.4. Events in the Secretory Granule

While some early cleavage events occur in the Golgi system, the secretory granule fraction is thought to be a major site of prohormone processing. The generation of the mature, secreted hormone occurs as a result of specific cleavages and modifications. The first events that take place are the proteolytic cleavages of the precursor peptide. The key enzymes involved in such processing include prohormone converting enzymes (PCs), a carboxypeptidase B-like enzyme (CPB), and possibly an aminopeptidase B-like enzyme (AP).

A purified protease which has an in vitro pH optimum of 4.0-5.0 and considered to be an aspartyl protease was proposed as a pro-hormone converting enzyme (PCE) cleaving pro-hormones at pairs of dibasic residues especially at lys-arg pairs (172). Recently, Estivariz et al (173) demonstrated that a specific aspartyl protease, associated with the membrane of secretory vesicles of bovine intermediate lobe cells, could cleave the 16K fragment of POMC at the arg_{49}-lys_{50} site to produce $16K_{1-49}$ and lys-γ_3-MSH, implying tissue specificity. Other studies also point to differences in processing in both lobes. It has been shown in AtT-20 cells, a mouse anterior pituitary cell line, transfected pro-renins are cleaved at lys-arg and arg-arg sites while mutants, containing the lys-lys and arg-lys dibasic pair were not processed (174). This confirms the cleavage specificity of endogenously expressed convertases by this cell line with respect to POMC processing (175). However, two new convertases, called PC-1 (also known as PC-3) and PC-2, have been cloned and sequenced using polymerase chain reaction strategies (176-179). Extensive homology was noted between these enzymes and the other members of the subtilisin family of serine proteases. They are proteases with neutral pH optima and are dependent on calcium for full activity. The convertase family includes the KEX-2 gene product (kexin) proven to be involved in α-factor biosynthesis in yeast (180) and furin, a Golgi-localized endoprotease expressed in many mammalian cells, which has been implicated in the processing of several constitutively secreted polypeptides and proteins (181). Kexin and furin are membrane-bound proteases bearing membrane-spanning sequences. These convertase enzymes act mainly within the constitutive pathway of secretion. Localization of PC-1/3 and PC-2 by in situ hybridization to distinct regions of the brain and pituitary has strongly implicated these proteases as important participants in prohormone processing (182,183). The distribution of the two convertases within

the mouse pituitary may provide the biochemical basis for tissue-specific processing of POMC. Using Northern analysis techniques, mRNA for PC-1/3 but not PC-2 has been localized to the anterior pituitary while mRNA for both PC-1/3 and PC-2 has been localized to the intermediate lobe (179). Expression of the two convertases in a variety of cell lines together with POMC has revealed much about their substrate specificity (184,185). Proinsulin processing can also be accounted for in terms of the actions of PC-1/3 and PC-2 (186-188).

In many of these studies, convertase and substrate have been coexpressed within model cell lines using the vaccinia virus system. PC-1/3 has been shown to be active with respect to POMC processing when coexpressed in BSC-40 cells, a constitutively secreting cell line lacking secretory granules (184,185). Cleavage of the precursor occurred at -lys-arg- and -arg-arg- sequences to yield products typical of anterior lobe processing (i.e. ACTH, ß-LPH, and ß-endorphin). When PC-2 was expressed with POMC in BSC-40, further processing of the ACTH domain to give $ACTH_{1-13}$ amide was evident. This particular cleavage event can be considered diagnostic for pituitary intermediate lobe processing. The cumulative evidence indicates that both PCs cleave predominantly to the carboxyl-terminal side of the pairs of basic residues (189). These amino acids are subsequently removed by the action of a carboxypeptidase. A critical role for PC-1 in POMC processing was evident when the expression of this convertase enzyme was suppressed in the mouse anterior pituitary AtT-20 cell line using antisense RNA technology (190). Suppression of PC-1 activity in this corticotroph cell line significantly inhibited POMC processing, resulting in the accumulation of uncleaved precursor. A critical series of experiments has recently been undertaken to determine whether PC-1/3 and PC-2 can account for all the cleavage events typical of intermediate lobe processing (191). Mutant strains of AtT-20 cells

were developed that stably expressed the PC-2 convertase. The mutant cells acquired the ability to undertake all the additional cleavages typical of the intermediate pituitary. This included the synthesis of lys$^{1}\tau_{3}$MSH, a product generated through cleavage of the 16K or amino-terminal frament of POMC as well as carboxyl-terminally truncated forms of ß-endorphin. These latter cleavage events are known to occur late in the biosynthetic processing of POMC. Thus the products of POMC processing and the kinetics of their generation that are typical of the intermediate lobe are duplicated in AtT-20 cells expressing PC-2 as well as the endogenous PC-1/3 convertase. These experiments and those from other laboratories suggest that the combined action of PC-1/3 and PC-2 will acccount for most of the cleavage events that gives rise to biologically active peptide hormones and neuropeptides. An endopeptidase activity, bringing about the cleavage of leu_{17}-phe_{18} of ß-endorphin to produce τ-endorphin, has also been described for POMC (192). Interestingly, a peptide corresponding to the 18-31 sequence of human ß-endorphin was purified from an extract of a pituitary adenoma assocaited with Cushing's disease (see section 3.1.1). The enzyme responsible for this would be a basic thiol endopeptidase, present in the pituitary.

Because of the nature of the cleavages due to PCE and the PCs, two exopeptidases are needed for the removal of the N and C terminal basic residues that are located on the cleaved peptides originating from the precursor. The first of these two enzymes is a carboxypeptidase B-like enzyme (CPB) and some of its characteristics include: stimulation by Ca^{2+}, optimal pH of 6.0, and being a thiol, metallopeptidase (193). This enzyme cleaves in a sequential manner any basic residues which are present at the C-terminal of the peptides formed from the action of PCE. An example of such processing may be $ACTH_{1-17}$ which, after the trimming of the

basic residues through the action of the carboxypeptidase B-like enzyme, will become $ACTH_{1-14}$. The CPB is most likely located in the secretory granules because evidence for its activity has been found in the secretory granule fraction of the intermediate and anterior lobe of the rat pituitary (194).

The second exopeptidase is an aminopeptidase B-like enzyme (AP) which has a pH optimum of 6.0, is a metallopeptidase that is enhanced in its activity by the presence of Ca^{2+} and Zn^{2+} and is membrane bound. Recent studies have shed some light on the specificity of this enzyme (195). First, the enzyme seems to have greater affinity for the cleavage of N-terminal arg than lys. Evidence for this comes from experiments looking at the generation of Met-enkephalin from either arg-Met-enkephalin or lys-arg-met-enkephalin. This demonstrated that the rate of cleavage by AP of arg-met-enkephalin was six times faster that leu-arg-met-enkephalin for the generation of Met-enkephalin. Also, incubation of Lys-γ_1-MSH in the presence of membranes from secretory granules which would contain AP, did not yield any detectable free lys. Another property of AP is that it will not cleave N-terminal arg residues if they are followed by proline (196). Thus the specificity of this enzyme probably explains why the major form of bovine and rat intermediate pituitary γ-MSH is in the form of lys-γ-MSH (the cleavage of the lys would not take place) (197), and it also would explain why CLIP retains its N-terminal arg (a proline residue follows the arg in the primary sequence). A carboxypeptidase activity, possibly distinct from the B-like enzyme discussed above, has also been implicated in the production of ß-$endorphin_{1-26}$ from ß-$endorphin_{1-27}$ (198); however, little is known about its enzymatic specificities.

Once these primary and secondary cleavages have occurred, certain non-proteolytic

enzymes modify the products arising from the cleavage of the precursor. The consequence of these modifications are often important for the function of the peptide. First, they may reduce the susceptibility of the peptide to degradation by extracellular aminopeptidases and carboxypeptidases and secondly, they may affect the biological activity of the secreted peptide.

An important enzyme with respect to α-MSH and ß-endorphin biosynthesis is N-acetyltransferase (NAT) found in the secretory granules of the intermediate pituitary. The rat and bovine enzyme has been shown to be soluble and to have an optimal pH of 7.0 although its range of action is from pH 5.7-8.0. As the name implies, it can acetylate the N-terminal of peptides and seems to have a preference for serine (α-MSH) and tyrosine (ß-endorphin) N-terminal amino acids (184). This NAT enzyme has been shown to modify both desacetyl α-MSH ($ACTH_{1-13}NH_2$) and ß-endorphin and produce α-MSH and acetylated ß-endorphin while using acetyl-CoA as co-substrate. Kinetic studies performed on this enzyme have demonstrated that in the case of $ACTH_{1-13}NH_2$ and ß-endorphin the K_m was similar while in the case of the α-MSH to α-N-O-diacetyl α-MSH reaction, the K_m was much higher (199). Moreover, competition studies support the conclusion that NAT is the same enzyme involved in the acetylation of the two peptides.

Some results indicate that a very small proportion of the ß-endorphin, found in the anterior pituitary (200-202) is N-acetylated, and may represent a different subset of anterior pituitary corticotrophs.

In considering the biological properties of the peptides due to this modification, evidence shows that the NAT enzyme inactivates ß-endorphin while conferring biological activity upon α-MSH. Therefore, the modifications due to the enzyme will either inhibit or stimulate the

biological activity of the peptides in question.

The other major processing enzyme located in the secretory granules is a peptidyl-glycine α-amidating monooxygenase (PAM). This enzyme has the ability to α-amidate neuroendocrine peptides (203). It has been found that for α-amidation to occur, a C-terminal glycine must be exposed following the action of the convertase and carboxypeptidase-B-like activities. Within the precursors for amidated neuropeptides, the motif X-gly-basic-basic is invariably encountered, where X is the residue that becomes amidated in the mature peptide and where the basic residues can either be lys or arg. Moreover, X is usually a neutral amino acid (for instance val, phe, gly), but charged amino acid like asp and lys can also get amidated. For instance, guinea pig ß-MSH has a C-terminal lys amide and, in the case of the acidic joining peptide from human, rat and mouse POMC, the amidated residue is glu. Glycine-extended structures are known to give rise to α-amidated carboxyl-termini in a two step process involving sequential actions of a peptidyl-glycine α-hydroxylating mono-oxygenase followed by a peptidyl-α-hydroxyglycine α-amidating lyase (204,205). Both enzymatic activities are encoded within a common precursor bearing a transmembrane domain at its carboxyl-terminus. Processing within the secretory pathway yields a soluble form of lyase enzyme (206). Studies on rat and bovine PAM revealed that the enzyme requires Cu^{2+}, ascorbic acid, and molecular oxygen in order to function and that its optimal pH is about 6.5 (206). Also, PAM is presumed to function is a similar manner to DBH (dopamine ß-hydroxylase) in the sense that the enzyme-bound Cu^{2+} undergoes repeated reduction by ascorbate and oxidation during the activation of molecular oxygen. This will lead to the oxidation of the substrate to produce the α-amide group and glycoxylate (207).

Amidation of neuropeptides is frequently an important modification for their biological

activity and it seems to confer added potency. An example of this is provided by corticotropin-releasing-factor(CRF) which has a diminished potency of at least 1000 fold when ala-NH_2 is replaced by ala. This therefore shows that α-amidation is an important process in the biosynthesis of peptides (171). Certain POMC peptides also get amidated. In the intermediate lobe, α-MSH is amidated while the acidic joining peptide(AJP) has been found to be amidated in both the anterior and intermediate lobes of the pituitary (208,209).

It should be noted that recently two forms of PAM have been isolated from bovine neurointermediate lobe. They are very similar to the original PAM and vary in MW from 37 kDa to 50 kDa. They are products of alternative mRNA splicing that yield forms of PAM with and without their C-terminal transmembrane domain. However, their physiological significance remains to be established (210).

Finally, another enzyme has been shown to be involved in the maturation process of proteins and peptides. Glutamine cyclotransferase has the ability to catalyse the formation of an N-terminal blocking group. In all cases, it brings about the cyclization of a glutamine residue at the N-terminal to form a pyrolidone carboxylic acid (ie. pyroglu) (211), and an example of this is provided by LH-RH, a hypothalamic hypophysiotropic hormone that regulates secretion of LH from the anterior pituitary. This modification seems to protect the peptide once it is released from the cell from degradation due to aminopeptidases present in the extracellular milieu.

CHAPTER 2: MATERIALS AND METHODS

2.1. Source of Tissues

Male Sprague-Dawley rats, 150-175g, and male Hartley guinea-pigs, 200-250g, were purchased from Charles Rivers Breeding Farm, St.Constant, Quebec, Canada.

An anterior pituitary adenoma, consistent with Cushing's Disease, was obtained after being surgically removed using the trans-sphenoidal approach. This procedure was performed by Dr. Gilles Bertrand at the Montreal Neurological Hospital on a 39-year-old Black female patient on 21/2/89.

2.2. Isolation and Purification of Peptides by High Performance Liquid Chromatography

2.2.1. Materials

Trifluoroacetic acid(TFA) was purchased from Baker Chemical Co., Philipsburg, NJ, USA. HPLC grade acetonitrile(MeCN) was obtained from Fisher Scientific Co., Montreal, Quebec, Canada. Heptafluorobutyric acid(HFBA) was bought from Pierce Chemical Co., Rockford, IL, USA. Octadecylsilyl-silica(ODS-silica) cartriges(C_{18} Sep-Paks), C_{18}μBondapak HPLC colums, and Gel Permeation columns were purchased from Waters Associates, Mississauga, Ontario, Canada. Vydac C_{18} HPLC columns were obtained from Chromatographic Specialties Scientific Co., Brockville, Ontario, Canada.

2.2.2. Extraction of Peptides

Anterior lobes or neurointermediate lobes of guinea-pigs were dissected in situ following decapitation. These tissues(15) or the anterior pituitary adenoma obtained from surgery were homogenized, using a glass homogenizer(Kontes, Toronto, Ontario, Canada) in an acidic extraction medium which consisted of 1N hydrochloric acid containing 5%(v/v) formic acid, 1%(v/v)TFA, and 1%(w/v) sodium chloride. The homogenates were centrifuged at 3000xg for 10 minutes. The supernatant was then collected and the pellet re-extrected. The supernatants from the original extraction and the re-extracted pellet were pooled together.

Before using the ODS-silica cartriges (C_{18}Sep-Pak)(5) which were connected in series.

by trimmed Eppendorf tips, they had to be pre-treated by "wetting" them first with 10 ml of 80%(v/v) acetonitrile containing 0.1%(v/v) TFA, followed by 20 ml of 0.1%(v/v) aqueous TFA. The supernatants were then passed over the C_{18} Sep-Paks using a polypropylene luer syringe. This was followed by a wash with 10 ml of 0.1%(v/v) TFA to remove the excess acidic extraction medium. Then, in order to elute the material retained on the cartridges, 6 ml of 80%(v/v) MeCN containing 0.1%(v/v) TFA was passed through and collected.

The homogenization of tissues using this acidic medium for the extraction of peptides has proven to be very efficient (212,213). The advantages of this extraction medium are that the low pH(< 1) prevents the action of peptidases, which would degrade the peptides to be studied, and the improvement of the adsorption on the ODS-silica cartridges. Also, the presence of the NaCl in the extraction medium prevents the peptides from adsorbing to large proteins. This procedure has been shown to give good yields, to produce eluates which are compatible with reversed-phase HPLC, and not to cause any damage to the peptides (212).

2.2.3. Preparation of Solvents for Chromatography

To prepare HPLC-grade water, deionized glass-distilled water was filtered through a Waters Associates filtering system with a (HFPA) filter of 20 μm. The purified water and the acetonitrile were then degassed for 20 minutes immediately before use.

Stock solutions of 1%(v/v, 0.08M) TFA, and 1.3%(v/v, 0.1M) HFBA were prepared using HPLC grade water. These stock solutions were then purified using ODS-silica cartridges. All high performance liquid chromatography was done using an aqueous solution(sol.A) of the counter-ion pairing reagent at concentrations of 0.1% TFA or 0.13% HFBA, and an organic solution(sol.B) of 80% acetonitrile containing the corresponding ion pairing reagent at the same concentration as the aqueous solution.

Since the TFA and HFBA solvent systems have a low pH, the ionization of the carboxyl groups on the peptides is suppressed. Chromatographic separation depends on the hydrophobic interactions of the peptides with the column. The use of hydrophobic ion pairing reagents, such as TFA and HFBA, brings about an increase in the retention times of positively charged peptides during RP-HPLC, thus facilitating the process of purification.

2.2.4. High Performance Liquid Chromatography

The high perfomance liquid chromatography system used consisted of two 6000A pumps(Waters Associates, Milford, MASS, USA) and a 660 solvent programmer. Column eluates were monitored for u.v. absorbance using a Waters Model 450 variable wavelength detector and a Beckman Model 330 (Beckman, Palo Alto, CA, USA) single wavelength detector.

They were connected in series for dual wavelength monitoring at 210 and 280 nm.

Samples were loaded onto the HPLC column after reducing the acetonitrile concentration to less than 5% of their original value. This was achieved either by drying the samples under vacuum in a Speed-Vac concentrator (Savant, Hicksville, NY, USA) or by diluting them with aqueous 0.1% TFA or 0.13% HFBA, depending on the acid used for chromatography. The samples were injected through a Valco injector (Chromatographic Specialties). Samples were loaded onto the HPLC column using the solvent A pump, followed by isocratic elution with solvent A until stable u.v. baseline was achieved.

Elution of the sample from the column was achieved by using a linear gradient of increasing acetonitrile concentration. The initial chromatography of all the tissue extracts was carried out using TFA as counter-ion and a gradient of either 0.33% or 0.66% acetonitrile per minute. Further purification steps employed TFA and HFBA in order to exploit the basic character of the peptides. Gel permeation HPLC was undertaken using two I-125 colums connected in series and the peptides were eluted isocratically with 40% acetonitrile containing 0.1% TFA at a flow rate of 1ml per minute. TFA was generally used for the final purification step since it is the cleanest solvent.

Fractions eluted from the column were collected in 12X75mm polypropylene conical tubes (Sarstedt). This was done either by hand to minimize the volume and to ensure minimal contamination from neighbouring peaks or by using an LKB Ultrorac Type 7000 (Fisher Scientific Co.) fraction collector.

2.3. Structural Characterization of Purified Peptides

2.3.1. Acid Hydrolysis and Amino Acid Analysis

Samples for amino acid analysis were dried in vacuo in 5X50mm borosilicate glass tubes using the Speed-Vac concentrator. 150μl of 6N constant boiling hydrochloric acid(Pierce Chemical Co., Rockford, IL, USA) containing a small crystal of phenol (to reduce the destruction of tyrosine) was added to the glass hydrolysis flask holding the sample tubes. Samples were hydrolysed in vacuo using a Waters Picotag work station. The hydrolysis was carried out for 2 to 18 hours at 110^0C. Shorter hydrolysis times preserved phosphoserine residues while 18 hours was the standard time for complete hydrolysis of all peptide bonds and to determine amino acid compositions.

The hydrolysates were dried and then taken up in 150μl of an amino acid analysis starting buffer. The sample tube were vortexed and each sample loaded individually onto the Beckman Model 6300(Beckman Instruments Inc., Fullerton, CA, USA) amino acid analyser. The cation exchange column was eluted in a stepwise manner with citrate buffers, whose exact compositon is not revealed by the manufacturer, using a combination of increasing gradients of molarity, pH, and temperature. At the beginning of each day, the analyzer was calibrated with a standard 1 nanomole amino acid mixture.

2.3.2. Enzymatic Digestion

Enzymatic digestions using diphenylcarbamyl chloride-treated trypsin(type IX) (Sigma Chemical Co., St. Louis, MO, USA) and S.aureus Vg protease (Miles Labs, Rexdale, Ontario, Canada) were performed on some of the isolated peptides in 100μl of 50mM TRIS buffer, pH 7.8, at 37^{0}C for 18 hours. The ratio of enzyme to peptide in these digestion was 1:50(w/w).

2.3.3. Mass Spectrometry

Ion spray mass spectra of purified guinea-pig peptides were obtained in the positive mode on a triple stage mass spectrometer Model API-III (Sciex, Toronto, Canada) located at the Montreal Biotechnology Research Institute of the National Research Council of Canada. Briefly, the lyophilized samples were redissolved in 10% acetic acid (v/v) and infused through a stainless steel capillary (100uM ID) at a flow rate of 1μl/min. A stream of air (pneumatic nebulization) was introduced to assist in the formation of submicron droplets (214). For a pure protein, each peak represents a differently charged species with a specific mass to charge ratio. The charge difference between contiguous peaks is one unit so the molecular weight of the protein can be calculated from a series of m/v values. The charge on each species can then be determined and used to calculate a mass estimate. A series of these mass estimates are then averaged to give an accurate molecular weight value. Each sample was scanned approximately 25 times, each scan being added to the next to give a cumulative signal.

2.4. Adrenal Cell Bioassay

2.4.1. Materials

The enzymes used for the cell dispersion buffer were collagenase (Boerhinger Mannheim, Montreal, Quebec, Canada) and DNAase I (Sigma Chemical Co., St.Louis, MO, USA). The medium employed in the bioassay was Ham's F-12 (Flow Laboratories, Mississauga, Ontario, Canada) which was already buffered and contained 1mM Ca^{2+}.Bovine serum albumin(BSA) from purified fraction V was purchased from Sigma Chemical Co., and used in the formation of the density gradient. It was also present in all the different buffers used in the bioassay. Calcium chloride was obtained from Fisher Scientific and utilized in the incubation buffers.

2.4.2. Cell Dispersion and Preparation of the Bioassay

The dispersed rat adrenal bioassay is a modification of the method described by Sayers et al (215). 10 male Sprague-Dawley rats, 150-175g, were decapitated and their adrenals carefully removed and decapsulated with very sharp tweezers. This allowed the separation of the

mineralocorticoid producing zona glomerulosa, which remained attached to the outer capsule of the adrenal gland from the glucocorticoid producing zona fasciculata and zona reticularis, found in the inner cortex of the adrenal gland. The cells from either the capsule or inner cortex were then dispersed in the dispersion medium, containing 15mg of collagenase, 7mg DNAase I, and 0.5% BSA per 8ml of Ham's F-12 medium, and shaken in the presence of 95% O_2/ 5% CO_2 at 37^0C in a Dubnoff Metabolic Shaking incubator(Precision Scientific Co., Chicago, IL, USA) for 1 hour in a 20ml polypropylene vial. The dispersal of the cells was favored by gently pipetting the medium up and down with a pasteur pipette several times.

The supernatant was transferred to a 15ml polystyrene conical tube and centrifuged at 1000xg for 10 minutes at 4^0C. The supernatant was discarded and the pellet resuspended in 10ml washing buffer (Ham's F-12 with 0.5% BSA), followed by a centrifugation at 1000xg. After a second washing step, the cells were resuspended in 2ml of incubation buffer and filtered either through a "pre-wetted" nylon gauze or 8-ply cheesecloth. The cells were layered on top of an 8ml 2.5%(w/v) BSA density gradient and centrifuged at 1000xg for 10 minutes. Only the bottom 4ml and the cell pellet were kept and resuspended with incubation buffer to a final volume of 10ml. An aliquot of 10μl of the cell suspension was taken and mixed with 10μl trypan blue, a vital cell coloring agent, and then counted on an Neubauer hemocytometer. Only single cells which contained lipid droplets and excluded trypan blue were considered to be viable.

The adrenal cells were suspended in the incubation buffer, composed of 0.5%(w/v) BSA and 7mM Ca^{2+}, in Ham's buffer, to make a final cell concentration of 3-4x10^5 cells/ml and

0.5ml aliquots were pipetted into 12X75mm plastic test tubes for incubation. Cells were pre-incubated in an atmosphere of 95% O_2/ 5% CO_2 for 1 hour at 37^0C in order to allow the cells to recover from the dispersion procedure. Test peptides were prepared in plastic polystyrene tubes in 1.2ml of incubation buffer from which 0.5ml aliquots were pipetted into the tubes containing the cells to obtain accurate duplicates. Cells were incubated for 2 hours at 37^0C with 95% O_2/ 5% CO_2 atmosphere in a metabolic shaker to prevent the cells from clumping together and adhering to the walls of the plastic tubes.

At the end of the incubation period, the cell supension was centrifuged at 3000xg at 4^0C for 10 minutes and the supernatant decanted into 2ml of methylene chloride, an organic solvent in which steroids are soluble. The solution was vortexed and aliquots of the methylene chloride extracts were used to determine the amount of steroids released into the medium by the adrenal cells.

The human ACTH standard peptide from Bachem (Torrance, CA, U.S.A.) was prepared from 2μg samples, diluted in 80% MeCN containing 0.1% TFA and divided into 120ng aliquots. These aliquots were dried in the Speed-Vac concentrator in 12X75mm tubes and dissolved in 3ml incubation medium. Decreasing concentrations of standard ACTH were prepared in duplicate or triplicate for each assay. Eight points with 1:3 dilutions from 20ng to 9pg were used for the $ACTH_{1-39}$ curve.

2.4.3. Steroid Radioimmunoassay

The quantitation of steroid formation from the bioassay was achieved using a radioimmunoassay(RIA). In the case of corticosterone, BSA-phosphate buffer was prepared by dissolving 2g of sodium azide, 18.0g of sodium chloride, and 13.8g of mono-basic phosphate in 1.8L of deionized distilled water. After adjusting the pH to 7.4 with 10N sodium hydroxide, the total volume was increased to 2.0L. The equivalent of 1g BSA per L was added to the phosphate buffer 1 hour prior to use.

Dextran-coated charcoal was prepared by mixing 625mg of charcoal (Fisher Norit-A, neutral) and 0.0625g of Dextran T-70 (Pharmacia) with 250ml of BSA-phosphate buffer.

Corticosterone tracer was prepared by drying down an appropriate amount of tritiated corticosterone (approximately 50 Ci/ mmol, from New England Nuclear, Boston, MASS, USA) under nitrogen. Phosphate buffer was then added to give a final concentration of 8,000-12,000 cpm/ 0.1ml.

Corticosterone antibody was purchased from Biomega, Montreal, Quebec, Canada. Each vial was reconstituted with deionized distilled water according to the manufacturer's direction. From this stock solution, aliquots of 100μl which could be used for approximately 100 assay tubes were stored individually at -20^{0}C until required. BSA-phosphate buffer was then added to

allow for 25 to 40% binding in 100μl of the antibody solution.

Corticosterone standards were prepared from a stock solution of 100mg/ 100ml ethanol. Eight standards between 25pg to 5000pg/ 100μl ethanol of corticosterone were made. 100μl duplicates of the standards were dried under vacuum in the Speed-Vac concentrator prior to the assay in 12X75mm borosilicate glass tubes. Aliquots of the methylene chloride extracts of steroids, released from the cells during the bioassay, were also taken to dryness in a vacuum oven attached to a water aspirator.

Assay buffer, tracer, antibody, and charcoal solutions were added to each tube. The "totals" tubes, which assess the amount of radioactivity added to each tube of the RIA, did not receive any charcoal or antibody, and the "NSB" tubes, representing non-specific binding due to the charcoal, did not contain antibody. The "O" tubes representing the corticosterone standards and the sample tubes("S") received a pre-determined quantity of all the various solutions of the RIA. The tubes were vortexed and incubated at room temperature (22^0C) for 2 hours.

250μl of Dextran-coated charcoal was added to all tubes except "totals" and "NSB", vortexed, and incubated for 10 minutes at 4^0C. The tubes were then centrifuged at 3000xg for 10 minutes at 4^0C and the supernatant was decanted into individual counting vials to which 4.5ml of scintillant (Formula-963, New England Nuclear, Boston, MASS, USA) was added. The vials were counted in an LKB 1214 Rack-Beta counter. The counting efficiency for tritium was

40.8%. The results were calculated, using RiaCalc program (LKB, Sweden), and corrected for the appropriate aliquot factor.

The aldosterone RIA follows exactly the same principle and procedure as the one for corticosterone (see beginning of section 2.5.3.). However, there were notable differences. The assay buffer contains 2.0g of gelatin, 2.0g of sodium azide, 18.0g of sodium chloride, 10.8g of mono-basic sodium phosphate, and 17.4g of dibasic sodium phosphate per 2L. Also, the specific amounts of the various solutions needed in the RIA are different from those found for the corticosterone RIA.

CHAPTER 3: RESULTS

3.1. Isolation of Peptides from a Human Anterior Pituitary Tumor Assocociated with Cushing' Disease

The human anterior pituitary adenoma (approximately 50mg wet-weight), obtained by the trans-sphenoidal approach, was extracted to purify and identify all the POMC-derived peptides present in the tissue. The use of an acidic extraction medium facilitated maximal yield of the biosynthetic products of POMC. The peptides were purified using reversed-phase HPLC alone, and were identified by amino acid analysis and mass spectrometry. This also offered us a tool to quantify the various POMC products produced by the tumor and determine the nature and extent of post-translational modification.

3.1.1. Purification of POMC-derived Peptides

As described in section 2.2.2., the pituitary tumor was extracted in an acidic extraction medium shortly after surgery. The supernatant was passed onto three ODS-silica Sep-Pak cartridges which retained the peptides of interest. The 80% MeCN in aqueous 0.1%(v/v) TFA (solvent B) eluates from the Sep-Paks, representing approximately 12 mls, were reduced by about 80% to a volume of 2.7 mls in a Speed-Vac concentrator. 3 mls of aqueous 0.1% TFA (solvent A) was added to the sample to dilute any residual acetonitrile that might be present. This was followed by injection of the sample onto the RP-HPLC column.

The initial HPLC run was performed on a Waters C_{18} μBondapak column that had previously been equilibrated with solvent A. Once the sample was loaded on the HPLC column, approximately 20 mls of solvent A, at a flow rate of 1.5 ml per minute, was passed though until a stable U.V. baseline was obtained. Column elution was achieved with a linear gradient of 1.6% to 61.6% acetonitrile over three hours at a flow rate of 1.5 ml per minute. U.V. absorbance was monitored at 210 nm. 1 minute fractions were collected. The initial chromatogram is shown in Figure 3.01.

The first peak, fraction 38, eluted at 12% acetonitrile in the initial HPLC run (Fig.3.01). Amino acid analysis of this apparently pure peak is of a peptide of 11 amino acids, having

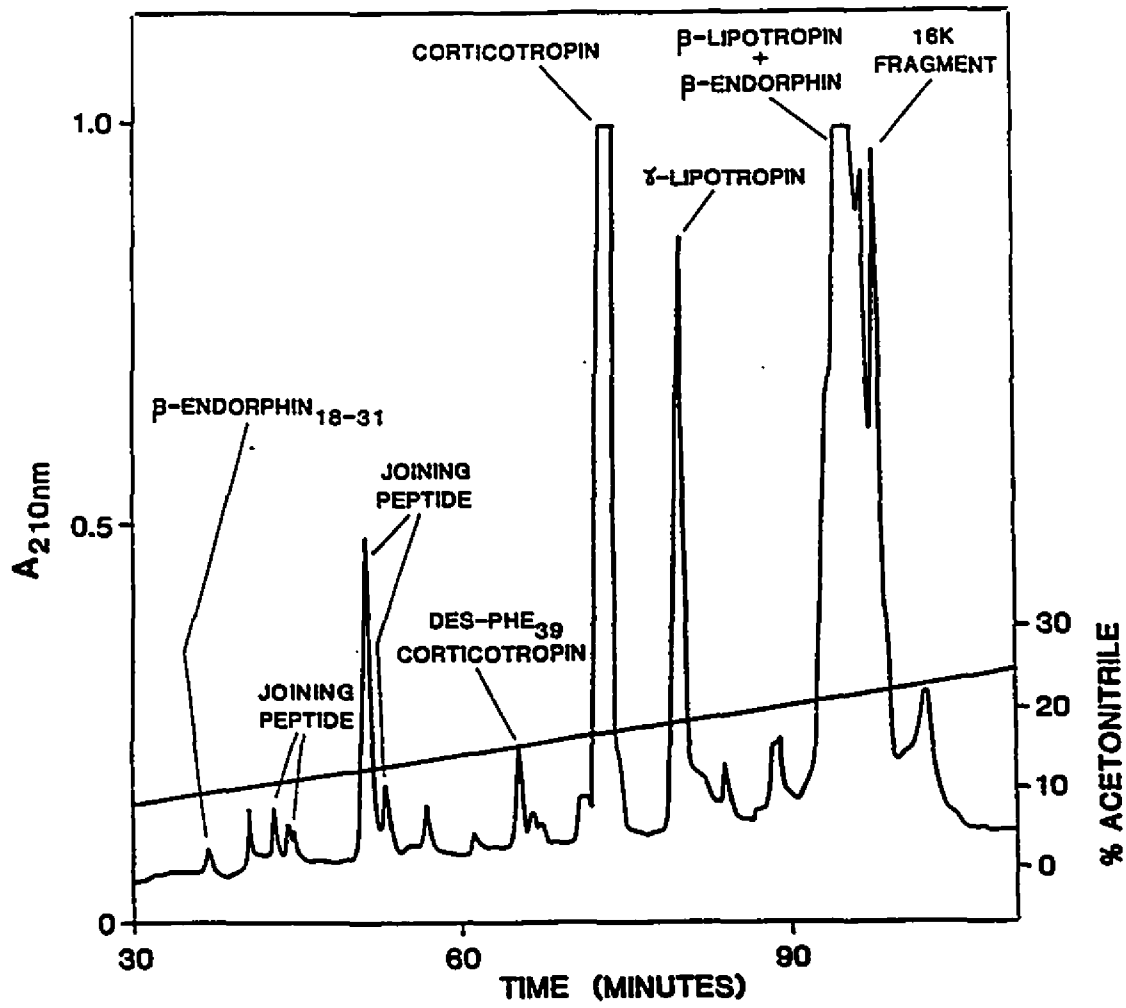


FIGURE 3.01 Reversed-Phase HPLC of an extract of a human anterior lobe pituitary adenoma causing Cushing's Disease. The extraction procedure is described in the Materials and Method(section 2.2.2.). The column was eluted at a flow rate of 1.5ml/min over 3 hours with a linear gradient of 1.6-61.6% acetonitrile containing 0.1% TFA throughout. The identities of the peaks can be found in Table 3.01.

a relatively high polar character. To determine the purity of this peptide, fraction 38 was reduced in volume to 300 μl in a Speed-Vac concentrator, and re-chromatographed on a Vydac C_{18} analytical column, using a linear gradient of 4 to 24% acetonitrile over 1 hour. Amino acid analysis of the resulting peak indicated the presence of 675 pmoles of a peptide, having the same composition as determined initially (see Table 3.01). The same peptide has recently been purified from an extract of normal human pituitaries and identified as ß-endorphin$_{18\text{-}31}$ by sequence analysis (Bennett, personal communication). This fragment is a product of endoproteolytic activity distinct from the pro-hormone convertases.

The following group of peaks contained peptides that had similar amino acid compositions after initial analysis, and were tentatively identified as acidic joining peptide(AJP). AJP eluted in three fractions (44,45,46) respctively at 14.2, 14.5, and 14.7% MeCN. Since the larger of these three AJP peaks had a composition which closely matched the theoretical composition of AJP (Table 3.01) and was apparently homogeneous, it was not re-chromatographed. However, since there was an overlap between the peaks contained in fractions 45 and 46, they were pooled and the volume was reduced to 500 μl. They were then loaded and re-run on a Vydac C_{18} analytical column, using a linear gradient of 15 to 40% B over 1 hour. This permitted the separation of two other forms of AJP from a contaminant and more accurate compositions and quantifications were obtained (Table 3.01).

Amino acid compositions of fractions 53-54 and 55-56 of

TABLE 3.01 Amino acid composition (molar ratios) of biosynthetic fragments of pro-opiomelanocortin from a human anterior lobe pitiutary adenoma.

Peptide[a]	xxx	AJP(M)	AJP(M)	AJP(M)	AJP(D)	AJP(D)	AJP(D)	des-Phe-ACTH	ACTH	γ-LPH	ß-LPH	ß-endo	16K[e]	16K
Asx	1.9(-)[b]	2.9(3)	3.4(3)	3.1(3)	2.7(3)	2.6(3)	2.5(3)	2.3(2)	1.9(2)	5.8(5)	7.4(7)	2.2(2)	7.5(8)	7.8)8)
Thr										0.9(2)	3.2(5)	2.6(3)	3.2(4)	3.5(4)
Ser		1.7(2)	2.5(2)	2.8(2)	2.0(2)	2.7(2)	2.9(2)	2.7(3)	3.1(3)[c]	2.0(2)	3.5(4)	1.8(3)	8.8(10)	7.1(10)
Glx	1.4(-)	4.2(5)	4.1(5)	4.1(5)	5.2(5)	5.2(2)	5.1(5)	5.1(5)	5.3(5)	7.7(8)	10.8(11)	3.6(3)	10.6(10)	11.5(10)
Pro		3.6(6)	2.4(6)	2.3(6)	5.7(6)	5.1(6)	5.2(6)	5.9(4)	3.8(4)	3.8(6)	5.8(7)	1.1(1)	5.5(5)	5.5(5)
Gly	2.0(-)	4.3(7)	4.8(7)	4.8(7)	5.8(7)	6.1(7)	6.1(7)	3.1(3)	3.0(3)	7.7(8)	10.0(11)	3.4(3)	8.5(7)	7.8(7)
Ala	1.1(-)	1.1(2)	1.5(2)	1.5(2)	1.9(2)	2.0(2)	2.1(2)	3.3(3)	2.9(3)	4.7(5)	7.0(7)	2.3(2)	3.3(3)	3.2(3)
Val		0.7(1)	0.7(1)	1.6(1)	1.0(1)	1.1(1)	0.9(1)	2.5(3)	2.6(3)	1.1(1)	2.4(2)	1.4(1)	1.3(1)	1.2(1)
Met								0.4(1)	1.0(1)	0.9(1)	1.5(2)	0.7(1)	1.5(2)	1.2(2)
Ile	0.8(-)										1.4(2)	1.4(2)	0.8(1)	1.0(1)
Leu		1.4(1)	0.8(1)	1.2(1)	1.2(1)	1.2(1)	1.1(1)	1.5(1)	1.2(1)	5.0(5)	7.0(7)	2.2(2)	5.9(6)	7.2(6)
Tyr	0.9(-)							1.1(2)	1.6(2)	0.9(1)	1.8(3)	1.4(2)	0.8(1)	0.9(1)
Phe								2.0(2)	2.7(3)	0.9(1)	2.8(3)	1.9(2)	2.6(3)	3.1(3)
His								0.6(1)	1.0(1)	1.8(2)	1.9(2)		2.2(1)	2.3(1)
Lys	2.6(-)	0.9(1)	0.5(1)	0.5(1)	1.0(1)	0.7(1)	0.9(1)	1.7(4)	3.5(4)	2.7(3)	7.6(9)	4.9(5)	2.0(1)	1.5(1)
Arg		1.7(2)	1.7(2)	1.7(2)	2.2(2)	2.2(2)	2.1	3.0(3)	3.3(3)	4.1(4)	4.8(5)		6.1(6)	6.1(1)
Trp	n.d.(-)[d]	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)	n.d.(-)
MW Obs.					6008.50				4541.52	6074.10	9804.03	3464.90	---[f]	
MW Exp.					6010.38				4542.14	6075.58	9806.97	3466.05	8470.37[g]	
YIELD	1.7	1.4	0.4	0.7	7.9	3.1	3.5	1.14	30.0	11.7	25.1	8.1	14.3	5.5

[a] The amino acid composition suggest the peptides indicated.
[b] The expected amino acid composition values for each fragment is shown in parentheses assuming the identity shown above each fragment.
[c] Amino acid hydrolysis of ACTH for 3h at 100°(--) indicated that the serine residue is not phosphorylated.
[d] Not determined because of destruction during acid hydrolysis.
[e] Cysteine was not quantitated although present.
[f] No signalprobably due to glycosylation.
[g] Nascent peptide chain, no sugars.

the initial chromatogram, also gave the same results as the three previous peaks (i.e. the compositon of AJP). However, these forms of the peptide eluted later at 16.5% and 17.0% MeCN. Fractions 53-54 were pooled and re-run on a linear gradient of 15 to 40% B over 1 hour. This revealed the presence of three different peaks (Figure 3.02) which all had an amino acid composition consistent with AJP (Table 3.01). Also, when fractions 55-56 were re-run using the same conditions as shown in Figure 3.02, two of the purified peaks had the amino acid composition of AJP and aligned with the last two AJP peaks of Figure 3.02. Since these peaks were collected by hand to increase their purity, they were individually re-chromatographed on the same day, using the same column and the same solvents. The results clearly indicate that three different species of AJP had been purified and separated (Figure 3.03). It should be noted that fraction #52 was later purified and revealed a single pure peak which had the composition of AJP and perfectly aligned itself with the last peak of Figure 3.02.

The purification and characterization of AJP clearly demonstrated six different forms of this peptide produced by the tumor. The elution positions of the acidic joining peptide on the initial chromatogram (Figure 3.01) indicated that the peptides formed two groups - one eluting at approximately 14.5% MeCN while the other eluted at 16.5% MeCN - even though the amino acid compositions of these peptides were very similar (Table 3.01).

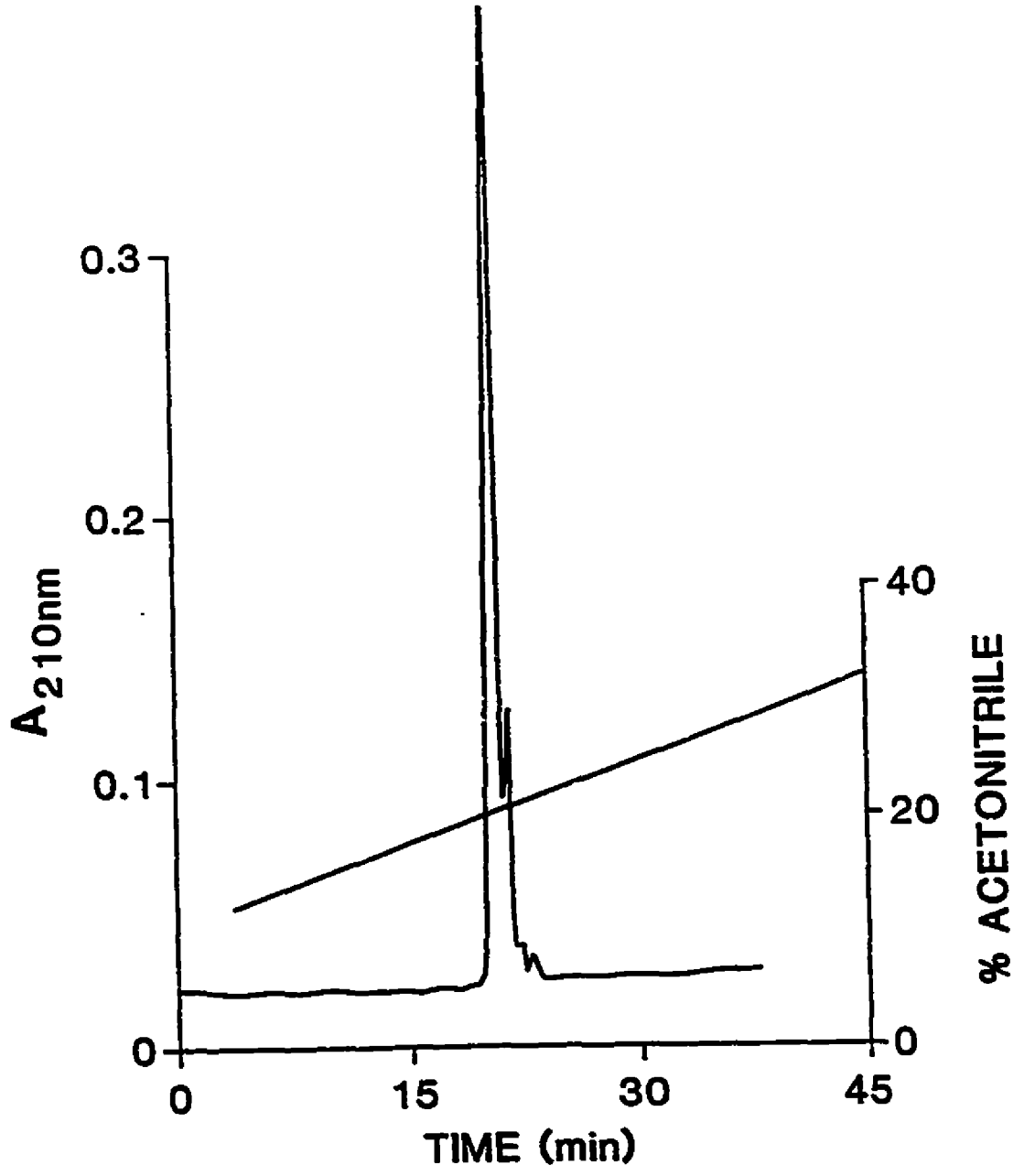


FIGURE 3.02 RP-HPLC of fractions 53 and 54 of the initial chromatogram. These factions were pooled and re-run on a Vydac C_{18} analytical column. A gradient of 12-32% acetonitrile, over 1 hour, containing 0.1% TFA was used. Three forms of AJP were identified according to their amino acid compositions.

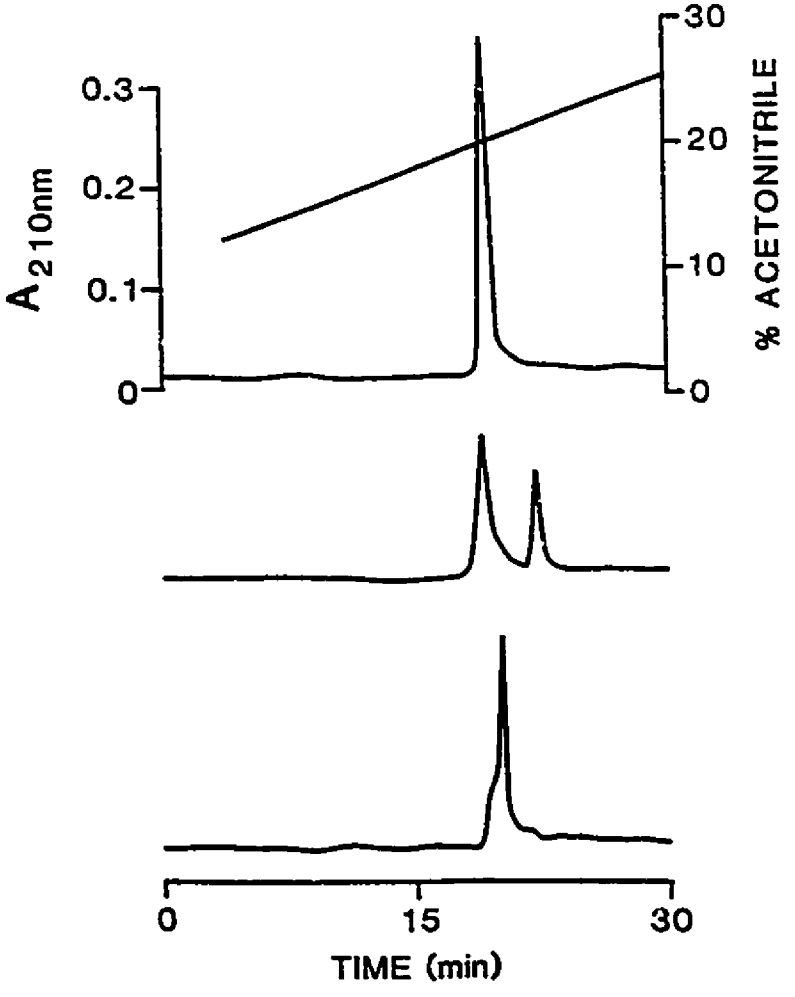


Figure 3.03 The alignment of three RP-HPLC runs of purified AJP. The exact conditions were used for each chromatogram (a linear gradient of 12-32% acetonitrile over 1 hour with 0.1% TFA throughout). Amino acid analysis identified them as three distinct forms of AJP.

Separation of peptides on RP-HPLC is based both on the chemical nature and on the size of peptides. However, gel permeation chromatography (GP-HPLC) separates peptides and proteins according to their molecular size, and this property was used to investigate the behaviour of the major components of both AJP groups which had been previously purified. Upon GP-HPLC the retention time of the AJP eluting at 16.5% MeCN was about one minute less than the one eluting at 14.5% MeCN (Figure 3.04). This indicated that this form of AJP had a larger molecular weight than the one eluting at 14.5% MeCN. The molecular weight estimates, using a standard curve obtained from standard peptides and proteins, established that the form of AJP present in fractions 53-54 was approximately twice the size of the one found in fraction 45. Therefore, this indicated that human AJP could be produced both as a monomer and a dimer in this anterior pituitary tumor. Also, based on amino acid analysis the dimeric form of AJP seemed to be the favored product since it represented 85.7% of the total AJP found in the tumor while only 14.3% of the AJP was found to be monomeric.

The next peak to be studied was fraction 67 of the initial chromatogram. This peak eluted at 21.4% MeCN (Figure 3.01) and amino acid analysis indicated the presence of a peptide which, from its amino acid composition, could not be readily identified as a product of POMC. However, further purification using a linear gradient of 15 to 40% B over 1 hour, revealed two peaks eluting at 31.7% MeCN and 32.5% MeCN respectively. One of the these purified

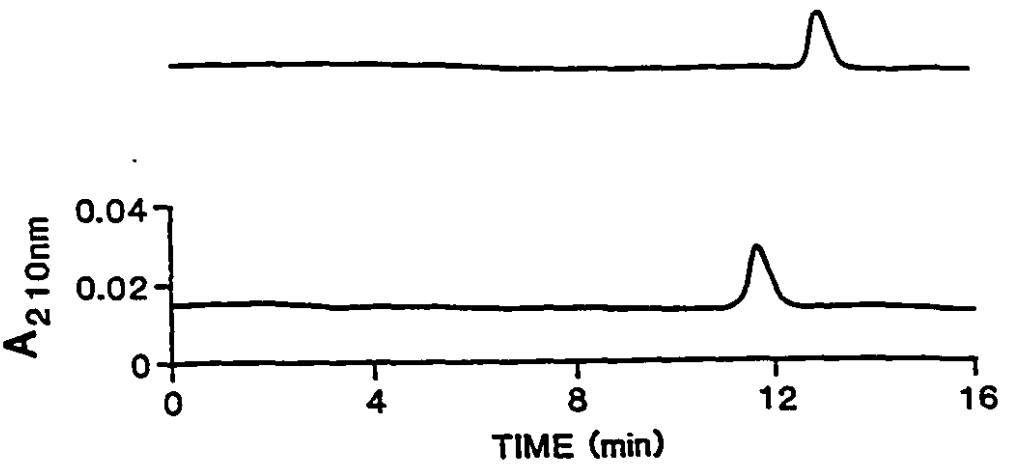


FIGURE 3.04 Gel permeation HPLC of two forms of AJP. 100μl of AJP, eluting at 14.5% acetonitrile on the initial chromatogram (fraction 45), was injected onto two I-125 columns connected in series and chromatographed using 40% acetonitrile containing 0.1% TFA. Another form of AJP eluting at 16.5% acetonitrile (fractions 53-54) underwent the same procedure. The AJP from fractions 53-54 eluted about 1 minute before the other form of AJP tested (fraction 45), indicating a larger molecular weight for this peptide.

peptides had an amino acid composition that tentatively identified it as the des-phe$_{39}$ form of ACTH (Table 3.01). The other peak that was purified had a composition that was similar but not identical to that of ACTH and it was repurified using the same linear gradient as the one used for fraction 67 in order to identify it more accurately. A single homogeneous peak, eluting at the same position as previously established was observed. However, not enough material was present to give a representative amino acid composition.

ACTH, the main hormonal product of POMC processing, was identified by amino acid analysis and found to elute at 24.0% MeCN in fractions 74 to 77 on the initial chromatogram (Figure 3.01). This peptide hormone is known to be phosphorylated in normal anterior pituitary tissue at serine residue 31 to an extent of approximately 30%. In order to determine if the tumour contained both phosphorylated and non-phosphorylated forms of ACTH, the ACTH fractions were dried down in a Speed-Vac concentrator and pooled. This material was then rechromatographed on a Vydac C_{18} analytical column, using a linear gradient of 25-50% B over 1 hour. This gradient would separate the the forms of ACTH, if present. However, ACTH eluted in a single homogeneous peak (Figure 3.05). An aliquot was subjected to mild amino acid hydrolysis for 2 hours at 110^{0}C conditions under which the phosphate group is not destroyed. Results indicated that the serine residue of this purified ACTH was not phosphorylated (i.e. absence of phospho-serine). 30.0 nmoles of ACTH was recovered from this pituitary adenoma.

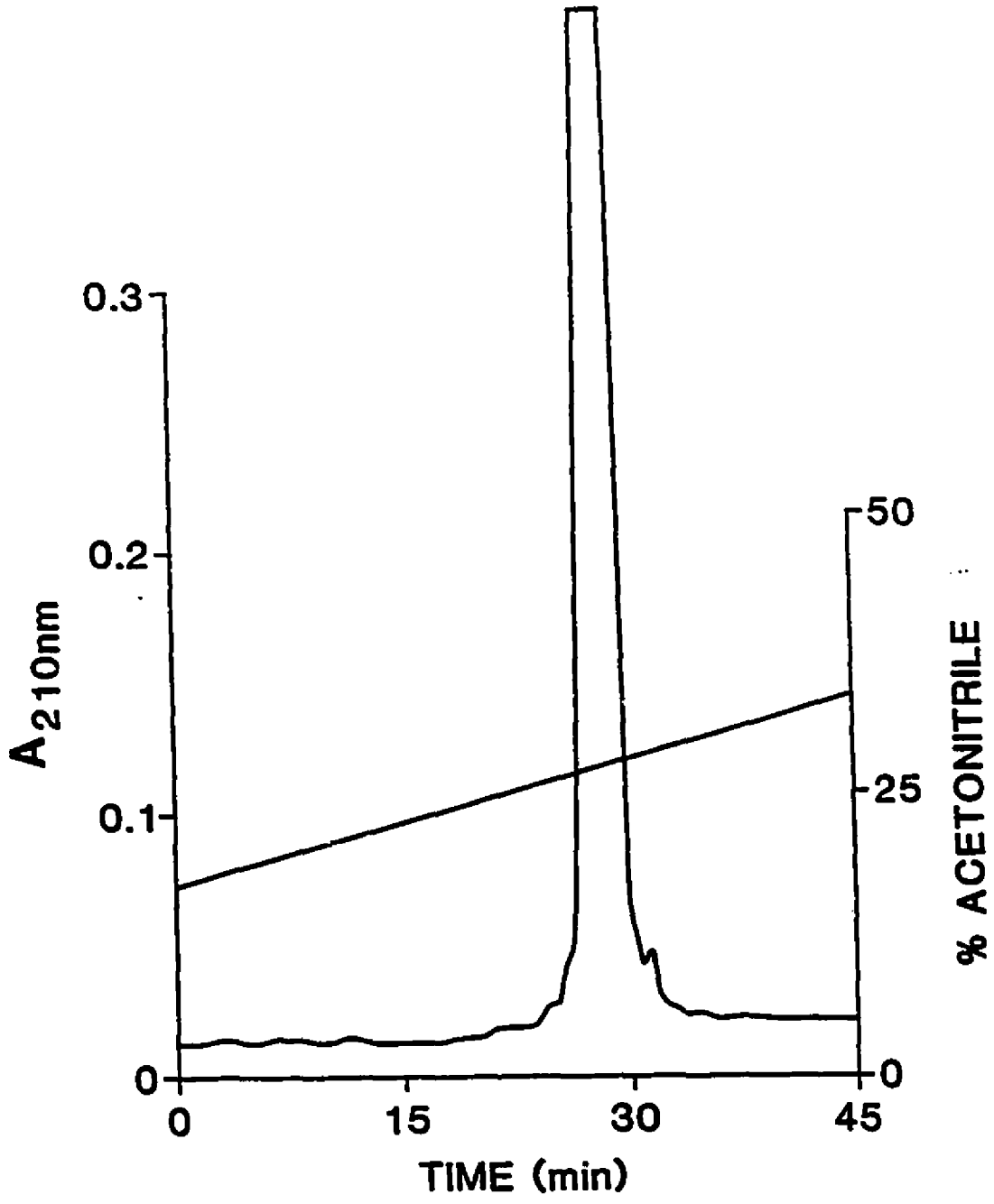


FIGURE 3.05 RP-HPLC of fractions 74-77 of the inital chromatogram. These fractions were pooled and re-chromatographed on a 20-40% acetonitrile gradient over 1 hour containing 0.1% TFA. Amino acid analysis of the purified peak identified it as ACTH.

The next peak which eluted at 26.3% MeCN on the initial chromatogram was found in fractions 80-83 and seemed pure. Amino acid composition identified it as τ-LPH. The fractions were pooled, dried, and the peptide was purified to homogeneity using a Vydac C_{18} column and a linear gradient of 30-55% B over 1 hour containing 0.1% TFA throughout. 11.7 nmoles of τ-LPH were recovered from this tumour.

The next major peak (fractions 94-98), eluting at 31.0% MeCN, was not pure. Its amino acid composition indicated ß-LPH, one of the biosynthetic products of POMC was present. The fractions containing ß-LPH were combined and reduced in volume in a Speed-Vac concentrator. Then the material was loaded on a Vydac C_{18} analytical column and subjected to RP-HPLC using a linear gradient of 35-60% B over 1 hour containing 0.1% TFA. Since this approach did not lead to the purification of ß-LPH, gel permeation chromatography was employed (see Material and Methods for protocol section 2.2.4). Two peptides were separated from each other using this method (Figure 3.06). The first peak, eluting at 10.8 minutes, was larger than the second. Amino acid analysis revealed the former peak to be ß-LPH. The second peptide, having a smaller peak height and lower molecular weight minutes (Figure 3.06), was identified by amino acid analysis as ß-endorphin 1-31. No further processing to ß-endorphin 1-27 was evident. No post-translational modifications of ß-LPH were revealed. The processing of ß-LPH into τ-LPH and ß-endorphin represented 31.8% of the ß-LPH production from the tumor (see Table 3.01 for values).

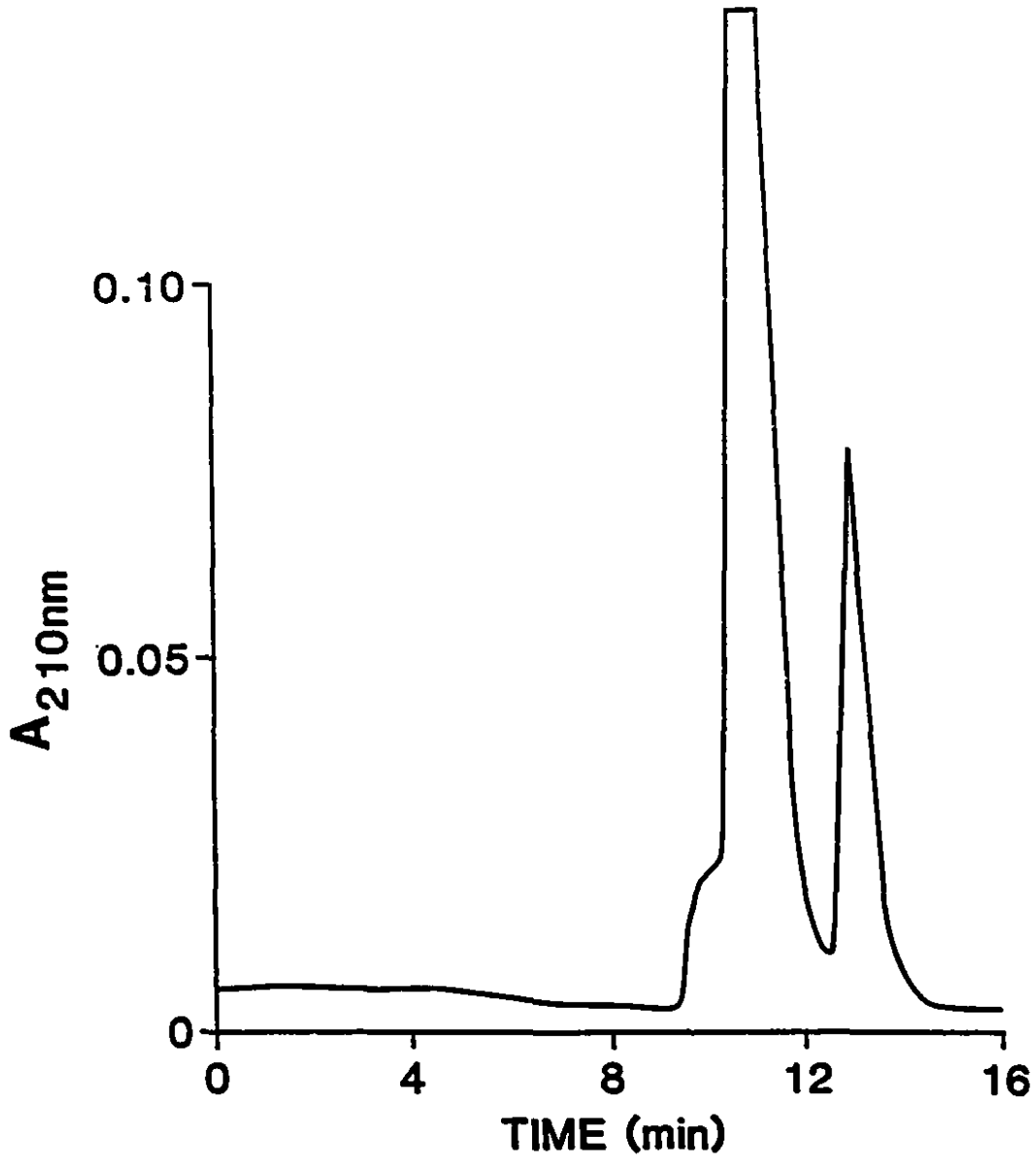


FIGURE 3.06 GP-HPLC of the major peak from an intermediate RP-HPLC purification step of ß-LPH. The peak that eluted earlier(10.8 minutes) was confirmed to be ß-LPH by amino acid analysis. The peak that eluted at 12.9 minutes was identified as ß-endorphin 1-31.

The last remaining POMC product found was the amino-terminal fragment (16K fragment), a glycosylated peptide. Although this peptide can be identified from its amino acid composition, the presence of sugar residues on the amino acid analysis facilitates identification. Fractions 99-102 from the initial chromatogram (Figure 3.01) all contained amino sugars. Amino acid compositions were similar to those expected for the amino-terminal fragment of POMC.

The peak, eluting at 31.8% MeCN, included in fractions 99-100 whereas peak, found in fractions 101-102, eluted at 32.1% MeCN (Figure 3.01). Both of these peptides were purified and from their amino acid compostion represented two forms of the amino-terminal fragment of POMC. Figure 3.07 shows the RP-HPLC of the 16K fragment eluting at 32.1%. Moreover, both of these peptides showed from, a qualitative point of view, the presence of the same amino sugars but in different proportions.

In order to determine on which residue(s) the peptide was glycosylated, an enzymatic digest was performed (see section 2.3.2.), followed by a RP-HPLC step in 0.1% TFA with a linear gradient of 2-52% B over 1 hour (Figure 3.08) to separate the resultant fragments. Several peptides from the amino-terminal fragment were identified by amino acid analysis (Table 3.02). These results were consistent with glycosylation of both threonine$_{45}$ and asparagine$_{65}$ of the amino-terminal fragment were glycosylated. It should, however, be noted that not all the possible products of the enzymatic digestion were identified. Also, the 1-49 fragment

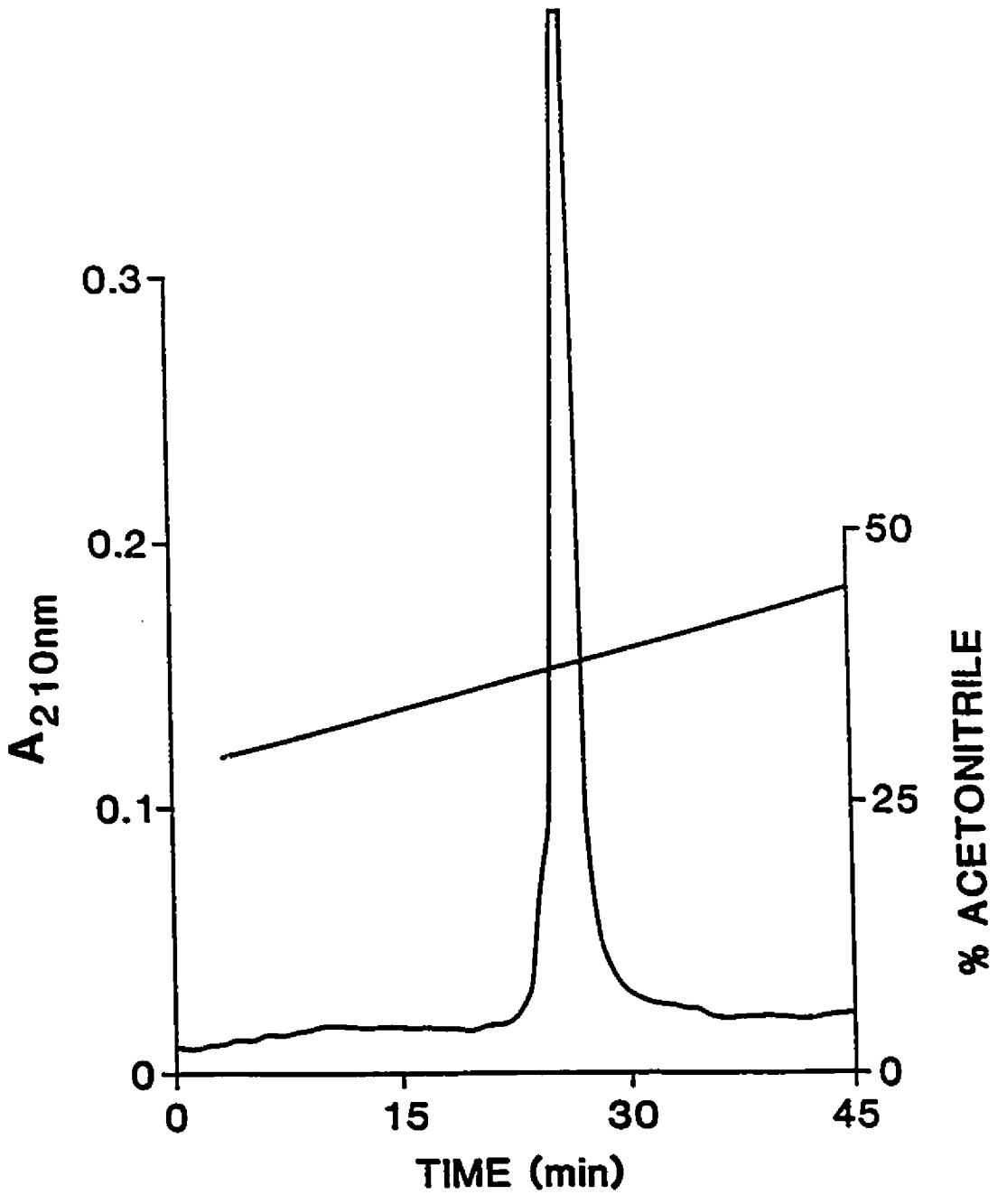


FIGURE 3.07 Purification of the peak eluting at 32.1% of the initial chromatogram (fractions 100-103). The isolated peak was identified using amino acid analysis and confirmed the presence of a second form of the amino-terminal fragment of POMC. A gradient of 28-48% acetonitrile over 1 hour with 0.1% TFA was used.

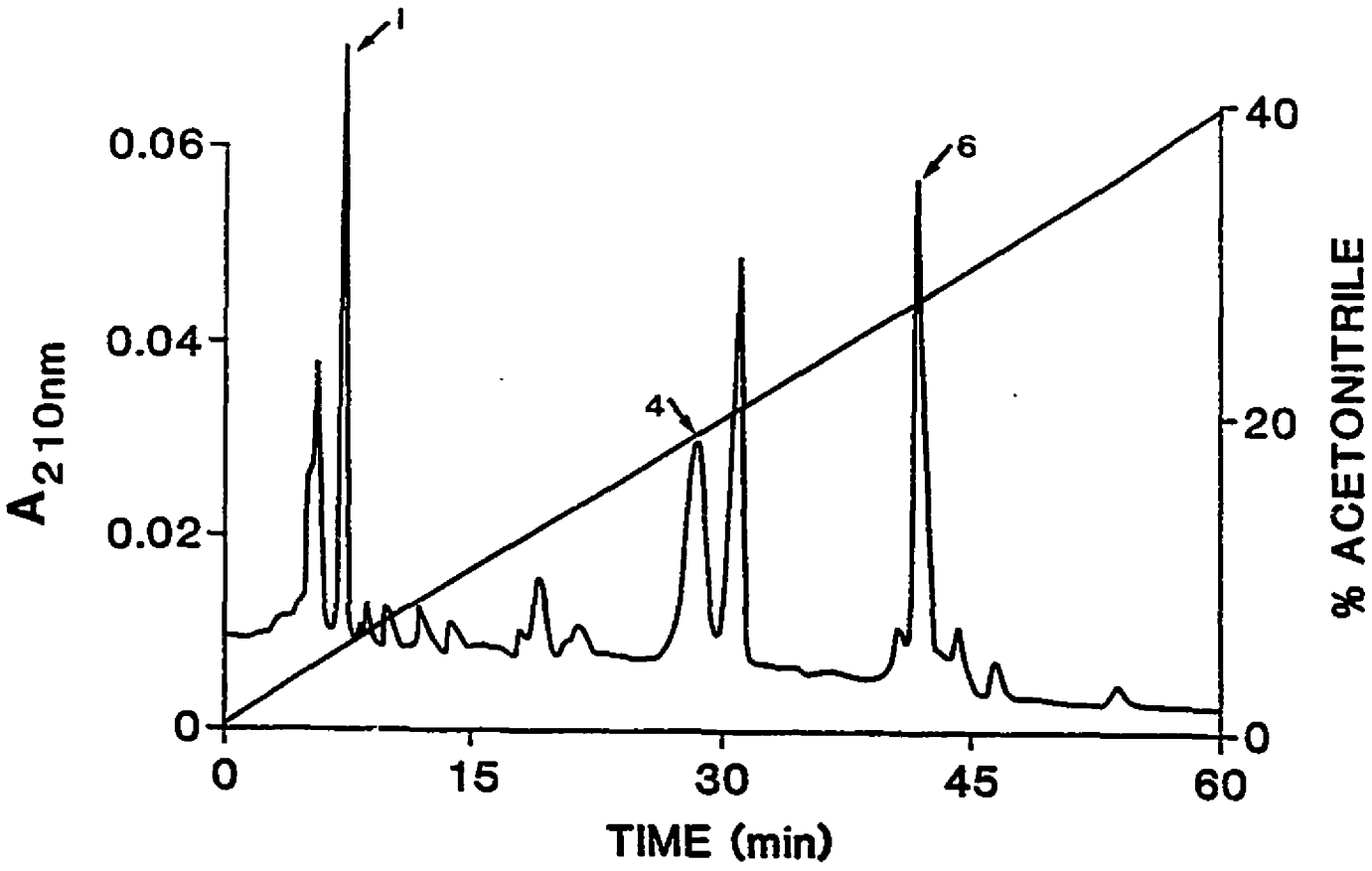


FIGURE 3.08 Digest of the amino-terminal fragment of POMC with trypsin and S.aureus V_8. 10 nmoles of the purified peptide were incubated with the two enyzmes simultaneously for 18 hours in 100μl of 50mM TRIS buffer, pH 7.8. The products were analysed by RP-HPLC using a gradient of 1.6-41.6% acetonotrile, containing 0.1% TFA. The peaks were collected by hand and aliquots taken for amino acid analysis. The identities of the numbered peaks can be found in Table 3.02.

TABLE 3.02 Amino acid composition (molar ratios) of biosynthetic peptides obtained from enzymatic digestion of the amino-terminal fragment of POMC. The amino acid sequence of the possible fragments is listed as follows (single letter code used for amino acid):

Fragment 1.	DSSSSGSSGAADKFGR
Fragment 2.	FGR
Fragment 3.	YVMGHFR
Fragment 4.	PDLSAETPMFPGDGDEEPLTEDPR
Fragment 5.	WDR
Fragment 6.	WCLESSQCQDLTTESNLLECIR

PEPTIDE[a]	Fragment #1[b]	Fragment #4[e]	Fragment #6[f]
Asx	1.9(1)[c]	3.7(4)	2.3(2)
Thr		1.4(2)	1.7(2)
Ser	5.7(6)	1.4(1)	4.1(3)
Glx	1.4(1)	4.6(4)	5.2(5)
Pro		4.4(5)	
Gly	3.2(3)	3.1(2)	
Ala	1.0(2)	1.9(1)	
Met		0.7(1)	
Ile			0.7(1)
Leu		2.1(2)	3.5(4)
Phe	0.5(1)	0.9(1)	
Lys	0.7(1)	1.2	
Arg	1.1(1)	1.3(1)	1.4(1)
Trp	n.d.[d]	n.d.	n.d.

[a] The amino acid compositions suggest the peptides indicated.
[b] Fragment #1 is glycosylated.
[c] The expected amino acid composition values for each fragment is shown in parentheses assuming the identity shown above each fragment.
[d] Not determined because of destruction during acid hydrolysis.
[e] Fragment #4 is glycosylated.
[f] Fragment #6 was found to contain some oxidized cysteine.

and lys τ_3-MSH which are the natural cleavage products of the amino-terminal fragment of POMC were not isolated from the tumor extract.

The yield of ACTH and the other POMC products produced by the tumor was very high, characterising the nature of this anterior pituitary adenoma. The total yield for all the AJP species was 17.62 nmoles and represented the lowest yield of all the POMC products. For the other biosynthetic products of POMC, 31.14 nmoles of ACTH (including des-phe ACTH), 36.80 nmoles of ß-LPH (including the amount purified for τ-LPH), and 19.80 nmoles of the amino-terminal fragment (both forms) were isolated from the pituitary tumor (Table 3.02 for specific yields).

3.2. Structural and Biological Characterization of Guinea-Pig POMC Products

The purification and characterization of guinea-pig POMC products was accomplished following the scheme described in section 2.2.3. Anterior and neurointermediate lobes of 20 guinea-pig pituitaries were separated and extracted. This was followed by RP-HPLC and the initial chromatograms for both lobes is shown in Figure 3.09. Column fractions were screened for their amino acid content in order to identify POMC-like peptides. A combination of RP-HPLC and GP-HPLC generated peptides of apparent homogeneity according to their amino acid content. An illustration of this is the purification of ACTH from anterior pituitary lobes.

After the screening of the initial chromatogram of the anterior pituitary extract, fractions 45-47 were identified as containing ACTH-like material according to amino acid analysis. They were dried in a Speed-Vac concentrator to 600μl per tube and pooled. This sample was then loaded on a Vydac C_{18} analytical column. The peptide was repurified using a linear gradient of 15-40% B over 1 hour. The peak was collected by hand and amino acid analysis was performed to determine the exact composition of guinea-pig ACTH (Table 3.03). The peptide eluted at 30.4% MeCN and 18 moles were purified. Moreover, mass spectrometry (see section 2.3.3.) of the purified guinea-pig ACTH established that the peptide was not phosphorylated.

Other peptides from the anterior and neurointermediate lobes were purified

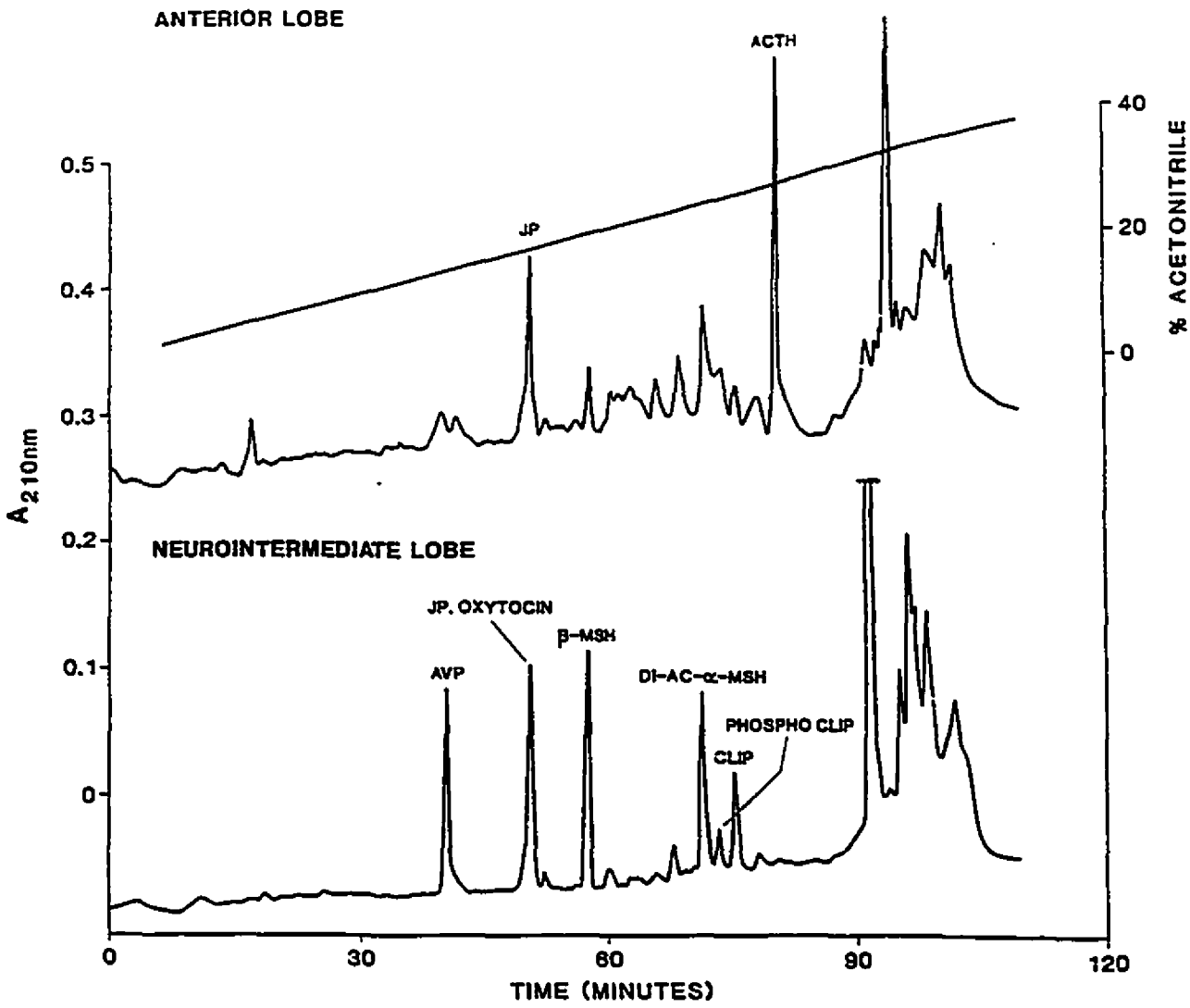


FIGURE 3.09 RP-HPLC of extracts of anterior lobe(a) and neurointermediate lobe(b) obtained from 20 guinea-pig pituitaries. Peptides were purified to homogeneity by a combination of RP- and GP-HPLC. Joining Peptide, ACTH, ß-melanotropin, di-Ac-alpha-MSH, phosphorylated and non-phosphorylated CLIP were characterized by amino acid analysis and mass spectrometry (see Table 3.03). Arginine vasopressin and oxytocin were derived from the neural lobe and were not the subject of this study.

Table 3.03. Amino acid compostion (molar ratios) and mass estimations of biosynthetic fragments of pro-opiomelanocortin from anterior and neurointermediate lobes of guinea-pig pituitary. The cDNA sequence for guinea-pig pro-opiomelanocortin (241) predicts the following sequences (single letter code used for amino acid).

Joining Peptide: EEEAAAADPGFHGDGVEPGLRED (JP)
Corticotropin[a]: SYSMEHFRWGKPVGKKRRPVKVYANGAEEESAEAFPLEF (ACTH)
ß-Melanotropin: DDGSYRMEHFRWGTPRK-NH_2 (ß-MSH)

Peptide[b]	JP (anterior lobe)	ACTH	JP (intermediate lobe)	ß-MSH	di-Ac-α-MSH	phospho-CLIP	CLIP
Asx	2.9(3)[e]	1.1(1)	3.3(3)	2.1(2)		1.1(1)	1.2(1)
Thr				1.0(1)			
Ser		2.7(3)		1.0(1)	1.8(2)	0.8(1)[f]	1.0(1)
Glx	5.2(5)	6.0(6)	5.0(5)	1.2(1)	1.1(1)	5.0(5)	5.3(5)
Pro	1.9(2)	2.4(3)	1.7(2)	1.0(1)	0.9(1)	1.7(2)	1.8(2)
Gly	4.4(4)	3.0(3)	3.9(4)	2.2(3)	1.1(1)	1.0(1)	1.1(1)
Ala	3.9(4)	3.7(4)	3.8(4)			4.0(4)	4.2(4)
Val	1.0(1)	2.8(3)	0.8(1)		1.0(1)	1.9(2)	2.0(2)
Met		0.9(1)		1.0(1)	1.0(1)		
Leu	1.2(1)	1.2(1)	1.2(1)			1.1(1)	1.2(1)
Tyr		1.6(2)		0.9(1)	1.0(1)	0.9(1)	1.0(1)
Phe	1.0(1)	2.7(3)	1.0(1)	1.1(1)	1.0(1)	1.9(2)	2.1(2)
His	1.0(1)	1.0(1)	1.1(1)	0.8(1)	0.8(1)		
Lys		3.8(4)		1.1(1)	1.1(1)	1.0(1)	1.0(1)
Arg	1.0(1)	3.3(3)	1.1(1)	3.2(3)	0.8(1)	1.2(1)	1.2(1)
Trp	N.D.(0)[g]	N.D.(1)	N.D.(0)	N.D.(1)	N.D.(1)	N.D.(0)	N.D.(0)
Yield (nmole per lobe)	0.82	0.9	1.0	0.94	1.8	0.63	0.36
Mass[c] (observed)	2368.32	4529.44	2368.65	2137.98	1706.65	2534.48	2454.0
Mass[d] (expected)	2368.42	4529.13	2368.42	2137.38	1707.95	2532.7	2453.7

a. The sequences of α-MSH and corticotropin-like intermediate lobe peptide (CLIP) are to be found within the structure of ACTH (ie. N-, O-Acetyl, ACTH (1-13)-NH_2 and ACTH (18-39) respectively).

b. The amino acid compositions and observed masses suggest the peptides indicated.

c. Observed masses are average values obtained from multiply charged ions.

d. Expected masses were calculated using average masses for each amino acid.

e. The expected amino acid composition values for each fragment is shown in parentheses assuming the identity shown above each column.

f. Amino acid analysis following hydrolysis of phospho-CLIP for 3 h at 100° indicated that the serine residue is phosphorylated.

g. Not determined because of destruction during acid hydrolysis.

in a similar fashion and their composition data can be found in Table 3.03. Their respective amino acid compositions corresponded closely to that predicted from the cDNA sequence for guinea-pig POMC (223), and their molecular weights were determind by mass spectrometry. The results illustrate the novel purification of guinea-pig POMC products from both the pituitary lobes and confirm the structure of guinea-pig ACTH as shown by others (231).

In a previous study, the potency of guinea-pig ACTH was compared to that of synthetic ACTH(1-24) in an isolated rat glomerulosa cell bioassay (231). Guinea-pig ACTH gave rise to supra-maximal secretion of aldosterone, indicating that the peptide was a superagonist in terms of the stimulation of the secretion of adrenal steroids. In order to confirm this result, the activity of the guinea-pig ACTH, purified from the initial chromatogram (Figure 3.09), was compared with synthetic human ACTH (1-39) in both isolated rat fasciculata-reticularis and glomerulosa bioassays in vitro (section 2.4.1.). The dose-response characteristics of both peptides in the bioassay systems are shown in Figures 3.10 and 3.11. The curves obtained for the different experiments indicate that guinea-pig ACTH (closed circles) is approximately two to three times more potent than human ACTH (open circles) in fasciculata-reticularis and glomerulosa cell bioassays respectively. However, both guinea-pig and synthetic human ACTH elicted similar activities in terms of maximal steroid output in the two bioassay systems, and thus, these results are not consistent with guinea-pig ACTH being a superagonist at least with respect to the human synthetic ACTH standard that was used.

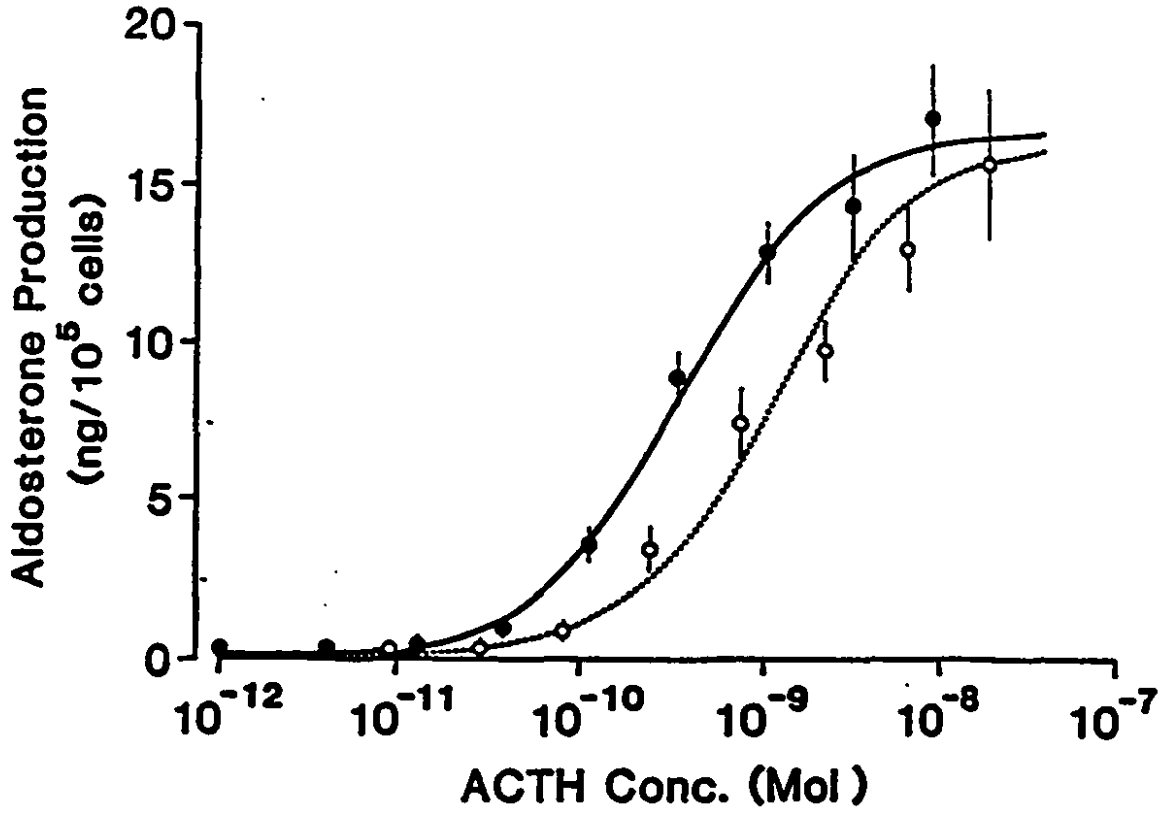


FIGURE 3.10 A comparison of the dose-response characteristics of guinea-pig ACTH (closed circles) and synthetic human ACTH(1-39) (open circles) in the dispersed rat glomerulosa cell bioassay system. Steroid output (aldosterone) is expressed as the average of three separate experiments ± standard deviation.

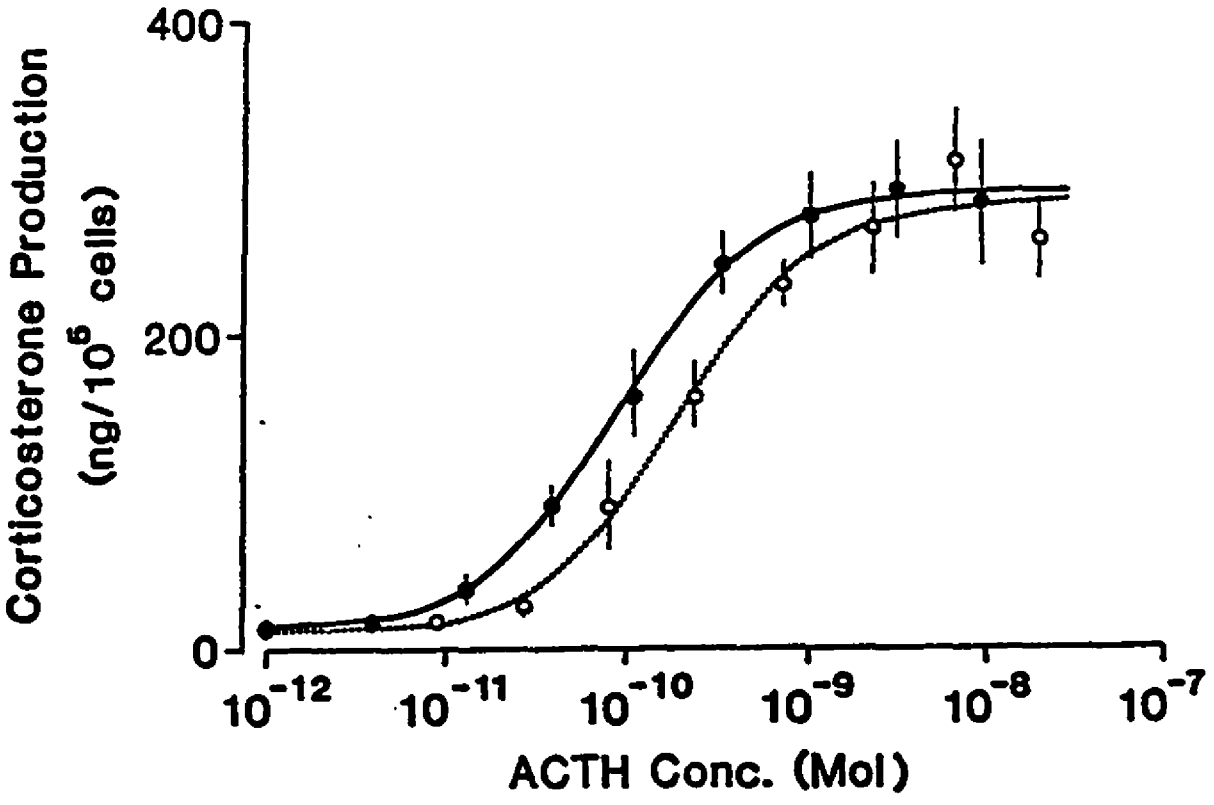


FIGURE 3.11 A comparison of the dose-response characteristics of guinea-pig ACTH (closed circles) and synthetic human ACTH(1-39) (open circles) in the dispersed rat fasciculata-reticularis cell bioassay system. Steroid output (corticosterone) is expressed as the average of three separate experiments ± standard deviation.

CHAPTER 4: DISCUSSION

4.1. Processing of POMC in a Cushing's Tumor

Under normal conditions, CRF stimulates pituitary ACTH secretion in a pulsatile manner(85). In Cushing's Syndrome, ACTH is also secreted episodically by pituitary adenomas(216,217); however, the circadian pattern of cortisol is absent because the negative feedback of ACTH by glucocorticoids is impaired(218). Most of the clinical abnormalities seen in Cushing's Syndrome are related to the excess of glucocorticoids, and the excess of androgen is usually proportional to ACTH and cortisol. It is implied that the cells involved in these pituitary tumors generate and process large quantities of POMC. Thus, it was of interest to biochemically characterize the different POMC products.

In biologically active peptides, structural integrity is under tight control(219). AJP is a low molecular weight peptide that is produced by POMC, and is structurally very heterogeneous among mammalian species(220-222). It has been proposed that AJP or a fragment of this peptide may be an androgen stimulatory factor(223) and thus, in conjunction with ACTH, may have a role in promoting some of the classical traits of Cushing's Syndrome such as hirsutism. However, recent work has clearly demonstrated the lack of activity of this peptide in androgen

production (224-226).

Previous work has shown that AJP can be found in human pituitary tissue either as a monomer or as a dimer(224,227,228). The formation of the AJP dimer probably arises through disulfide bridging since human AJP has a lone cysteine residue at position nine. The AJP dimer, isolated from this pituitary adenoma associated with Cushing's Syndrome,constitutes at least 87% of the total AJP production(Table 3.01). This indicates that the biosynthetic machinery of the tumor gives preference to dimer formation rather than to monomer formation, which seems prevalent in normal pituitary tissue(227). The presence of three forms of both the monomer and of the dimer, as indicated by the different elution positions of AJP, is compatible with other post-translational modifications that can occur within this peptide i.e. carboxyl-terminal α-amidation and amino-terminal pyroglutamate formation(168,229). It should however be noted that the modifications could not be identified in this study because of the low yield of the different forms of purified AJP.

Phosphorylation is a significant post-translational modification of POMC. In human anterior pituitary tissue, ACTH bears a site of phosphorylation, shown to be at ser_{31}(229). This residue is part of the ser/thr-x-acidic residue sequence which is the general recognition site for physiological casein kinases, a member of a class of cAMP-independent protein kinases responsible for catalysing the phosphorylation of POMC in the human pituitary gland(230). An intriguing finding is that all the ACTH present in the tumor is in the unphosphorylated form, while in normal anterior pituitary tissue, 30-40% is so modified(229). This may mean that there is no active physiological casein kinase-like activity present in these cells. Phosphorylation is known to regulate the activities of many enzymes and proteins(230,231). The apparent lack of

physiological casein kinase activity may influence other cellular events in these tumor cells.

The partial processing of ß-LPH into γ-LPH and ß-endorphin is often seen in normal anterior pituitary tissue in mammals(152). The form of ß-endorphin found in the tumor (i.e. ß-endorphin$_{1\text{-}31}$) is consistent with POMC processing in corticotrophs. Two forms would have been found in the intermediate lobe pituitary cells of other species where further cleavage occurs at a pair of lysine residues to yield ß-endorphin$_{1\text{-}27}$ which is processed further to ß-endorphin$_{1\text{-}26}$. These latter two forms are essentially devoid of opiate activity. Since truncated forms of ß-endorphin are a consequence of the action of the pro-hormone convertase PC-2, this particular tumor was most likely devoid of this enzyme. ß-LPH has been shown to be post-translationally modified in bovine neurointermediate pituitaries with the formation of an amino-terminal pyroglutamate residue(168). The pyroglutamate in this case is derived from an amino-terminal glutamic acid residue rather than glutamine. The biochemical basis for this modifcation is not clear at the present. Since this also occurs with bovine AJP and the tumor produced several different forms of this peptide(assessed by their elution position), the possibility of the presence of a modified ß-LPH in the tumor existed. However, careful HPLC and amino acid analysis revealed only one form of ß-LPH, which was apparently not modified.

The 16K fragment was also produced by the tumor. It has been postulated that this peptide is involved in steroidogenesis by stimulating cholesterol ester hydroxylase actvity(232) or promoting compensatory adrenal growth(233). It is highly conserved among all mammalian species and several forms have been found depending on the degree of glycosylation. Two forms of the 16K fragment were isolated and both had N- and O-linked sugars, indicating that thr$_{45}$ and asn$_{65}$ underwent glycosylation, the two sites previously identified as glycosylation sites in

this peptide from other mammalian species. The difference between them thus depended on the extent of glycosylation as determined by amino sugar composition. The fact that thr_{45} was O-glycosylated may explain the absence of both the 1-49 fragment and γ_3-MSH. Glycosylation at thr_{45} may not allow the dibasic pair at residues 49-50 to be proteolytically cleaved as it is in the intermediate lobe of the pituitary.

Post-translational modifications such as amino-terminal acetylation, carboxyl-terminal amidation, and tyrosine sulfation are essential for the full biological activity of many peptide hormones(170,234). Other modifications can also be present (glycosylation, phosphorylation) but their biological significance remains unclear(170). In the present study, the tumor cells involved in Cushing's Syndrome demonstrate classical anterior lobe pituitary processing with no intermediate lobe derived POMC peptides. This ACTH-secreting tumor seems to be lacking the enzymatic ability to post-translationally modify some of the peptide hormones produced such as ACTH which is not phosphorylated. Certain enzymes, for instance, casein kinase involved in the phosphorylation of various proteins may have important functions in normal cellular activity and thus their absence may help explain the aberrant regulation involved in this type of tumor.

4.2. Charcterization and Purification of Guinea-pig POMC Products and the Biological Activity of Guinea-pig ACTH

The purification and characterization of guinea-pig peptides originating from pituitary

POMC permitted an in depth look at its processing both in the anterior and neurointermediate lobes. Using techniques including amino acid analysis, HPLC, and mass spectrometry, post-translational modifications were identified in these peptides. In addition, the biological characteristics of ACTH purified from the anterior lobe were determined with respect to stimulation of adrenal steroids.

The joining peptide was the first POMC product isolated (Figure 3.09). It is found in both lobes of the guinea-pig pituitary, and the mass and amino acid composition of these two peptides correspond closely to that predicted by the cDNA sequence for guinea-pig POMC. As in all species of POMC, the joining peptide domain immediately precedes ACTH and is thought to function primarily as a structural element within the prohormone, as is the case of the C-peptide domain of proinsulin (235). The joining peptide is one of the least structually conserved sequences within POMC but some sequence homology between guinea-pig, bovine, and human JP exist. However, unlike human JP, the guinea-pig JP does not contain cysteine and thus cannot be found as a dimer. Moreover, it is apparent that guinea-pig has little similarity to corresponding sequences found in mouse and rat (236,237). This observation supports the recent phylogenetic reassignment of the guinea-pig as evolutionarily distinct from the other rodents (238).

In the intermediate lobe of the pituitary, the ACTH domain of guinea-pig POMC is processed, as in other species, into α-melanotropin (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP). The amino acid composition of α-MSH was found to be identical to the equivalent peptide foumd in many other species since no amino acid substitutions are present in that region of the ACTH domain of the POMC sequence. The observed mass of this peptide

clearly indicates that the amino-terminal serine residue is subject to both N-and O-acetylation, a result that is consistent with the known processing of other α-MSH species within the intermediate lobe of the pituitary (239).

The other biosynthetic product originating from the processing of the ACTH domain in the intermediate lobe, CLIP, was found in two forms in the guinea-pig. Amino acid analysis and mass determination were performed on both forms of CLIP and indicate that the more polar peptide was phosporylated (Table 3.03).This post-translational modification has been observed in many other species and is dependent upon the presence of the physiological casein kinase phosphorylation site ser_{12}-ala_{13}-glu_{14} (240). Mass spectrometry confirmed the presence of the unusual alanine for proline substitution in both forms of CLIP at position 7, as seen in the corresponding sequence of ACTH.

Mass determination of guinea-pig ACTH within the anterior lobe indicated the absence of phosphorylation of the ser_{29} site. In other species where the phosphorylation site is present (i.e. rat, mouse, and man), at least partial phosphorylation of the purified peptide can be found (240), although the purified ACTH from the Cushing's tumor was not phosphorylated (section 3.1.). the degree of phosphorylation of the guinea-pig ACTH may be influenced by the tertiary structure of POMC within the two lobes of the pituitary and its transit time through the Golgi compartment, the probable site of the kinase activity (240). Alternatively, the kinase activity may be absent from the Golgi apparatus of guinea-pig anterior lobe corticotrophs.

ß-MSH was also found to be a major product of the biosynthetic processing of guinea-pig POMC within the intermediate lobe (Table 3.03). This peptide requires the presence of a lys-lys cleavage site within the ß-LPH domain of POMC and is absent from anterior lobe extracts. This

explains why ß-MSH is not found in the pituitaries of rat and mouse since the paired lysine cleavage signal to the terminal side of the peptide is not present (236,237). Amino acid analysis and mass determination of guinea-pig ß-MSH strongly implies that the peptide is carboxyl-terminally amidated. This would be the first example of a ß-MSH that would be post-translationally modified in this way. Study of the cDNA sequence of guinea-pig POMC shows that proteolytic cleavage at the lys-arg dibasic pair to the carboxyl-terminus of the ß-MSH domain should reveal a lys-gly amino acid pair (241). Glycine-extended sequences have been shown to be substrates for the enzyme system responsible for carboxyl-terminal amidation of peptides (205). These glycine residues are cleaved to give rise to an α-amidated carboxyl terminus in a two step enzymatic process involving the sequential actions of a peptidyl-glycine α- hydroxylating mono-oxygenase followed by a peptidyl-α-hydroxyglycine α-amidating lyase. Moreover, these enzymatic post-translational modifications of ß-MSH would occur in the secretory vesicles of the Golgi apparatus. Structure-activity studies of the α-amidating system in vitro have shown that peptides with polar residues amino-terminal to the glycine are poor substrates for the mono-oxygenase enzyme(242,243). In the present study, the results indicate that the in vivo efficiency of amidation of guinea-pig ß-MSH approaches 100% despite the potentially unfavorable nature of the substrate.

The yield of each peptide on a per pituitary basis reveals that there is a stoichiometric relationship between each domain when proteolytic processing is complete (Table 3.03). Previous studies have revealed that within the intermediate, there is a sub-stoichiometric cleavage of the amino-terminal or 16K fragment domain of POMC. Thus examination of extracts of the neurointermediate lobes of bovine, mouse, and rat pituitaries have indicated that approximately

30% of the amino-terminal is cleaved to form lys-γ_3-MSH (154,163). This peptide elutes close to ß-MSH in the RP-HPLC system used in this study(244). However, no evidence of a γ-MSH-like peptide was found. The presence of an O-linked oligosaccharide at thr_{45} within the amino-terminal domain has been implicated in determining the extent of proteolytic cleavage of the -arg_{49}-lys_{50}- sequence that gives rise to lys-γ_3-MSH. Such sugars appear to limit the production of lys-γ_3-MSH at least within the intermediate lobe(163). The cDNA sequence for guinea-pig POMC shows the presence of a thr_{45} residue, implying the potential for O-linked glycosylation(241). The lack of lys-γ_3-MSH production suggests that glycosylation at this site is essentially complete, preventing any further biosynthetic processing.

Guinea-pig ACTH was compared with synthetic human ACTH in both the isolated rat fasciculata-reticularis and glomerulosa cell bioassay in vitro. In both systems, the guinea-pig and synthetic ACTH showed similar activities with respect to maximal steroid secretion (Figures 3.10 and 3.11). No superagonism of guinea-pig ACTH was observed although it was 2 to 3-fold more potent than human ACTH in both bioassay systems. Sequence variations within the carboxyl-terminal domain of ACTH have been shown to alter the observed steroidogenic potency. For instance, dogfish ACTH which displays little similarity with human ACTH within the carboxy-terminal domain has approximately 15% of the steroidogenic potency of human ACTH in an isolated rat adrenal cell bioassay(245). Absence of the carboxyl terminus causes ACTH(1-24) to be up to ten times more potent than human ACTH in vitro (246,247). However, dogfish ACTH and ACTH(1-24) were observed to be full agonists relative to synthetic human ACTH standard. In view of the sustitution of alanine for proline at residue 24 within guinea-pig ACTH, a change in potency might be expected. However, an increase in potency in terms of maximal steroid output would not be expected. The results indicate that guinea-pig ACTH is a full agonist relative to synthetic human ACTH in both bioassay systems and that no evidence for

superagonist activity was observed. In a previous study, the activity of guinea-pig ACTH was compared to that of ACTH(1-24) in an isolated rat glomerulosa cell bioassay and results indicated that guinea-pig $ACTH_{1-39}$ had superagonist activity(248). Metabolism of peptides during isolated adrenal cell bioassays has been shown to decrease the steroidogenic potencies observed(249). ACTH(1-24) is particularily prone to proteolytic cleavage in this bioassay and this may account, in part, for the discrepancy between our observations and the results obtained in the previous study(248).

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The main contributions are:

1. The first report of the full characterization of the biosynthetic products of POMC produced by a human pituitary adenoma in Cushing's Disease.
2. The partial characterization of five different forms of AJP and the existence of this peptide predominantly as a dimer rather than a monomer.
3. The identification of unphosphorylated ACTH, implying an apparent lack of physiological casein kinase activity.
4. The fact that this pituitary adenoma, involved in Cushing's Disease, demonstrates classical anterior lobe processing.
5. Identification, purification, and characterization of the major biosynthetic POMC products from both the anterior and the neurointermediate lobes of the guinea-pig pituitary.
6. The first report of a ß-MSH (guinea-pig) that would be carboxyl-terminally amidated.
7. No superagonism of guinea-pig ACTH was observed although it was 2 to 3-fold more potent than synthetic human ACTH(1-39) in the production of aldosterone and corticosterone.

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