PURIFICATION AND CHARACTERIZATION OF THE N-TERMINAL PEPTIDES RELATED TO PORCINE PRO-OPIOMELANOCORTIN

by

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List of abreviations

ACTH	Adrenocorticotropic hormone
β-END	Beta-endorphin
CLIP	Corticotropin-like intermediate lobe peptide
CMC	Carboxymethylcellulose chromatography
CNBr	Cyanogen bromide
HMrpNT	High molecular weight porcine amino terminal
HPLC	High performance liquid chromatography
LPH	Lipolytic hormone or lipotropin
MB-LYS-C	Myxobacter Lysine-C protease
MSH	Melanocyte stimulating hormone
Mr	Molecular weight
pJP	Porcine Joining peptide
pNT	Porcine amino-terminal segment of POMC
POMC	Pro-opiomelanocortin
PTH	Phenylthiohydantoins.
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
V8	Staphylococcus aureus protease

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Abstract

Adrenocorticotropic hormone (ACTH) is synthesized in the pituitary gland as part of a larger precursor molecule. This precursor is characterized by the fact that it contains within its structure multiple chemical messengers with quite different biological activities. Among them are included the melanotropins (MSH), the beta-lipotropin (β-LPH) and the endorphins. To account for the major biological activities of its components, the name pro-opiomelanocortin (POMC) has been proposed for this common precursor. Characterization of this molecule in pituitary tissues including both the anterior and intermediate lobes as well as the mouse anterior pituitary tumour cell line AtT-20/D16v, established its apparent molecular weight (Mr) to be around 30 000 Daltons with the presence of carbohydrate side chains attached to a peptide backbone. This latter aspect is considered partly responsible for the heterogeneity observed in both the molecular weight and the charge of the molecule. Nucleotide sequencing of the cDNA coding for POMC has predicted the whole primary structure. ACTH and β-LPH together account for only half the size of the precursor, the other half being located on the amino-terminal side. This cryptic segment as ACTH and β-LPH contains an MSH-like sequence called γ-MSH. Pulse and pulse-chase experiments indicated that this cryptic segment of the POMC has an apparent molecular weight of 16 000 to 19 000 Daltons and that it is an important maturation product.

In order to completely characterize this cryptic segment we dissected porcine pituitary glands into both the anterior and neurointermediate lobes. Our objective was to determine the molecular form of the amino terminal segment of the porcine POMC (pNT) in the anterior lobe using a chemical approach. We extracted the peptides from the anterior lobes and chromatographed them on various systems including molecular sieving, ion-exchange and high performance liquid chromatography. Complete primary structure of the pNT revealed that the major form is a glycopeptide of 80 amino acid residues with at least two glycosylation sites. Two other peptides of 107 and 61 amino acid residues were isolated. We proposed that they are members of the genealogic tree of the pNT, the 107 residue being the whole cryptic segment preceding ACTH and the amidated 61 residue being a maturation product of the major secretory form. We also isolated a peptide which links the 80 residue glycopeptide to the ACTH in the POMC; it will be called the joining peptide (pJP).

Résumé

Il est maintenant bien établi que l'hormone adrénocorticotrope (ACTH) est biosynthétisée à partir d'un précurseur de haut poids moléculaire. Des approches chimiques et immunologiques ont permis d'établir que ce précurseur contient à l'intérieur de sa structure plusieurs peptides candidats à diverses fonctions endocriniennes. Parmi ces peptides, mentionnons outre l'ACTH, les mélanotropines (MSH), les lipotropines (LPH) et les endorphines. Le nom pro-opiomelanocortine (POMC) est employé pour souligner la diversité des actions biologiques des composants du précurseur commun. La structure primaire du POMC déduite à partir de la séquence de nucléotides d'un ADNc a permis d'établir que l'ACTH et la β-LPH ne représentent que la moitié du précurseur. Un segment cryptique situé à la partie amino-terminale de la molécule représente l'autre moitié. Des expériences de cinétique de marquage ont permis d'établir que ce segment cryptique représente un important produit de maturation du POMC dans le lobe intermédiaire du rat et dans une lignée tumorale de cellules adénohypophysaires de souris. Pour ce travail, nous avons utilisé des lobes antérieurs d'hypophyses de porc pour caractériser les peptides de la portion cryptique du POMC. A l'aide de techniques chromatographiques incluant les tamis moléculaires, les échangeurs d'ions, le chromatographe à haute performance en phase liquide et l'électrophorèse à une et deux dimensions, nous avons isolé et purifié quatre

peptides associés au segment amino-terminal du POMC. La structure primaire de chacun d'eux a été déterminée par séquence selon le procédé d'Edman. Le principal peptide isolé est composé de 80 résidus d'acides aminés avec au moins deux sites de glycosylation. De par sa localisation à la partie amino-terminale du POMC, nous l'avons nommé le "porcine N-terminal" (pNT). Deux autres peptides respectivement de 107 et 61 résidus ont été isolés, en quantité mineure, et seraient reliés généalogiquement au pNT. Enfin, un quatrième peptide de 25 résidus représentant le lien entre le pNT et l'ACTH a été isolé et séquencé, il sert à relier le pNT à l'ACTH et il a été dénommé "Joining peptide" (pJP).

INTRODUCTION

The pituitary hormones corticotropin (ACTH) and β-lipotropin (β-LPH) have been shown to be synthesized from a common precursor glycoprotein in mouse pituitary tumor cells (24, 152) and in rat pars intermedia cells (165), called pro-opiomelanocortin (159). The mRNA for this precursor has been isolated from bovine pituitary intermediate lobes and its cDNA copy has been cloned in Escherichia coli and sequenced (18). This study revealed the complete primary structure of the common precursor. It appears that the peptide consists of three repetitive units each containing a homologous melanotropin-like (MSH) heptapeptide. The -MSH and β-MSH are in ACTH and β-LPH respectively whereas γ-MSH is located in the cryptic portion of the POMC. Each repetitive unit, as well as the core heptapeptides, is flanked by pairs of basic amino acid residues which have been proposed to be cleavage sites for maturation enzyme(s) (12, 13). More recently, investigations on the genomic DNA structure of human (185), bovine (187) and rat (104) POMC were published confirming the findings of Nakanishi et al. (18). The glycopeptide nature of the POMC was demonstrated in mouse ATt-20/D16v cells by incorporation of radioactive glucosamine into the 31K common precursor (56, 60). The length of the signal peptide of the POMC was determined to be 26 amino acid residues (182). The deduced amino acid sequence of

the bovine POMC revealed that the non-ACTH, non-LPH segment accountsfor almost half the molecule.

Pulse and pulse-chase experiments in rat pars intermedia (176, 177) have shown that during the maturation of POMC the processing of the POMC goes further than in the anterior lobe and yields β-endorphin and -melanotropin instead of β-LPH and ACTH. Also in the intermediate lobe there is evidence that the same maturation process yields two additional end maturation peptides with apparent molecular weights of 17 000 and 19 000 Daltons (165), respectively. Such peptides are considered to be homologous with the 16 000 Dalton glycopeptide corresponding to the amino-terminal segment of the precursor molecule (149). These results suggest that the N-terminal portion of the POMC, or a very large fragment of it, would be a major maturation product. However the exact size has never been accurately determined and there are some discrepancies among the different reports (202, 204, 208, 210).

Preliminary characterization of this N-terminal fragment isolated from whole pituitary glands of several species including man (210) indicated that it could represent the segment from the N-terminal tryptophan, after removal of the signal peptide, up to almost the ACTH molecule. It was found that considerable sequence homology is maintained between species and in all of them an MSH-like structure is present (210).

The existence of new potentially active peptides in the structure of the POMC is the subject of much interest. In order

to obtain information about the nature of the peptides related to the N-terminal portion of the POMC, we dissected fresh porcine pituitary glands into the anterior and neurointermediate lobes, and characterized these molecules in the former tissue, hoping to clarify the controversies.

In this work we present the complete chemical characterization of three forms of the N-terminal fragment of the porcine POMC and of a peptide obtained after maturation of the N-terminal. It was also possible for us to identify two sites of glycosylation within the amino terminal one being an N-glycosylation type and the other an O-glycosylation. The three forms of the amino-terminal are composed of 107, 80 and 61 amino acid residues with respective apparent Mr of 21 000, 17 000 and 13 000 Daltons, as determined on SDS-PAGE. The latter is an amidated peptide and represents the smallest N-terminal we were able to isolate so far from the anterior pig pituitary. The 107 amino acid residue N-terminal probably represents the whole cryptic segment of the POMC up to the putative pair of basic residues sequence preceeding the ACTH sequence (18, 104, 185). Based on primary structure we proposed that this 107 residues molecule is processed into an 80 amino acid residues glycopeptide plus a 25 residues joining peptide with the removal of the pair of basic residues -LYS - ARG located between them. This 80 residues may then be processed to a 61 residues amidated glycopeptide.

REVIEW OF THE LITERATURE

Biosynthesis of proteins and polypeptides

Much of our knowledge of secretory proteins is based on their primary structure as determined by their amino-acid sequence. The chemical structure of the secreted protein is then deduced but we cannot tell if this protein is the initial translation product or if a precursor of the secreted form is initially synthesized.

Many proteins undergo a variety of specific post-translational modifications as they acquire biological activity within the living organism. Such modifications include the cleavage of peptide bonds to convert long-chain generally inactive precursors into shorter and sometimes active peptides (1, 2, 3, 4). Many other mofidications such as glycosylation, amidation, phosphorylation and sulfation may occur on a peptide chain before the secretion of the maturation product.

The maturation of peptide hormones is characterized by the fact that these hormones are processed within their cells of origin. The prefix "Pre" has been reserved for the whole primitive ribosomal translation product which includes the signal peptide originally proposed by Blobel and Dobberstein (5, 6). In the signal hypothesis (5, 6) it was proposed that the initial event leading in translocation was interaction of the signal sequence with a signal recognition protein (SRP) (7) present in the cytoplasm and may be attached to the ribosome (8). When

cytoplasmic SRP recognizes the signal sequence further translation is prevented. This block persists until the nascent chain-SRP-ribosomal complex reaches and binds to the RER. Only then can translation continue and transfer through the membrane commences. Binding to the RER membrane involves the interaction of the complex with an endoplasmic reticulum-specific SRP receptor, the docking protein (DP) (9). Then the block is removed, translation resumes and translocation commences. As a result of this interaction a functional ribosome-membrane junction is formed resulting in the formation of a pore through which the nascent peptide chain passes into the cisternal space. The cleavage of prepeptides has been postulated to occur as a cotranslational event as the nascent peptide chain enters the cisternae of the RER (10). Many indications accumulated so far including primary and tertiary structure of the signal and the absence of the signal in the ovalbumin argue against the possibility that highly specific converting enzymes exist for each presecretory protein (11).

Once the signal peptide has been removed, the remainder of the molecule which may undergo some modifications is designated as the pro-peptide. One remarkable fact emerges from the survey of the structures of some of the pro-peptides characterized so far. Most contain paired amino acid basic residues at the sites of cleavage (12) and it seems that arginine rather than lysine is preferred on the carboxyl side of the pair (13). This hypothesis is confirmed in the case of the pro-insulin (14), pro-gastrin

(15), pro-somatostatin (16), pro-glucagon (17), pro-opiomelanocortin (18), pro-enkephalin (19), pro-parathyroid hormone (20) and pro-albumin (21).

Some of the conversion of the pro-hormone to the active form of the molecule seems to occur into the cysternal space of the RER in its transport to the Golgi apparatus (22, 23, 24). The nature of processes involved in the pro-hormone conversion is far from fully resolved. During the biosynthesis of functional proteins stepwise modification of the primary translation products often occur such as proteolytic cleavage, glycosylation and hydroxylation which must be carried out by enzymatic systems located near the ribosome membrane junction (25-29). Other modifications such as removal of peptide segments, addition of peripheral sugars to the oligosaccharide core added contranslationally, phosphorylation or sulfatation can follow (30-35). Two such modifications are of particular interest for this work: covalent addition of oligosaccharide residues and their subsequent maturation (36) and the proteolytic cleavage of the polypeptide backbone of a protein (37).

The dolichol-bound oligosaccharide core is located in the endoplasmic reticulum (38). This initial oligosaccharide core is rich in mannose and undergoes one or more obligatory trimming reactions before further coupling of sugars unique to specific glycoproteins (39) is seen. Presumably these reactions occur later in the secretory pathway through the smooth endoplasmic

reticulum and Golgi fractions. Dunphy et al. detected alpha-1.2-mannosidase activity, an enzyme involved in oligosaccharide trimming, in Golgi-like membranes (40). A trypsin-like protease activity was found in the nascent granules in the Golgi apparatus (41). A chymotrypsin-like activity was also described in some granules (42). So within the mature granules a mixture of fully processed, partially-processed and eventually non-processed precursors exists. The mixture will be released into the extra-cellular fluid where, in some cases, maturation of the peptide may continue.

Chemistry of ACTH and related peptides

Adrenocorticotropic hormone (ACTH)

The pituitary gland contains a complex mixture of cell types synthesizing and secreting various peptide hormones. Adrenocorticotropic hormone (ACTH), a linear nonatriacontapeptide, carries vital biologic information from the anterior lobe of the pituitary to the adrenal cortex and the other parts of the mammalian body. This peptide, in all species studied, presents a highly conserved amino-terminal sequence with species differences in the carboxy-terminal (43-47). The amino terminal portion of the molecule contains the essential information for corticotropic effects while the carboxy terminal is responsible for its stabilization in the body (48, 49).

Molecular forms of ACTH

Evidence that ACTH is synthesized from a larger precursor has come indirectly from radioimmunoassays of gel filtration fractions. The first evidence was reported by Orth et al. (50) who showed that a cloned, functional murine pituitary adenocarcinoma cell line, AtT-20/D-1 secreted two biologically active ACTH molecular forms by gel exclusion chromatographic analysis. One of these eluted in the same fraction as highly purified human pituitary ACTH while the larger appeared to have a molecular weight (Mr) of about 7 800 Daltons. These two forms were immunologically indistinguishable from human ACTH in a radioimmunoassay employing an antibody directed against the ACTH 1-24 sequence (51). They were designated "Little" and "Intermediate" ACTH by Coslovsky et al. (52) who also described a "Big" ACTH that eluted in the void volume of the Sephadex G-50 column and, thus, appeared to have a Mr of around 20 000 Daltons or more. Both "Big" and "Intermediate" ACTH maintained their apparent Mr when rechromatographed in 8M urea. The "Intermediate" ACTH was found to be the major ACTH form in at least three species (mouse, rat and rabbit) in which corticosterone is the major glucocorticoid (53).

Using the D-16v clonal line of the AtT-20 tumor cells Herbert et al. (54-55) confirmed that the "Intermediate" ACTH is the predominant molecular form in tissue culture medium and that it is biologically active. They used a dual approach consisting of the classical immunological characterization of the secretory

products coupled with the translational studies of the corresponding mRNA in a heterologous system. In addition to molecular sieving, they performed a further separation with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and found three forms of ACTH, one 4,5-5,0 K (Kilodalton) unit which was biologically active and another two, 6,5-9,0 K and 20-30 K which were only mildly active. Subsequently they were able to find 4 forms having apparent Mr on SDS-PAGE of 4,5 K, 13 K, 23 K and 31 K respectively when the material studied was obtained following incorporation with radioactive amino acids. Using incorporation of tritiated glucosamine Eipper and Mains found that the larger molecules are glyco-proteins (56-60).

Alpha-melanocytes stimulating hormone (α-MSH)

This molecule, first isolated in 1955 (61), is composed of the first 13 residues of ACTH. The primary structure of the porcine molecule was determined by Harris and Lerner in 1957 (62). It was later found in other species to be chemically identical to the porcine molecule (63-65). The ACTH 1-39 molecule cleaves into α-MSH 1-13 and a corticotropin-like intermediate peptide or ACTH 18-39 that are primarily products of the intermediate pituitary (66). Further chemical characterisation of the α-MSH revealed that the amino-terminal of the molecule may be either mono, di-acetylated or non-acetylated (67). The acetylation of the serine portion affects the melanophore-stimulating activity or may have other functional significance (67).

Corticotropin-like intermediate lobe peptide (CLIP)

This peptide consisting of residues 18-39 of ACTH (68) was first isolated in 1974 by Scott et al. (69). Based only on primary structure analysis, it was suggested that both α-MSH and CLIP are maturation peptides of ACTH (66). Immunocytologic studies have demonstrated that most of the cells of the intermediate lobe stained strongly with antisera crossreacting with the 17-39 portion of the molecule but much less so with an anti-ACTH 1-24 antiserum (70). Physiological studies demonstrated marked discrepancies in bioactive versus immunoreactive ACTH content of the neurointermediate lobe with much lower bioactive content (71). From these studies it is suggested that the processing of the ACTH molecule in the anterior lobe differs from that in the intermediate lobe (66). It was proposed that in the intermediate lobe, ACTH was cleaved into CLIP and α-MSH while no such cleavage occured in the anterior lobe. In support of this last hypothesis, one can point out that α-MSH and CLIP are found only in very small amounts in the adult human pituitary which lacks an intermediate lobe (72). Pulse and pulse-chase experiments however have not proven inconclusively that CLIP is produced from such a cleavage, nor that it is formed as a separate entity and in large amounts in the intermediate lobe of the rat pituitary (73). Many chemical forms of CLIP have been isolated by high performance liquid chromatography (HPLC) by Bennett and coworkers (74). These forms

include post-translational modifications as phosphorylation and glycosylation of the molecule.

The human fetal pituitary, which has a pars intermedia, contains, in addition to ACTH, large concentrations of material resembling α-MSH and CLIP (75). The ratio of α-MSH and CLIP to ACTH seems to be related to the onset of parturition, for at this point, there is a dramatic decrease in the relative amounts of CLIP and α-MSH with a concomitant increase in ACTH. These authors associate this switch in peptide synthesis in the fetal pituitary at term with a functionally related metamorphosis of the human fetal adrenal gland, CLIP and/or α-MSH being the tropic hormone for the fetal zone of the adrenal cortex. Another potential biological activity for CLIP has been reported which involves the porcine molecule or synthetic ACTH 17-39, in the genesis of hyperinsulinaemia in obese mice (76).

Beta-lipotropin, gamma-lipotropin and β-melanotropin

Preliminary indications that the pituitary gland contains lipolytic substances were found in the early thirties (77). The complete chemical characterization of two of these factors was first done in ovine pituitaries by Li et al. in 1965 (78), Chrétien and Li in 1967 (79), and later reviewed by Graf and Li in 1973 (80). These molecules were called beta- and gamma-lipotropin. The former consists of 91 amino-acid residues of which

the first 58 residues comprise the sequence of gamma-lipotropin (79). Gamma-LPH contains at its COOH-terminal the complete structure of beta-melanotropin (β-MSH), a substance isolated from the pituitary in a number of species. The first primary structure of this molecule was published in 1956 (81-82). All non-human β-MSH isolated to date have 18 residues, which correspond to the sequence 41-58 of their homologous β-LPH's (81-87). Human β-MSH seems to have 22 amino-acid residues which corresponds to residues 37-58 of human β-LPH (88-89). It is however still difficult to determine whether this 22-residue peptide is a natural or a degraded product. Extraction procedures of human pituitary using diluted acetic acid generated β-MSH immunoreactivity with elution characteristics on gel filtration consistent with molecular forms the size of β-MSH and a concomitant decrease in LPH fractions (90-91). These authors thus concluded that human β-MSH found in earlier studies had been an artefact formed during extraction at pH values which aid not abolish enzymatic activity. This hypothesis was ruled out by Chrétien and Gilardeau (92) when they showed that β-LPH is not broken down chemically or enzymatically during the isolation process used for the isolation of β-MSH.

The complete primary structure of human β-LPH was published in 1972 (93) four years after its isolation (94). Some revision of its structure was done by Li and Chung in 1976 (95). Comparison of the structure of LPH revealed that the 35-residue amino-terminal shows greater heterogeneity among species than the carboxy-terminal. This observation indirectly suggests that the NH_2-terminal region may not carry a specific biological function.

Based on the primary structure of these molecules, Chrétien and Li proposed that the two smaller peptides γ-LPH and β-MSH are formed from β-LPH by a specific proteolytic cleavage (12). They proposed the theory that β-MSH could be derived from β-LPH by enzymatic cleavage with the initial production of γ-LPH as an intermediary. To strengthen this hypothesis, Chrétien's group started a series of *in vitro* labelling experiments in fresh whole bovine pituitary glands (96-99). These experiments showed the *de novo* biosynthesis of β-and γ-LPH but the authors were unable to isolate newly synthesized β-MSH. Chrétien stated that it might well be true that human β-MSH contains 18 residues and that the 22 amino-acid molecule is either an intermediate in the maturation process or else an artefact of purification (81). Incorporation studies in human fresh pituitary glands with complete chemical chracterisation of immuno-precipitable material with a specific antibody will be needed to solve this problem. Seidah *et al.* (101) have shown that rat β-MSH is different from other mammalian β-MSH because it lacks methionine^{7}. Some further characterisation carried by Robert *et al.* (54) has shown that Lys^{6}-Met^{7} sequence is replaced in the rat by Arg^{6}-Val^{7}. Still in the same species Seidah *et al.* (101) and Gianoulakis *et al.* (102-103) have demonstrated that the amino terminal of the rat γ-LPH is different from that of other species. Complete nucleotide sequencing of the rat genomic DNA coding for the common precursor of the ACTH/LPH carried out by Drouin and Goodman (104) have shown that the

predicted rat β-MSH sequence is similar to that of bovine β-MSH but it does not have the pair of basic residues that precedes the bovine sequence. These authors concluded that unless a different type of processing is involved in the rat, the only β-MSH sequence that could be produced would be the amino-terminal fragment of 38 residues (γ-LPH) and the β-LPH, as it was proposed in man by Bloomfield et al. (91). As related to the biologic properties of β-LPH, it has been known for a long time that the melanocyte stimulating and lipolytic activities of the parent molecule are due to the β-MSH region (residues 41, 58) of its structure (105, 106). Lipolytic activity was estimated by the release of free fatty acid from perirenal adipose tissue of rabbit, in vitro, exposed to different fragments of the β-LPH molecule (94). Among other biological activities of beta-and gamma-LPH there is: induction of hypocalcemia (107), hypercoagulability (108), glucose oxidation (109, 110) and stimulation of stretching and yawning (111). In most instances β-MSH was 50-100 times more active than either of the LPH's.

β-LPH precursor hypothesis

The isolation of γ-LPH by Chrétien and Li (79) provided a molecule possessing the structure of an intermediate product between two other known substances, β-LPH and β-MSH. At the time

they proposed that a larger mother molecule, in this case β-LPH, is synthesized and later enzymatically split into smaller fragments in the pituitary cells. This hypothesis was based on primary structure evidence. Concomitant with this observation, Steiner et al. working on the biosynthesis of insulin, another peptidic hormone, published that the active insulin was synthesized from a larger and relatively inactive precursor (112) they called pro-insulin. This latter molecule was completely sequenced in 1968 (114, 113). Comparison of the structure at the postulated sites of cleavage in β-LPH and in pro-insulin added strong support to Chrétien's working hypothesis, namely that a pair of basic amino acids is present at each side of the cleavage. Such strong leads directed Chrétien's group to concentrate their efforts on the biosynthetic processing of β-LPH in bovine pituitary and in rat pars intermedia cells.

Endorphins

The fact that morphine produces analgesia in animals by binding to a specific receptor led to the search for endogenous morphine-like substances in mammalian brain extracts. Criteria essential to the demonstration of specific binding to opiate receptors in neuronal membranes was proposed by Goldstein et al. in 1971 (114). Two years later Pert et al. (115) and Terenius et

al. (116) successfully demonstrated the presence of stereospecific opiate binding sites in brain tissue. Late in 1975, Hughes et al. (117) working on pig brain, isolated, characterized and synthesized two opiate-like pentapeptides which they named Met^5-enkephalin and Leu^5-enkephalin respectively. These peptides were active in the opiate receptor assay and caused inhibition of the contraction of the electrically stimulated longitudinal muscle of guinea pig ileum (118) and of the mouse vas deferens (119). Hughes et al. also noted that the structure of methionine-enkephalin is identical with residue sequence 61-65 of ovine β-Lipotropin (117, 78). In search of β-LPH from camel (Camaleus bactrianus) pituitaries, Li and Chung (120) reported the isolation, characterization and amino acid sequence of an untriakontapeptide which they named β-endorphin (β-END). Its structure corresponds to sequence 61-91 of β-LPH. This newly isolated peptide however was not tested in opiate assay before the structure of the enkephalins became known. β-END was then rapidly obtained from porcine (121, 122), ovine (123), bovine (124), rat (125) and human pituitaries (123, 126).

Guillemin's group have successfully isolated and completely characterized several endorphin-like substances from pig hypothalamus-neurohypophysis extracts. These molecules were designated alpha-endorphin, which are homologous with sequence 61-76 of β-LPH, and gamma-endorphin, homologous with sequence 61-77 of β-LPH (127-129). Smyth's group, searching for β-LPH maturation products,

isolated two substances, one corresponding to segment 61-87 of β-LPH and named C'-peptides (121, 130, 131). This C'-peptide was also isolated by Guillemin's group (129) and named delta-endorphin.

When the primary structure of all the opiate peptides was available, it soon became obvious that β-LPH could be the putative precursor of many types of molecules including the β-MSH's, γ-LPH, endorphins and enkephalins. Structure alone could not account to explain a maturation process. Sequence homology between a smaller peptide and its possible parent molecule is like a still picture from which it is difficult to extrapolate a movie of the maturation process. Other types of experiment are necessary to prove a causal-effect relationship in a biosynthetic pathway.

The ACTH/LPH common precursor

Before the discovery of methionine-enkephalin and the endorphins, interest in the LPH-related molecules centered on their precursor role since the lipolytic activity was doubtful (105-111) while the melanotropic activity has little biological importance. With the discovery of endorphins and their possible role in the pain control mechanism and drug addiction, much interest in β-LPH developed among many research groups. Careful immunohistochemical studies with specific antisera to various ACTH and LPH family of peptides indicated that scattered cells in the *pars distalis* and almost all the cells in the *pars intermedia* of

the pituitary contained both ACTH-related and LPH-related peptides (132, 133). Some other groups have confirmed that β-END is also located in these same cells (134, 136). At the electron microscopy level, immunochemical studies indicate that ACTH and LPH are found in the same secretory granules (132). Studies on the release of both ACTH and LPH related peptides have always indicated equimolar release of these peptides from normal pituitary, pituitary tumors and ectopic ACTH producing tumors (137-141). Given these observations it was tentative to conclude that the two peptides, ACTH and LPH must be synthetized as part of the same parent molecule. This hypothesis was presented by the group of Herbert in Oregon (142-144).

Biosynthesis of the ACTH/LPH precursor

Studies on the biosynthesis of ACTH were heavily influenced by prior work defining the biosynthetic pathways for insulin (145) and parathyroid hormone (23, 146). Preliminary studies suggested that some ACTH forms were substantially larger than the product peptide. By 1973 Scott et al. (66) suggested that processing of the ACTH molecule differed in the anterior and intermediate lobes. It was proposed that in the intermediate lobe ACTH was cleaved to CLIP and α-MSH while no such cleavage occured in the anterior lobe. Differential processing of β-LPH in the two lobes has also been suggested by studies in which β-MSH has been identified in the intermediate but not in anterior lobe (72). The ratio

of β-LPH to β-END is quite different in the two lobes, β-LPH being predominant in the anterior lobe whereas β-END is more important in the intermediate (86, 125, 147, 148).

Two approaches both using the mouse anterior pituitary tumour cell line AtT 20/D16v were used to confirm and expand the concept of the same precursor. High molecular weight forms of ACTH in these cells had been reported (51, 52). Eipper and Mains (57) using reducing agents and denaturants such as sodium dodecyl sulfate (SDS) and guanidine hydrochloride established that these high molecular weight forms of ACTH were not artifacts.

The experimental procedure of both models involved radioactive amino-acid incorporation followed by immunoprecipitation with antisera directed against some specific region of the putative precursor. Eipper and Mains (56, 60, 148, 149) and Mains and Eipper (24, 142, 150, 151) have shown by radioimmunoassay (RIA) using a middle ACTH antibody that four forms of ACTH-related material could be detected in AtT-20 cells extracts. Their apparent molecular weight on SDS-PAGE were 31 000 Daltons (31 K), 22 K, 13 K and 4,5 K - this last form having the same apparent molecular weight as $ACTH^{1-39}$. By incorporating tritiated glucosamine into the 31 K precursor they also established the glycoprotein nature of this molecule (56, 60). An 8-hours pulse with tritiated glucosamine followed by an immunoprecipitation and molecular weight determination showed that all three high molecular weight forms of ACTH were labeled with the radioactive carbohydrate. The 4,5 K

ACTH was not labeled. The three high molecular weight forms of ACTH were also labeled with radioactive mannose, fucose or galactose (149) and showed the same consistent results. The same conclusion holds true for normal mouse and rat pituitary cells (151). Further analysis based on the tryptic and chymotryptic peptides of 13 K ACTH labeled with a number of different amino acids and sugars, compared with both chymotryptic and tryptic peptides of 4,5 K ACTH, demonstrated that 13 K ACTH is simply a glycosylated form of 4,5 K ACTH or $ACTH^{1-39}$ and no extension beyond $ACTH^{1-39}$ could be found (60).

Roberts and Herbert (133) using an mRNA-dependent reticulocyte cell-free system under the direction of mRNA from AtT-20/D16v cells successfully isolated the initial gene product containing ACTH and LPH immunoreactivities of apparent molecular weight of 28 500 Daltons. A similarly sized ACTH-containing cell-free product was observed on translation directed by mRNA isolated from bovine anterior and neurointermediate lobes (153). Roberts and Herbert (144) immunoprecipitated their 28 500-Dalton precursor with both an anti 1-24 corticotropin and an anti-β-endorphin antibody. Tryptic analysis of these 28 500 K corticotropin and β-lipotropin molecules demonstrated the same lysine, methionine and tryptophan peptides. They concluded that the cell-free product contains the sequences of both ACTH and β-LPH and that there is only one copy of each sequence present. The relative position of the β-LPH and ACTH in the common precursor was determined by a

polysome runoff experiment in which the polysomes were incubated with labeled amino acids in the reticulocyte system in the presence of aurintricarboxylic acid at 0.1 mM to inhibit chain initiation (144). Results from this experiment suggest that β-LPH is located at the carboxyl terminal region of the precursor and that ACTH is located somewhere near the middle. All the non ACTH non LPH peptides appear to be located at the N-terminal to ACTH. The spacer peptide between the ACTH and the LPH could have a length from 0 to less than 20 amino acids. The difference in the molecular weight of the common precursor as determined by Roberts and Herbert and Eipper and Mains may be due to the glycosylation of the molecule in the latter group's experimental procedure.

This common precursor was first called 31 K precursor (54, 57), the ACTH/β-LPH precursor (18, 142, 143, 152-156), Propiocortin (157, 158) and Pro-opiomelanocatin (159) (POMC), names which account for the structural organization of the molecule.

Biosynthesis of ACTH and related peptides

Although structural studies indicated that the POMC could serve as a biosynthetic precursor for ACTH and related peptides, including β-LPH, endorphins and MSH's, kinetic studies were needed to demonstrate more directly the role of this precursor. Tager et al. (160) and Chrétien and Seidah (100) proposed a number of criteria needed to be met before the existence of a biosynthetic precursor can be established with certainty. Among these the

precursor-product relationship must be established by pulse-labeling and pulse-chase experiments, immunoprecipitation by the same antibody of the higher and lower molecular weights of the peptides, peptide mapping of the precursor in which the fragments of the precursor must be found in some of the lower molecular weight molecule and finally, and most important, the analysis of the putative precursor through amino acid or nucleotide sequencing. The fact that sets of molecules are related because of similarity in their primary structure or because immunostaining occurs on the same cells and even in the same secretory granules does not necessarily imply that they are synthesized as part of the same precursor (161, 162). Experiments to determine the maturation of ACTH and related peptides were done in the mouse pituitary tumor cells AtT-20/D16v (24, 56, 60, 148, 151) and in the rat pars intermedia (73, 163-166).

Processing of POMC in mouse AtT-20/D16v cells

When mRNA from AtT-20/D16v cells is translated in an mRNA dependant reticulocyte cell-free system the 28 500-Dalton precursor containing a single copy of each ACTH and β-LPH is synthesized (152). Roberts et al. used an approach which determined the order of proteolytic cleavage events and the order of addition of sugars to the carbohydrate side chain. In summary, the protocol consists of labeling the AtT-20/D16v with radioactive amino acid or sugar followed by an immunoprecipitation with antiserum specific to the

ACTH, β-endorphin or the non-ACTH, non-LPH region of the precursor, the immunoprecipitate was separated on SDS-PAGE and the proteins further characterized by peptide mapping.

With results from pulse labeling and pulse-chase studies considered together with peptide mapping of the ACTH and its related peptide Roberts et al. (167) were able to describe the initial events of the translation and detected three forms of the precursor with apparent Mr of 29 K, 32 K and 34 K all having a similar peptide backbone. They also showed that the smallest Mr translational form of the precursor, the 29 K is glycosylated. Taking into account that the cell-free translational product has a molecular weight of 28,5 K with the signal peptide the 29 K glycoprotein could represent the first translational product with excision of the signal peptide and addition of a sugar side chain. Evidence was insufficient to conclude that the 32 K and 34 K forms are generated from the 29 K form by the addition of one or two sugar side chains or by the addition of peripheral sugars to the pre-existing side chain in the 29 K. These authors also raised the hypothesis that three different precursor proteins are made each with a different amino acid sequence (168). Proteolytic processing of these glycoproteins leads to the formation of β-LPH and several different glycosylated ACTH intermediates. The ACTH intermediates are then processed to a glycosylated and unglycosylated form of $ACTH^{1-39}$ of 13 K and 4,5 K apparent Mr respectively and two forms of the amino terminal segment of the precursor(s)

with apparent Mr of 16 K and 18 K. In pituitary cell cultures β-LPH is then processed to β-endorphin. This processing scheme is valid for the muridae only since in other mammalian species such as bovine, porcine, ovine and human no glycosylated form of ACTH has yet been found (18, 169, 170).

When AtT-20 cells are incubated with radioactive sugars in presence of tunicamycin, an antibiotic that blocks the synthesis of the lipid-linked carbohydrate intermediate involved in the glycosylation process of asparagine residues (171-174), the precursor proteins have an apparent Mr of 25 to 26 K. This latter form does not contain glucosamine but has the same labeled tryptic peptides as the 29 K. The molecular weight difference between the 28,5 K cell-free product and the 25-26 K form made in tunicamycin treated cells is 2,5-3,0 K and could be an indication that the size of the signal peptide is around 25 amino-acid residues long.

Maturation of POMC into active hormones is achieved through proteolytic processing of the precursor which lead to the formation of β-LPH and several different glycosylated ACTH intermediates. These intermediates are then processed to 13 K and 4,5 K ACTH and to two or more N-terminal glycopeptides. β-LPH is further processed to β-END. There was no evidence that the Met-enkephalin is produced through processing of the β-END in the mouse pituitary tumor cells (55, 150, 167). Herbert et al. (168) have demonstrated that the conversion of the POMC into ACTH and β-LPH is not appreciably altered by blocking glycosylation of the

precursor although the processing appears slower in the tunicamycin treated cells. In the intermediate lobe of the frog, Xenopus leavis, the processing of POMC is altered by tunicamycin treatment (175).

Biosynthesis of β-END in mouse pituitary tumor cells AtT-20/D16v

Eipper and Mains demonstrated that ACTH is synthesized in the mouse pituitary tumor cells through a complex biosynthetic pathway beginning with the synthesis of a 31 K Mr precursor (24). Using the same experimental approach previously described they later found evidence that following a 15-minute pulse, cell extracts contain only the ACTH/END common precursor. Following a 30-minute chase, an 11,7 K endorphin molecule began to appear. A labeled β-END-like material appeared in the cell extract after a 60 and before a 240 minute chase (150). Using a concomitant immunoprecipitation with both anti-ACTH and anti-β-END, they showed that 23 K ACTH and 11,7 K β-END appears simultaneously and that following this first proteolytic cleavage step the ACTH and β-END regions are produced. Further characterization of the 11,7 K fragment indicates that the β-END like material is situated as expected at the COOH-terminal of the molecule and that the peptide derived from the amino terminal of the 11,7 K is shorter than β-LPH 1-65 peptide (60, 142, 148-150). As mentioned earlier the 4,5 K and 13 K represents the non-glycosylated and glycosylated forms of ACTH 1-39 (60, 149). When all the ACTH and β-END

containing molecules are removed from samples of culture medium by immunoprecipitation, a glycopeptide with an apparent Mr of 16 K on SDS-PAGE is found which should account for the non-ACTH region of the biosynthetic intermediate or the non-ACTH, non-β-LPH like region of the 31 K (149).

This peptide with a mass of 16 K plus the mass of the 4,5 K or 13 K fully accounts for the mass of the biosynthetic ACTH intermediate. Analysis of this 23 K with carboxypeptidase A and chymotrypsin or with cyanogen bromide indicated that its carboxy-terminal region is identical to ACTH 1-39, leaving the 16 K at the amino terminal of the 23 K (149). By analysing a minor cleavage product of the POMC, the 17 K segment, they determined that the β-LPH should be located at the COOH-terminal of the POMC and that no sizeable peptide extension separates the 23 K from the 11,7 K (149).

After all components of the precursor have been put together a working model for the processing of the POMC was proposed maintaining that at least two proteolytic cleavages are required to excise an ACTH-like peptide from POMC and that a β-END-like molecule could be released from the common precursor in a single step. However a β-LPH-like molecule appears to be an obligatory biosynthetic intermediate in the production of β-END in the AtT-20/D16v. From these experiments, they also observed that the 16 K fragment glycopeptide is secreted in large amounts by the tumor cell.

Biosynthesis of β-END in normal pituitary cells

Crine et al. (176, 177) were the first to show the active biosynthesis of β-END in the bovine pituitary. Because of the heterogeneity of the peptide families in the anterior pituitary, the intermediate lobe, where all the cells seem to be able to synthesize ACTH, was preferable for carrying out further characterization of the maturation process.

In a preliminary series of experiments (163) with a chase up to 120 minutes it was shown that β-END is released preferentially while β-LPH remains at a low concentration. In this paper the authors also observed that the 30 K precursor was degraded into several smaller peptides including β-END and β-LPH and a 18 K peptide tentatively identified as a large molecular form of ACTH. It was concluded in this paper that the 30 K gives rise to the 18 K and β-LPH, this latter being subsequently cleaved almost completely into β-END.

In the same tissue Crine et al. (73) were able to characterize two forms of the common precursor. These molecules were resolved by SDS-PAGE and their apparent molecular weight determined to be 34 K and 36 K respectively. When biosynthetic studies were performed in the presence of tunicamycin both the 34 K and 36 K forms were replaced by a single peptide with an apparent Mr of 32 K. Analysis of the 34 K and 36 K reveals that each contains within its sequence the antigenic determinants and tryptic cleavage fragments characteristic of both ACTH and β-LPH. It was there-

fore concluded that these two forms represent two glycoprotein variants of similar polypeptides differing in the number of asparagine-linked carbohydrate moieties. Chase incubations also released peptides with apparent Mr of 16 K to 20 K which were not further characterized. Two other major end products of the maturation process have been characterized. They have apparent Mr of 19 K and 17 K and correspond to the glycoprotein variants of the amino-terminal fragment of the precursor.

The lower molecular weight precursor and precursor synthesized in the presence of tunicamycin have a shorter half-live (73, 175) suggesting that the carbohydrate moieties may protect the precursor from proteolytic enzymes.

Futher characterisation of these two precursors by Crine and co-workers (165) showed that their forms are similar or identical polypeptides differing mainly in the number of linked carbohydrate side chains. However results from these experiments did not completely exclude the possibility that the 34 K form of the precursor lacks a polypeptide fragment present in the 36 K form. Intracellular processing of these two precursors is very similar giving the same non glycosylated end products, chemically characterized as being α-MSH, γ-LPH and β-END.

Glycosylated peptides which appear during the processing are the 27 K and 25 K, two "big corticotropins", produced respectively from the 36 K and 34 K. These "big corticotropins" give

two large glycopeptide with apparent Mr of 19 K and 17K which are stable even after a chase of up to 4 hours.

Structure of Pre-Pro-opiomelanocortin

The complete structure of the common precursor was tentatively determined using the protein characterization approach by Udenfriend and co-workers (178). From camel pituitaries, they isolated two proteins, one with an apparent Mr of 33 K and a minor component with an apparent Mr of 35 K.

Chemical characterization of the 33 K consisting of amino acid analysis and tryptic peptide mapping indicated that this peptide contains about 245 amino acid residues and contains the β-LPH 61-69 tryptic fragment.

Studying the incorporation of radiolabeled amino acids by rat pars intermedia cells, Gossard et al. (160) determined the identity of 19 of the first 30 amino-terminal residues of the precursor.

Another approach using mouse mRNA encoding for the precursor isolated from mouse AtT-20/D16v tumor cell line as a probe for in vitro incorporation studies allowed the isolation of a translational product from which 12 of the first 26 residues were identified (168).

The mRNA that encodes the precursor was also purified from the anterior and neurointermediate lobes of bovine pituitaries (153). In both tissues it directs the synthesis of a 31 000 Mr

product containing both the ACTH and the β-END sequence (153). From this mRNA a double-stranded c-DNA was synthesized and cloned in Escherichia coli (154). The nucleotide sequences coding for ACTH and partially for β-LPH reveal that on the c-DNA a 6-base-pair sequence coding for lysine and arginine links the carboxy-terminal of ACTH to the amino terminal of β-LPH (154). This experiment also indicated that β-LPH is located at the carboxy-terminal of the precursor. A few months later the same research group reported the complete nucleotide sequence of a cloned c-DNA for the bovine ACTH/β-LPH precursor (18).

From this sequence it was proposed that the bovine β-LPH should be 93 amino acid residues instead of 91, that there is no peptide extension beyond the carboxy-terminal of β-LPH, that the amino-terminal of ACTH, like the carboxy-terminal, is connected to the adjacent peptide by the pair of basic lysine-arginine residues and finally that a third MSH sequence is present in the cryptic segment of the precursor. Because of the primary structure homology between α-MSH, β-MSH and this new sequence, Nakaniski et al. (18) proposed to name this peptide γ-MSH. This latter putative peptide shares with the known MSH, tyrosine and methionine residues as well as the characteristic tetrapeptide sequence HIS-PHE-ARG-TRP. These results however did not determine the exact translational initiation site of this mRNA. Because the initiation of a protein must start with a methionine, the cryptic portion of the molecule could be 53, 110 or 131 amino acid

residues, including the signal peptide, based on the methionine position determined by the mRNA sequence. The authors decided to consider the methionine 110 as the most probable initiation site. This will give a precursor with a Mr of 29,259 Daltons including the signal peptide, which according to the literature should be around 20 amino acid residues (161, 180-182). The complete purification procedure of this mRNA was published later (183). It consists approximately of 1360 nucleotides with a Mr of around 450,000 and contains a polyadenylate sequence with an average length of 68 nucleotides.

Within the bovine pituitary, this messenger is located both in the anterior and the intermediate lobes and is essentially non-detectable in either the neural lobe or in the stalk (184). Results have demonstrated that the specific activity of this mRNA is 20-fold higher in the intermediate lobe than in the anterior giving a total activity 2-fold higher in the intermediate lobe (184).

Roberts et al. (54) isolated from mouse pituitary tumor cells, AtT-20/D16v, a messenger from which a cDNA fragment was synthesized. It is about 1200 bases, of which approximately 450 bases are not translated. The nucleotide sequence of this fragment corresponds to β-LPH 44-90. These data showed that the linkage between β-MSH and β-END is a lysine-arginine sequence.

Studies of the genomic DNA library for rat and human species have led to the complete characterization of the precursor

in these two species. The structural organization of the human genomic DNA encoding the POMC peptide was carried out by Cohen's group (185).

The predicted amino acid sequence for human POMC extends 92 residues before ACTH to the last amino acid of the β-LPH. This partial sequence has enabled identification of interspecies divergence and conservation within the POMC. The length of the peptide encoded by the POMC DNA segment extending from the 3' end of a possible intervening sequence to the poly-A is similar in the bovine and human species (18, 185).

The few base substitutions that do occur fail to alter dramatically the protein sequence. There was however some difference found between the reported human β-LPH (95) sequence and the predicted sequence from the nucleotide. Owerbach et al. (158) demonstrated that the POMC gene is located in the distal region of the short arm of the chromosome 2 in man.

The rat genomic DNA encoding POMC was sequenced by Drouin and Goodman (104). The rat POMC DNA has the same overall organization as the bovine cDNA (18). By comparing the rat sequence with those of the bovine and the human, they concluded that in the human and the rat, the cDNA starts encoding at the 19th codon of the precursor and there must be an intervening sequence between the amino acids 18 and 19. In these three species three domains of the POMC are highly conserved. They are the γ-MSH, the ACTH and the β-MSH-β-END domains.

Nucleotide sequencing definitely proved that in the rat ACTH an asparagine at position 29 included within the sequence ASN-GLU-SER is likely to be the site of glycosylation (148, 167). Two regions of POMC, those preceding and following ACTH, have diverged considerably between the rat and bovine sequences. In the first region, the rat sequence is 8 amino acids less than the bovine with no pair of basic amino acid that deliniates -MSH; in the second the rat γ-LPH is 22 amino acids shorter than its bovine homologue (186). As in the human, most of the coding region of the rat POMC has only one intervening sequence (104, 185).

To complete the knowledge about this precursor one needs to investigate the protein coding sequence in its gene. Nakanishi et al. (187, 188) isolated and characterized bovine genomic DNA fragments encoding this precursor protein and demonstrated that the protein sequence is encoded by two non-consecutive DNA segments. An intron of approximately 2,2 kilobase pairs separates an exon encoding mostly for the amino terminal 44 amino acids including the signal peptide from a second exon which contains the gene sequence for most of the protein structure including ACTH, β-LPH, the amino-terminal segment of the POMC and the poly-A tail. The larger exon is homologous in the bovine (187, 188) rat (104) and human (185) species. Structural organization of the bovine precursor gene revealed by nucleotide sequencing of the whole m-RNA-coding segments established that this precursor gene is approximately 7300 base pairs long and codes for the mature

mRNA of 1098 nucleotides (188, 189). The different domains of this mRNA coding sequence are distributed as follows: two large introns divided the sequence into three exons. Exon 1 encodes for all but the distal 20 nucleotides of the 5' untranslated sequence and is separated from exon 2 by intron A of around 4000 base pairs; exon 2 is separated from exon 3 by intron B of approximately 2200 base pairs. Most of the precursor protein is encoded by exon 3 (188).

Studies in a human ectopic ACTH producing thymic carcinoid tumor led to the identification of an mRNA coding for the POMC with around 1100 nucleotides length which directs the synthesis of a 38,000 Mr peptide that was reactive both with antibody to ACTH and with antibody to β-END (156). Miller et al. (190) have reported that the POMC encoded by the mRNA from a pancreatic carcinoma is indistinguishable from that synthesized in a pituitary adenoma in organ culture.

Presence of a signal peptide

The amino acid sequence predicted from the nucleotide sequence of the cDNA coding for the bovine intermediate pituitary precursor molecule (18) also indicates that a signal peptide - a hypothesis proposed by Blobel (5) - is present. Even though the complete structure of the precursor is known, it is impossible to establish the exact length of the signal peptide from the predicted amino-acids sequence alone. This pre-sequence, an

extension of 15 to 30 amino acids that is removed during synthesis of the secretory protein in the cell has a characteristic arrangement of hydrophobic and hydrophylic amino acids (191).

The bovine intermediate pituitary, the mouse tumor and the rat pars intermedia precursors are similar enough to predict the signal peptidase cleavage site. In the rat an automatic Edman degradation of the two forms of the common precursor gave a partial amino acid sequence of the N-terminal segment of the molecule (179, 192). Comparison with the predicted bovine pars intermedia precursor indicates the presence of a signal peptide of 26 amino acids (179, 192). In the mouse AtT-20/D16v cell line, comparison of the automatic Edman degradation of the RNA directed cell-free form of the precursor radiolabeled with various amino acids with the amino acid sequence of the 29 K obtained via pulse and pulse-chase experiments led to the conclusion that there exists a pre-sequence of at least 25 residues (168). Based on this affirmation the bovine signal peptide fits the usual pattern of a hydrophilic amino-terminal region, a hydrophobic middle region and a hydrophilic carboxy-terminal region (193-194). As in the case of the parathyroid hormone (195) the signal peptide is cleaved very rapidly after or even during translation in the rat pars intermedia where it is released within minutes (73, 163, 179).

The amino-terminal segment of POMC

As it is clearly established that ACTH and β-LPH account for only part of the mass of the precursor, a sizeable peptide should be present ranging from amino acid residue number 1, after excision of the signal, to the ACTH. Such a peptide, comprising the non-ACTH non-β-LPH portion of POMC, has been previously identified in mouse pituitary tumor cells and in culture medium (148-149) and its nature as a glycosylated peptide has been established. Crine et al. (144) have also identified an 18 000 Mr peptide generated after a 20-minute chase in rat pars intermedia cells. Although this peptide was not completely characterized it was considered to be a high molecular weight form of ACTH because of its cross reactivity with anti-ACTH antibody. Eipper and Mains raised an antibody against the cryptic portion of the POMC by injecting crude mouse tumor cell secretory products from which all the ACTH and endorphin related material was removed by immunoprecipitation (148-149). Material immunoprecipitable with this antibody during pulse experiments has an apparent Mr of 16 K and has been called the 16 K fragment (151). Its real molecular weight as determined by gel filtration in the presence of guanidine hydrochloride is between 10 000 and 12 000 Daltons (149). Basal secretion of the 16 K fragment-related material is equivalent to secretion of molecular forms of ACTH in mouse AtT-20/D16v tumour cells (150).

In the early 1970s Hakanson et al. (196-198) reported that the pituitary corticotropic cells contain large amounts of at least three different polypeptides having tryptophan in the amino-terminal position with an apparent Mr of 14 000 Daltons. These peptides are stored in the same secretory granules as ACTH (198) and are released with some ACTH related peptides such as β-LPH (199).

Processing of the amino terminal segment of POMC

Pulse-chase experiments using both rat and mouse pituitary cells showed the gradual maturation of the common precursor to proceed via the initial cleavage of the carboxy-terminal β-LPH followed by the release of ACTH, leaving an amino-terminal extension which does not seem to undergo further maturation (102, 142, 144, 163). Further characterization in the rat intermediate lobe has shown that during maturation of the POMC, the N-terminal portion of the molecule is released as two glycosylated peptides with apparent Mr of 17 000 and 19 000 Daltons (73, 165, 179, 200). Gossard et al. (179) reported sequence heterogeneity at position 1 of the rat POMC glycopeptide where both tryptophan and arginine were found (179). Results from this last experiment also favor the hypothesis of one peptide backbone or at least a common amino-terminal region for both previously isolated precursors. Chrétien and Seidah proposed that the presence of two different amino acids at position 1 suggests that the rat pituitary might have two genes encoding for the POMC (201). Crine et al. (200)

proposed that the 17 K and 19 K are the N-terminal segments of the 34 and 36 K forms, respectively, of the precursor and that they represent end products of the maturation process in the rat intermediate lobe. These peptides are considered to be homologous with the 16 K peptide of Eipper and Mains (151). The heterogeneity in their selective masses is thought to be due to differential glycosylation of very similar peptides and to the poor resolution by polyacrylamide gel electrophoresis for determination of molecular mass (73, 177, 200). Pulse experiments followed by a 2-hour chase demonstrated the metabolic stability of these N-terminals and showed that these molecules and not the γ-MSH heptapeptide (200) are the true biologically active species released by the rat intermediate lobe.

Preliminary results of the structure of the N-terminal

Even though Nakanishi et al. predicted the complete primary structure of bovine POMC (18), data that compare whole N-terminals between different species are lacking. In the human (185), rat (104) and mouse (168) species, genomic work predicted the primary structure of the N-terminal from the 19th amino acid. Automatic Edman degradation of radioactive peptides obtained from mRNA directed synthesis in mouse AtT-20/D16v (168) and from rat intermediate lobe labeled peptides (179) allowed a comparison of these peptides with the predicted bovine sequence (18). Sequence data showed that good homology between the three structures begins at

position 27 i.e. the first residue after removal of the signal peptide (179). Some of the difference between the bovine and the rat N-terminals are non-conservative such as substitution of phenylalanine for glutamic acid at residue 3 and tyrosine for glutamic acid at residue 14 (179). Nevertheless there is a great homology between the three species studied so far.

Preparation of milligram amounts of 16 K related material was performed by Keutmann et al. (202). They found that this 16 K fragment when chromatographed on G-75 Sephadex in 6 M guanidine HCl was resolved into two proteins with molecular weights of 12 500 and 10 200 Daltons. Each protein has an apparent Mr of 19,8 K and 17 K on SDS-PAGE. Based on analysis of tryptic fragments obtained from the separated 17 K and 19,8 K, they concluded that these peptides are indistinguishable. The cause for size and charge heterogeneity may be explained by differences in carbohydrate side chains and/or micro-heterogeneity within the peptide backbone (202). A preparation of a 16 K fragment was labeled with tritiated iodoacetic acid and the peptide submitted to automatic Edman degradation for 29 cycles. Counts were released at cycles 2, 8, 20, 24 as predicted by the bovine cDNA sequence (18). In this same experiment tumor cells were incubated with tritiated tryptophan and the labeled 16 K peptide submitted to sequencing. Results clearly indicated the presence of tryptophan at cycle 1 only for the 19 cycles performed.

Further characterization of the non-ACTH, non-LPH segment of

the POMC was performed by Hakanson et al. (203) from fresh pig whole pituitaries. This group had already isolated and purified a family of peptides having the common feature of a tryptophan at the amino-terminal (186-189). Automatic Edman degradation of one of these peptides with an 11 000 Dalton apparent Mr peptide as determined by gel filtration, which had not been recognized by antisera either to ACTH 1-24 or to α-MSH, gave the sequence for 33 out of the 35 first amino acid residues of the molecule (203). The amino acid composition of this peptide established that it is made up of 103 residues and clearly demonstrated the presence of glucosamine and galactosamine. Sequence homology between the predicted sequence of bovine cDNA and that of this peptide showed it to correspond almost exactly to the amino-terminal of the bovine POMC. The differences may be ascribed to the fact that two animal species are in consideration. The apparent Mr of this 103 residue peptide on SDS-PAGE is a little over 15 000 Daltons.

Preliminary characterization of the human homologous peptide was carried by Chrétien's group (204). From whole pituitaries they isolated a peptide with an apparent Mr of 18 K. Automated microsequencing of this peptide labeled with ^{14}C-iodoacetamide allowed the identification of cysteine residues at cycles 2, 8, 20 and 24. These are exactly the cysteine positions that would be expected for the bovine POMC (18). The total amino acid composition of the purified peptide gives a total of 103 amino acid residues (204). As in the case of the porcine N-terminal, the human

N-terminal does not cross-react with antibody to ACTH 1-24. Amino acid composition of tryptic and chymotryptic fragments of the human N-terminal, cross-reactivity with the bovine synthetic γ-MSH raised antibody and co-elution of some tryptic fragments and synthetic γ-MSH on HPLC indicated that this 18 K Mr peptide contains within its structure the γ-MSH (203). A more advanced characterization of this peptide was performed by Seidah et al. (205). Direct sequencing of the first 40 residues showed only 2 substitutions with the bovine homologue and about 95% sequence homology (4 variations) within the first 70 residues between the two species. The majority of species differences occurs within the C-terminal 24 amino acid residues. The authors also concluded that the γ-MSH sequence is maintained, and proposed the asparagine 65 as a probable site for glycosylation (205). At the same time Guillemin et al. (206) isolated from bovine pituitaries, three peptides which cross-reacted with γ-MSH antibody with apparent Mr of 13 000, 8 800 and 4 500 Daltons. They proposed that the native γ-MSH-like peptides contain carbohydrate which can be linked to the asparagine at site 65 and and/or at the serines 67, 68, 69, 70 (206).

Amino acid sequence of a crude preparation of 16 K from mouse AtT-20/D16v cells allowed the assignment of 27 of the first 29 residues as well as threonine 32 and alanine 34 (207). The presence of methionine at site 27 as proposed by Herbert et al. (168) was not confirmed (207).

Purification and characterization of the peptide N-terminal isolated from the anterior lobe of porcine pituitaries (208) estimates an amino acid composition of 103 residues, with the presence of glucosamine and galactosamine. Based on the tryptic digest of this peptide, two glycosylation sites were proposed, one at threonine 45 and a second at asparagine 65 (208). The complete sequence of the γ-MSH is also present within the molecule.

Complete primary sequence of the N-terminal segment of POMC

Complete chemical characterization of the amino-terminal segment of POMC was achieved in the human and porcine species. In the human pituitaries the major secretory peptide of the cryptic portion of the POMC consists of 76 amino acid residues (209). Within this peptide there are two sites of glycosylation, an O-glycosylation at threonine 45 and an N-glycosylation at asparagine 65 (210). There are also two disulfide bridges, one between cysteine residues 2 and 8 and another between cysteine residues 20 and 24 (210). These works clearly demonstrated the presence of γ-MSH sequence within the human N-terminal (209-210).

In the porcine species the N-terminal is a slightly longer glycopeptide with 80 amino acids and, again, 2 sites of glycosylation (208, 211). In this species the γ-MSH sequence is also present within the N-terminal.

Presence of a joining peptide between the N-terminal and ACTH within POMC

Based on the nucleotide sequence of the human genomic DNA encoding for POMC (185) and on primary structures obtained from Edman degradation of the N-terminal (210-211), it was deduced that the segment ranging from tryptophan residue number one to the expected glycine located before the LYS-ARG dibasic residues and preceding ACTH should be 109 amino acid residues. The isolation of a 76 residue glycopeptide which according to the predicted gDNA sequence is followed by a LYS-ARG sequence in the POMC molecule indicates the presence of a peptide which should be 31 residues long after removal of the dibasic residues preceding and following it (12, 13). Such a peptide was isolated from human pituitaries (212). Because of its location within the whole cryptic fragment of the POMC, it was named the "Joining peptide" (212). An homologous peptide was isolated from porcine anterior pituitaries (213). It consists of 25 amino acid residues and its identity is based on sequence homology between bovine (18), human (195, 209, 210, 212) and murine species (104).

Characterization of multiple forms of the porcine anterior pituitary POMC N-terminal glycopeptide

Chromatography on a molecular sieve column of the porcine N-terminal glycopeptide resulted in the isolation of three forms of the molecule with respective apparent Mr of 21 K, 17,5 K and

13,5 K on SDS-PAGE (214). Determination of the amino acid composition of each peptide revealed that the 17,5 K corresponds to the PNT 1-80 (211). The forms with Mr of 21 K and 13,5 K correspond respectively to longer and shorter forms of the N-terminal glycopeptide. The high molecular weight form contains 107 amino acid residues. Sequencing of the fragments obtained after cleavage of the 21 K with Myxobacter Lys-C protease indicated that an extension of 27 amino acid residues is linked to the 17,5 K through a LYS-ARG sequence. The 13,5 K corresponds to the first 61 residues of the 17,5 K. Pronase digestion of the peptide and dansylation of the digest revealed the presence of phenylalanine amide at position 61 (214).

Biological activity of the cryptic segment of POMC

Now that it has been clearly established that the ACTH related family of peptides is biologically processed from a common precursor and that many of these peptides have a specialized biological activity, it is of interest to consider a possible function for the amino-terminal segment of POMC. Sequence homology of the N-terminal between species (18, 104, 155, 210, 211) and conservation of the γ-MSH sequence (18, 104, 155, 204, 210, 211) could indicate a possible biological function as a chemical messenger within mammalian species studied so far.

Preliminary reports show a correlation between the occurence of immunoreactive calcitonin and immunoreactive ACTH in

hormone producing tumors (215, 216). Immuno-histochemical staining has shown the co-existence of these two hormones in the same cells of the anterior and intermediate pituitary (217, 218). These observations suggest that the sequence of calcitonin is part of the common precursor to ACTH and β-END (142, 149, 150). Weber et al. (219) demonstrated that neither the 16 K fragment nor its tryptic fragments contain antigenic determinants against calcitonin-antiserum and they concluded that calcitonin is not contained within POMC.

It has been proposed that the forms of ACTH secreted by the pituitary determines the type of glucocorticoid produced by the adrenal glands (220). To verify this hypothesis Gasson (221) incubated cortical adrenal cells in the presence of high molecular weight ACTH and $ACTH^{1-39}$. All high Mr ACTH, including 31 K, 23 K and 13,5 K are capable of stimulating the same maximal level of steroïdogenesis as human $ACTH^{1-39}$. Nevertheless the 31 K and 23 K forms are respectively 300 and 100 fold less potent than native ACTH. Glycosylated ACTH is equipotent with non-glycosylated ACTH. All of the different forms of ACTH stimulate the synthesis of corticosterone and related steroids without significant production of cortisol or aldosterone. This author also observed that the 16 K did not stimulate or interfere with steroïdogenesis. Other in vitro observations on fetal sheep adrenal cells show that high Mr forms of ACTH block the action of $ACTH^{1-39}$ (222, 223).

Guillemin's group (224) chemically synthesized four possible γ-MSH peptides derived from the proposed bovine nucleotide

sequence (18) named acetylated-γ_1-MSH, γ_1-MSH, γ_2-MSH and γ_3-MSH. Bioactivity of these peptides determined in an *in vitro* MSH assay was very weak compared to that of the γ-MSH. None of these peptides was able to release anterior pituitary glycoprotein, FSH, LH, TSH or GH and PRL at a dose as high as 100 ng. An antibody was raised against γ_3-MSH (225) which allowed the identification of three immunoreactive γ-MSH peptides in bovine whole pituitary extract with apparent molecular weights of 15 K, 8 K and 4,5 K. The presence of γ-MSH in plasma was not reported by these authors.

Pedersen and Brownie (226-227) used 16 K isolated from mouse AtT-20/D16v and 16 K tryptic fragment and observed that tryptic fragments, more than the native peptide, potentiate the action of ACTH 1-24 which results in an increase in serum corticosterone concentration. Using purified porcine N-terminal 1-80 glycopeptide from Chrétien's group, Lis (228) demonstrated the release of aldosterone by a human adrenal adenoma *in vitro*. The human homologue was evaluated under the same experimental conditions by Chrétien *et al*. and showed to be equipotent with porcine N-terminal and ACTH in stimulating the release of aldosterone from a human aldosteronoma *in vitro* (229). The possibility that primary hyperaldosteronism could be related to a pituitary adrenal dysfunction was raised following these observations (230).

The human N-terminal$_{1-76}$ glycopeptide which is inactive in maintaining adrenal weight and possesses weak melanotropic activity (231) has recently been found to potentiate the ACTH-induced

secretion of glucocorticoids and mineralocorticoids from the adrenal cortex by increasing RNA synthesis (232, 233). Estivariz et al. (234) described the situation where the N-terminal region of the POMC and ACTH, provides a variety of peptides capable of affecting different aspects of the adrenal cortex. Human N-terminal$_{1-28}$, hN-terminal$_{2-59}$ and ACTH stimulate DNA synthesis, hN-terminal$_{1-76}$ potentiates the action of ACTH by increasing RNA synthesis (232-233), hN-terminal$_{51-76}$ activates cholesterol ester hydrolase (227) required for the initiation of corticosteroidogenesis. Lowry et al. (235) reported that neurally mediated proteolytic cleavage of the circulating inactive precursor (hN-terminal$_{1-76}$) at the adrenal gland is the major mechanism of control of compensatory growth.

As mentioned earlier, POMC related peptides are synthesized both from the anterior and intermediate lobe give rise to a variety of corticotropin and melanotropin-related peptides (236) but the N-terminal unlike the rest of the POMC is only partially processed, at least in the rat (166). Bromocriptine and dexamethasone were used to respectively inhibit secretion from the pars intermedia and pars distalis to determine the source of peptides involved in compensatory adrenal growth after unilateral adrenolectomy (235). Results obtained in this experiment suggests that the N-terminal and ACTH related peptides of the pars intermedia are mitogenic in the adrenal whereas that the pars distalis corticotrope is the primary source of peptides involved in adrenal

hypertrophy and hyperplasia (235). These authors suggest that adrenal hypertrophy and adrenal hyperplasia are mediated by neural activation of proteolytic activity, which cleaves the N-terminal$_{1-74}$ to give rise locally to N-terminal$_{1-48\ 49}$, an adrenal mitogenic hormone, and N-terminal$_{50-74}$, an adrenal hypertrophic hormone. The whole N-terminal$_{1-74}$ is considered as being an adrenal growth factor (235).

Measurement of N-terminal of POMC in plasma

A radioimmunoassay developed against mouse 16 K proved suitable to detect this immunoreactivity in human plasma and was used to demonstrate that a peptide similar to 16 K varied in parallel with plasma ACTH (237). Similar results were subsequently obtained by Hope et al. (238) using a radioimmunoassay directed toward purified human N-terminal. Other groups reported that IR-γ_3-MSH, using a radioimmunoassay developed against chemically synthesized γ_3-MSH was present in human plasma but neither its exact nature nor the possible cross-reactivity of the human N-terminal was adequately assessed (239-241). Bertagna et al. (237) using three different radioimmunoassays directed toward γ_3-MSH, hN-terminal, and m16K, showed that immunoreactive γ_3-MSH is indeed identical to hN-terminal in pituitary-derived materials. Chrétien et al. (242) using a sensitive and specific homologous radioimmunoassay for hN-terminal demonstrated a strong correlation between IR-hN-terminal and IR-ACTH in plasma of normal subjects

during insulin-induced hypoglycemia as well as in plasma of patients with various disorders of pituitary-adrenal axis. Their results also indicates that hN-terminal$_{1-76}$ is the predominant circulating form in human plasma (242). Mean basal immunoreactive hN-terminal in normal subjects was less than or equal to 100 pg/mL (242).

MATERIAL AND METHODS

Porcine pituitaries

Fresh porcine pituitary glands were obtained from a local abattoir during the months of June, July and August. The animals consisting of females and castrated males in equal ratio were between 5 and 6 months old and weighed around 100 kilos. They were killed by electric shock or narcosis with carbon dioxide, followed by exsanguination through the anterior vena cava. Within 30 minutes after death, the animal was decapitated and the head was cut either sagitally (fig. 1A) or frontally (fig. 1B) to expose a large part of the brain and adjacent structures. The encephalon was removed leaving the pituitary in the sella turcica on the floor of the skull. The whole pituitary was then easily removed with a small dissection spatula. The pituitaries were immediately dissected into the anterior and neurointermediate lobes (fig. 2) and frozen either on dry ice or in liquid nitrogen when available. This material was kept at -60°C until extraction of the proteins, which usually took place within one week after collection.

Histologic examination of tissues

Some freshly dissected anterior and neurointermediate lobes were immediately fixed in 10% formaldehyde for 72 hours. The tissue was then embedded in wax, cut in 6 μ slices and stained with hematoxyline.

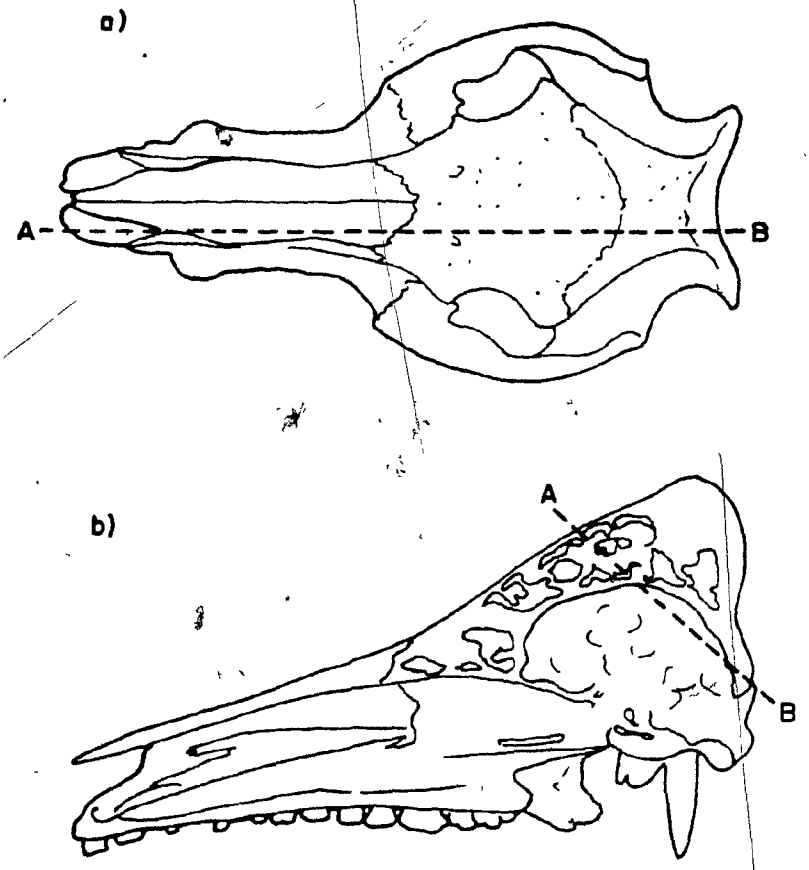
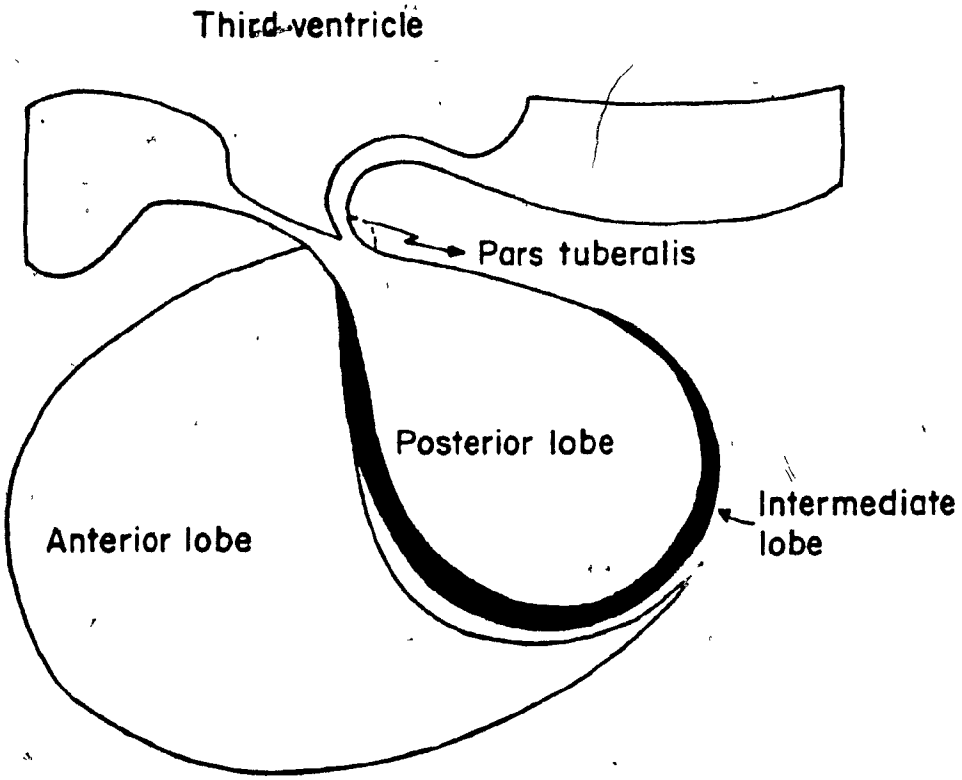


Figure 1. Schematic representation of the section of the pig's head used to expose the brain and adjacent structures including the hypothalamic-pituitary complex.



Schematic Representation of the Pig Pituitary.

Figure 2. Schematic representation of the pig pituitary.

Protein extraction procedure

The preliminary steps of this technique were mainly performed according to the procedure developed by C.H. Li and co-workers for the purification of ACTH and β-LPH (49). The anterior lobe was extracted in 0,2 M HCl at 4°C in the ratio of 7 mL acid to one gram of frozen tissue. The mixture was homogenized with a "polytron" for ten to fifteen minutes, until it took on the appearance of a rich pink cream. No enzymatic inhibitor was used. The homogenate was then diluted in technical grade acetone containing 25 mL HCl/L at 4°C in the ratio of 3 mL acetone by mL of homogenate. The mixture was kept stirring for one hour, then centrifuged for 30 minutes at 10,000 rpm. The supernatant was then poured into five times its volume of -20° C technical grade acetone. The proteins were left to precipitate for 16 hours at 4°C without any stirring. The proteins settled to the bottom of the jar, and after aspiration of the supernatant, were solubilized in water. The pH of the solution was adjusted to 3,0 with 5,0 N sodium hydroxide. Then a first precipitation in 6% sodium chloride solution was performed. The reaction was left to continue for 12 to 16 hours at 4°C and the mixture was centrifuged for 30 minutes at 8 000 rpm. The precipitate, called the "Prolactin Fraction", was separated from the supernatant and then saturated with crystaline NaCl at the concentration of 360 g NaCl/liter of the supernatant. This reaction was also left to continue for 12 to 16 hours at 4°C.

The mixture was then centrifuged for 30 minutes at 8 000 rpm, the supernatant was discarded and the precipitate, which contains many of the ACTH related peptides, was desalted by ultrafiltration through a 500 Mr cutoff dialysis membrane. The retained material in the ultrafiltration cell will be designated throughout this work as "fraction D".

Carboxymethylcellulose chromatography

The material already defined as "fraction D" was chromatographed on a cation exchange resin, carboxymethylcellulose CM-23 from Whatman Scientific. The resin was equilibrated in 0,01 M ammonium acetate pH 4,6 and filled in a chromatographic column of 16 cm x 100 cm. All chromatography was performed at 4°C. We usually poured 10 mg of "fraction D" per milliliter of resin. The proteins were dissolved in the starting buffer to a concentration of 25 mg/mL. The column was eluted with ammonium acetate in a gradient of salt from 0,01 to 0,2 M and a gradient of pH from 4,6 to 6,7. The mixing flask filled with the starting buffer was equal to ten times the volume of the resin.

The first step of the chromatography consisted of elution of the proteins with ammonium acetate 0,01 M, pH 4,6, for 2 times the bed volume. The unretained material was identified as "Peak A". The elution gradient was then started and consisted of ammonium acetate 0,1 M, pH 6,7 for six times the volume of the resin. In the third stage the elution gradient was finally increased by

adding ammonium acetate 0,2 M, pH 6,7 to the mixing flask, for six times the bed volume. Finally the resin was washed in 1 M ammonium acetate pH 6,7. The volume of the collected tube was equal to 0,075 times the bed volume. Absorbance was usually read at 280 nm. Fractions corresponding to elution peaks were collected and lyophilized three to four times.

Molecular sieving

Most of our molecular weight separation procedure was performed on a Sephadex G-75 superfine dextran gel from Pharmacia. The column was 160 cm long by 1,25 cm diameter filled with 180 mL resin swollen in 1 M acetic acid. The calibration kit from Pharmacia suitable as standards for molecular weight determinations of globular proteins by gel filtration contain the following markers: Dextran 2000, Ribonuclease A (Mr 13 700), chymotrypsinogen A (Mr 25 000), Ovalbumin (Mr 43 000), Albumin (Mr 67 000) and finally radioactive iodine as a salt marker for determination of the total volume of the filtration bed. The eluent buffer was 1 M acetic acid and the volume of the fractions was 0,5% the volume of the filtration bed. The absorbance of each tube was read at a wavelength 280 nm. The experiments were performed either at room temperature or at 4°C.

High performance liquid chromatography

Apparatus

High performance liquid chromatography (HPLC) was used for determination of phenylthiohydantoins (PTH) and for purification procedures of many peptides throughout this work.

We had at our disposal two systems, one from Waters Scientific and the other from Beckman. The Waters model 204 liquid chromatograph included: two model 6000A pumps, a Model U6K injector, a Waters intelligent sample processor (WISP), a Waters 720 system controller, a Model 450 variable-wavelength detector and a Model 730 data module. The Beckman system consisted of two Model 100A Preparative pumps, a Model 155-40 variable-wavelength detector, a Model 421 CRT controller, a Model 210 sample injector and a Waters Model 730 data module. Two types of columns were used, one for PTH determination, an Altex 5 µ ultrasphere ODS column kept at 45°C in a water jacket, and one for peptide purification, a Waters µ-Bondapak C18 (30 cm x 4 mm) analytical column run at room temperature.

HPLC solvents for peptide purification

Triethylamine phosphate buffer (TEAP) was obtained by bringing the pH of 0,2 N phosphoric acid to 3,0 with triethylamine (Pierce). The aqueous buffer consisted of 0,02 M TEAP pH 3,0 obtained by diluting the stock solution ten times. The organic

phases consisted of 90% acetonitrile or 90% 2-propanol (Burdick and Jackson Labs) and 10% 0,2 N TEAP pH 3. The water used in these preparations was first distilled, run through a Millipore system, redistilled, passed through an ion-exchange resin and then stored at 4°C in a glass container. In our results the gradient will always be indicated as the percentage of the organic phase.

Reduction and alkylation of peptides

In order to open disulfide bridges and to detect the presence of S-carboxymethylated cysteine on the amino acid analyser protein reduction and alkylation had to be performed. The reduction was performed in 10 mL of 0,1 M trismabase pH 8,2 to which we added 5 µL of β-Mercaptoethanol plus 4,8 g of ultrapure urea. The solution was flushed under nitrogen for 5 minutes. Then 600 µL of this solution was added to 100 µg of peptide to be reduced. The reaction was left to occur for at least 4 hours, in the dark, at room temperature. The alkylation was performed by reaction of the reduced material with iodoacetic acid (Kokak) in the ratio of 67:1 (W/W) with the peptide or with C^{14} iodoacetamide (New England Nuclear) in the ratio of 200 µL/100 µg of peptide. After 2 to 4 hours of reaction the mixture was desalted in Sephadex G-15 using 1 M acetic acid as the eluent buffer. The peptide (hot or cold) was lyophilized and kept under vacuum.

Enzymatic and chemical cleavages

In order to achieve the complete sequence of the newly isolated high molecular weight peptides, we had to perform cleavage of the molecule. Many enzymes were used including trypsin (Miles laboratories), Myxobacter Lys-C protease (Mannheim-Boehringer), and V8 (kindly provided by Dr. G. Drapeau). The chemical cleavage involved a cyanogen bromide reaction (Kodak laboratories). Almost all the digestions wereperformed on a reduced and alkylated peptide.

Tryptic digest and mapping using HPLC

Tryptic digest was performed at 37°C in ammonium bicarbonate 0,1 M buffer, pH 8,0. We used DCC-treated trypsin in a final weight to weight ratio of enzyme to substrate of 1:200. The reaction was performed in a reaction vial, containing 1 mL of the buffer. First we added half the required dose of the enzyme and allowed the reaction to proceed for 2 hours at 37°C in the dark. Then we added the other half of the enzyme and again left the reaction to occur for another 12 hours. After that period of time the content of the reaction vial was injected in the HPLC without any lyophilization and dissolution in the starting buffer for the HPLC run.

The peptide mixture was analyzed by HPLC using 90% double-distilled water and 10% 0,2 N TEAP pH3 as the inorganic phase and 90% 2-propanol (or acetonitrile) and 10% 0,2 N TEAP pH3 as the organic phase. Chromatography was performed on a Waters u-Bondapak C18 (30 cm x 4 mm) analytical column, at room temperature.

Absorbance was read at a wavelength 210 nanometers. The chromatographic program consisted of elution of the column at a flow rate of 1 mL/min. The percentage of the organic solvent was kept at 0% for ten minutes then raised to 50% within 160 minutes. Each peak was collected, repurified under the same conditions and submitted to acid hydrolysis in 5,6 N HCl at 110°C for 24, 48 and 72 hours.

Myxobacter Lys-C protease cleavage and mapping using HPLC

Myxobacter Lys-C protease was added in a ratio of substrate to enzyme of 50 to 1 (W/W). The digestion was carried out at 40°C in 1 mL of 20 mM TRIS HCl, pH 8,8 for 6 hours. The resulting peptides were purified using HPLC with the same solvents and column as described in the previous section for tryptic digest. The following elution program was used: flow rate of 1 mL/min and 5% organic solvent for 5 minutes then raised to 65% within 60 minutes. Absorbance was read at a wavelength 235 nanometers. Each peak was collected, repurified, hydrolysed and submitted to amino acid analysis.

Cyanogen bromide cleavage and mapping using HPLC

Peptides to be cleaved were reacted with cyanogen bromide (CNBr) in 1 mL of 70% formic acid and a molar ratio of 1000:1 (CNBr:peptide). The reaction proceeded in the dark at room temperature for 18 hours. The fragments obtained were purified using HPLC with the same solvents as described for tryptic mapping. The

program consisted of a 1 mL/min flow rate and the percentage of 2-propanol was maintained at 5% for 3 minutes then raised to 95% within 90 minutes. Absorbance was read at a wavelength of 235 nm. Each peak was collected, repurified under the same conditions, lyophilized, hydrolyzed and submitted to amino acid analysis.

V8 cleavage and mapping using HPLC

Digestion with protease from Staphylococcus aureus was performed in 1 mL 0,05 M ammonium acetate buffer, pH adjusted to 4,0 with 1 M acetic acid. The reaction was left to take place at 37°C for 18 hours in a ratio of enzyme to substrate (W/W) of 1:50. The purification of the fragments obtained using HPLC was done as for the tryptic fragments.

Digestion with pronase

An aliquot (100 µg) of the peptide to be digested was dissolved in 400 µL of 0,1 M ammonium acetate pH 7,5 containing 10 mM $CaCl_2$. Pronase (Calbiochem-Behring Corp.) was added in a ratio of enzyme:substrate of 1:10 and the digestion performed at 37°C for 48 hours. The digest was dried by evaporation.

Radioimmunoassay

Iodination procedure

Peptides were radioiodinated by the chloramine "T" method (243). Radioactive iodine was purchased from New England Nuclear

or from Union Carbide. Usually 10 µg of peptide was iodinated with 1 mCi of 125-iodine in 0,5 M $Na_2 HPO_4 \cdot 7H_2$ buffer in the presence of 10 µg of chloramine T (Kodak Laboratories) for 30 seconds. The reaction was stopped with 100 µg of sodium metabisulfite. Separation of iodinated peptide from 125-iodine was performed on G-25 Sephadex (Pharmacia) in a disposable 10 mL plastic pipette eluted with 0,5 M $Na_2 HPO_4 .7H_2$, pH 7,6. The column and the resin were coated with 20 mg of bovine serum albumine diluted in the elution buffer. The fractions collected were counted using a Beckman gamma counter. Radioiodinated peptides were kept at 4°C in 0,5 M $Na_2 HPO_4 \cdot 7H_2$, pH 7,6.

Competitive binding assay

Antibody buffer and non radioactive antigen were mixed and radioactive antigen added after a slight delay, giving the unlabelled antigen an opportunity to bind to the antibody first. The buffer consisted of 0,5 M $Na_2 HPO_4 \ 7H_2$, pH 7,6 in the presence of 15% NaCl 1 M, 25% EDTA 0,1 M and 1% 1 M sodium azide. Bovine serum albumin was added to reach a concentration of 1 g/100 mL buffer. All assays were performed at 4°C with an incubation time of 72 hours. Separation of antibody-bound and free antigen was also performed at 4°C by successive addition of bovine γ-globulins and 30% poly-ethylene-glycol. Each tube was vigourously shaken, left to rest for one hour, then centrifuged in an Eppendorf for 15 minutes at 10 000 rpm. The supernatant was aspirated and the precipitate counted using a Beckman gamma counter.

Immunization procedure for antibody production

Native peptidic antigens were used for the production of specific antibodies. Highly purified peptides, both used for amino acid analysis and sequence determination were injected intradermally in New Zealand white rabbits, weighing around 2-3 kilos. All injections were done on the back of the animal, we did not use toe pad injections because they caused discomfort to the animal. The initial injection was with antigen in complete Freund's adjuvant (Difco Laboratories); 400 µg of antigen were injected per rabbit. The antigen and the Freund's complete or incomplete adjuvant were mixed directly in the syringe with a sonicator, this procedure gave excellent homogenisation without any major loss of the antigen.

The intradermal injections were done with a 25 gauge needle. Booster injections contained incomplete Freund's adjuvant and were done at 2-week intervals. Each rabbit received with the three booster injections the following quantities of antigen: 200 µg, 120 µg, 100 µg. Before the experiment and before every antigen injection 1 mL of blood was removed from the rabbit's ear. The blood was left to coagulate, then centrifuged to separate the clot from the serum. This latter was aspirated and kept at -60°C till titer determination. When antibody titer was better than 1: 10,000, booster injection was given every 8 weeks with 40 mL bleedings taken every two months. Bleeding was done through cardiac puncture with a 20 mL syringe and a 22 gauge, 2-inch long needle. Serum was kept in 1-mL fractions at -180°C in liquid

nitrogen. The serum was used as such without any antibody purification for all radioimmunoassay. Titer was determined for each bleeding and specificity determined when titer was judged satisfactory.

Antibody specificity

Antibodies raised against the amino-terminal segment of the POMC were characterized to determine their specificity. Antigens used were from the ACTH related peptides and included ACTH 1-24, γ-MSH, β-MSH, β-endorphin, β-LPH, α-MSH, CLIP, PNT 1-80, HNT 1-76. Iodinated PNT 1-80 was used as a tracer.

Amino acid and hexosamine analysis

For amino acid analysis the native peptide was first reduced and carboxymethylated. Triplicate analysis of the 5,7 N HCl hydrolysates (for 24, 48 and 72 hours hydrolysis at 105°C) was performed with an updated Beckman 120 C amino acid analyser modified to allow up to 0,5 nmol detection sensitivity, equiped with a model 126 computing integrator. The separation of the amino acids was done on a W3 resin (Beckman) which allows the separation of all amino acids and the hexosamines, glucosamine (GlcN) and galactosamine (GalN). Determination of these sugars was not corrected for hydrolysis loss at 24 hours. Determination of tryptophan residues were done by sequencing because they are destroyed upon 5,7 N HCl hydrolysis. Cysteine residues were determined as

S-carboxymethylcysteine after reduction with mercaptoethanol and alkylation with iodoacetic acid.

Sequence determination

The sequence determination was performed in a Beckman 890B sequanator equiped with a cold trap and a Sequamat P6 auto converter. Sequence was performed using 0,3 M Quadrol pH 9,0 as sequences buffer (Beckman) and adding 3 mg polybrene (Pierce) plus 100 nmoles of a dipeptide Leu-Val to the cup and performing 7 dummy cycles. The peptide to be sequenced was then added and double coupling was performed for the first cycle only. The program used is a Beckman's 0,1 M Quadrol with S1 + S2 wash (12.11.78).

All conversion was done automatically immediately following the cleavage step using 1,5 HCl/MeOH at 65°C. The phenylthiohydantoin amino acids (PTH) were separated by HPLC following the procedure published by Somack C.J. (58). Separation was performed on an Altex 5 u ultrasphere ODS column (0,46 x 25 cm) run at 45°C, using a 18 mM sodium acetate/tetrahydrofuran (THF) pH 4,73 as the aqueous phase and acetonitrile as the organic phase. This technique allowed the separation of all PTH amino acids (fig. 3) as previously reported except that PTH-Glu, PTH-Asp and PTH-S-carboxymethyl cysteine were detected as their respective methylesters and PTH-Nor-leucine was used as an internal standard. For all runs the detector was set at a wavelength of 269 nanometers.

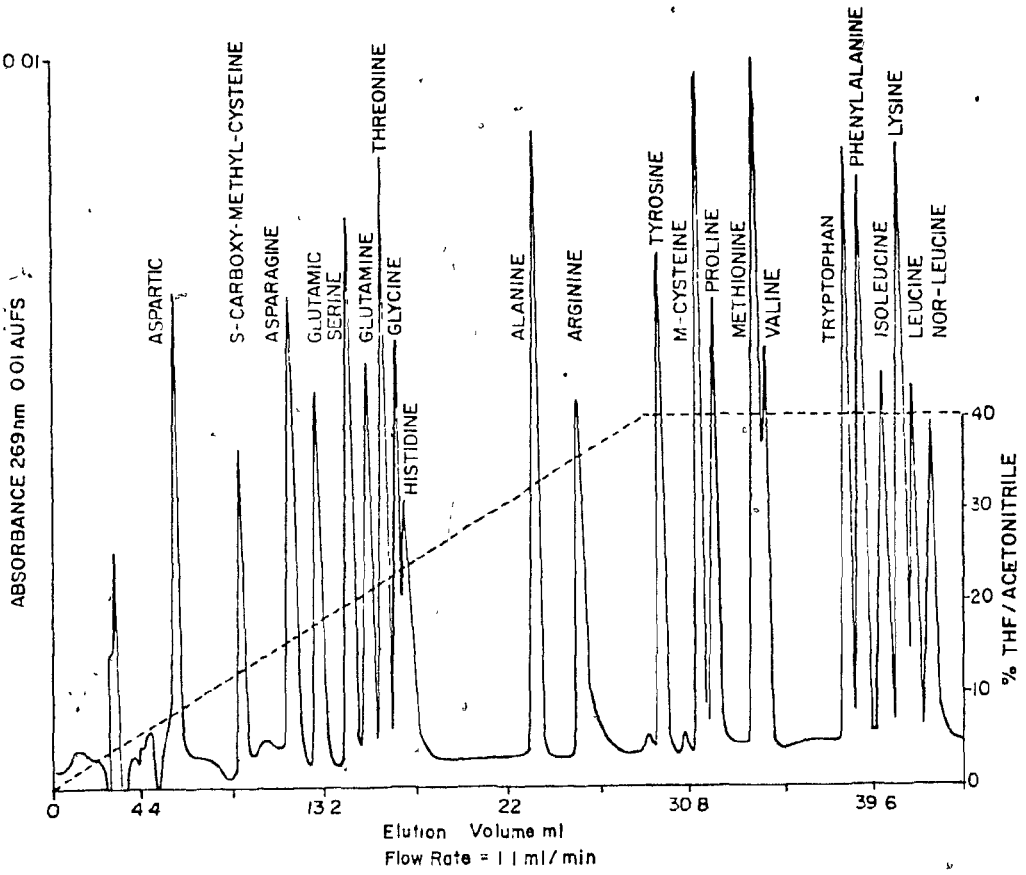


Figure 3. Phenylthiohydantoins separation on an Altex 5 μ ultrasphere ODS column (0,46 x 25 cm) run at 45°C. Aqueous phase consists of 18 mM sodium acetate-tetrahydrofurane pH 4,73. The organic phase consists of acetonitrile.

Assistance by the members of the laboratory staff

A great deal of collaboration was needed to dissect the some 30 000 pig pituitaries used in this work. This task was performed mostly by undergraduate students during their summer vacation. This group also performed most of the extraction of both the anterior and neurointermediate lobe. These two steps were done under my supervision. I personally made arrangements with the slaughter house management, established the procedure for dissection of the hypothalamic pituitary complex, and acquired competence in the extraction procedure to guide these students in their work.

I performed all the chromatographic procedures including gel filtration, cation exchange and high performance liquid chromatography of pituitary peptides. I also performed all the cleavage of peptides and the purification of the peptides which will be described, and their use as antigen for rabbit immunization. Purification of the antibodies produced and their characterization are also part of my work.

Hydrolysis of peptides for amino acid analysis and calibration of the analyser was done by the staff of the laboratory. I personally analyzed all data resulting from these experiments.

Sequence determination was performed under the guidance of Dr. Nabil Seidah by the personnel of the laboratory. I was nevertheless always a member of the team needed to perform such experiments.

To characterize the multiple forms of the peptides to be described later, we engaged in very close collaboration with Dr. Guy Boileau who confirmed my working hypothesis that heterogeneity within the molecules is caused by differend lengths of the peptide backbone.

All the people who specifically collaborated in the realisation of this work are duly mentioned in the acknowledgement.

RESULTS

Histologic examination of tissues

Photomicrographs of pig pars distalis (fig. 4) revealed that within one hour after death the tissue is in excellent cyto-architectural condition and there is no evidence of cell lysis. The coloration allowed the identification of major anterior pituitary cell lines, by light microscopy and distinguished the acidophils, basophils and chromophobes. Sections indicated that the pars distalis, hand dissected, is an homogenous tissue not contaminated by cells of either the pars intermedia or the pars tuberalis. These two components of the pituitary remain attached to the pars nervosa as revealed by examination of the sections of the neuro-intermediate lobe (fig. 5). Architectural organization of cells and staining characteristics allowed identification of these four types of tissues: pars distalis, pars nervosa, pars intermedia and pars tuberalis. So the pars distalis is dissected as is; the pars nervosa with the pars intermedia are grouped into a tissue we consider to be the neuro-intermediate lobe.

Purification and characterization of the Porcine N-Terminal 1-80 glycopeptide

Carboxymethylcellulose chromatography

Carboxymethylcellulose chromatography, CMC, a cation exchange resin chromatography, was used as a first separation procedure for the multiple proteins and peptides present in the "fraction D" of porcine pars distalis. To locate the amino-

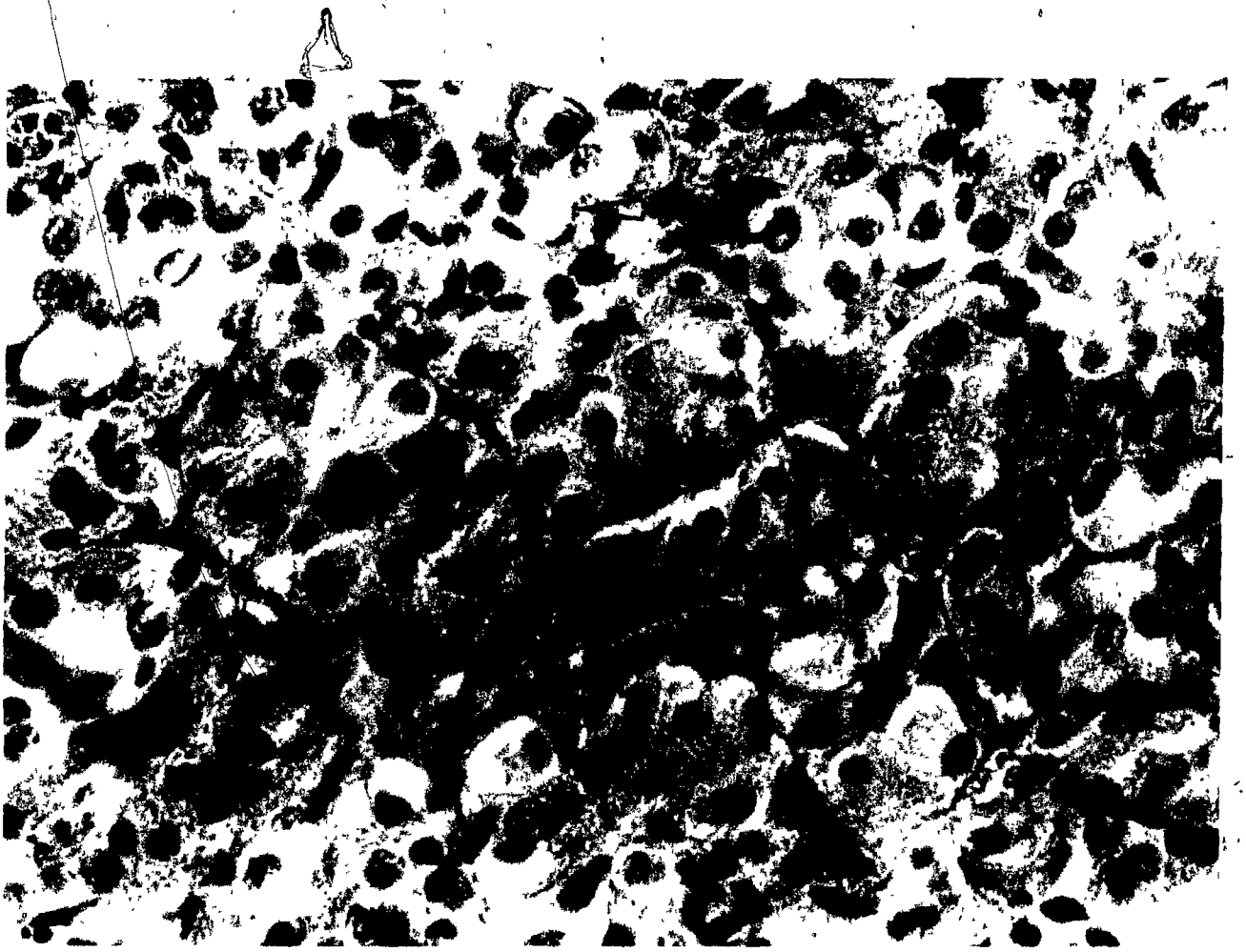


Figure 4. Photomicrograph of pig pars distalis.
C= Chromophobe cell

Figure 5. Photomicrograph of pig posterior lobe.

IL: Pars intermedia

PI: Processus infundibuli

terminal segment of POMC in the eluent on this type of chromatography, we performed a standard elution using a CMC column on which we poured 20 000 CPM of radio-active rat 16 K fragment obtained from pulse-chase experiments in rat pars intermedia (Kindly furnished by Dr. Francis Gossard). Almost 90% of the material was not retained by the resin and so was eluted by 0,01 M ammonium acetate pH 4,6 in a fraction already identified as "peak A".

This preliminary indication oriented us in preparing large amounts of "peak A". Although variations exist between CMC chromatographies of different "fraction D", one can always identify homologous peaks. As can be seen in figure 6, where a typical chromatographic profile on CMC of porcine pars distalis "fraction D" is presented, the flow through material is always important and is easily identifiable. In fact this material represents between 10 and 40 percent of the material poured on the column. After repetition of this chromatographic procedure on 4 batches of "fraction D", 4,89 g of "peak A" was prepared. This material was ultrafiltered through a 10 000 molecular weight cutoff dialysis membrane. The material retained in the dialysis cell was lyophilized and kept in a vacuum jar until further purification procedure.

Identification of the amino-terminal of POMC in flow through material of CMC

In our search for the amino terminal segment of POMC we used the method of labeling cysteine residues with ^{14}C-iodoacetamide followed by automatic Edman degradation in order to follow

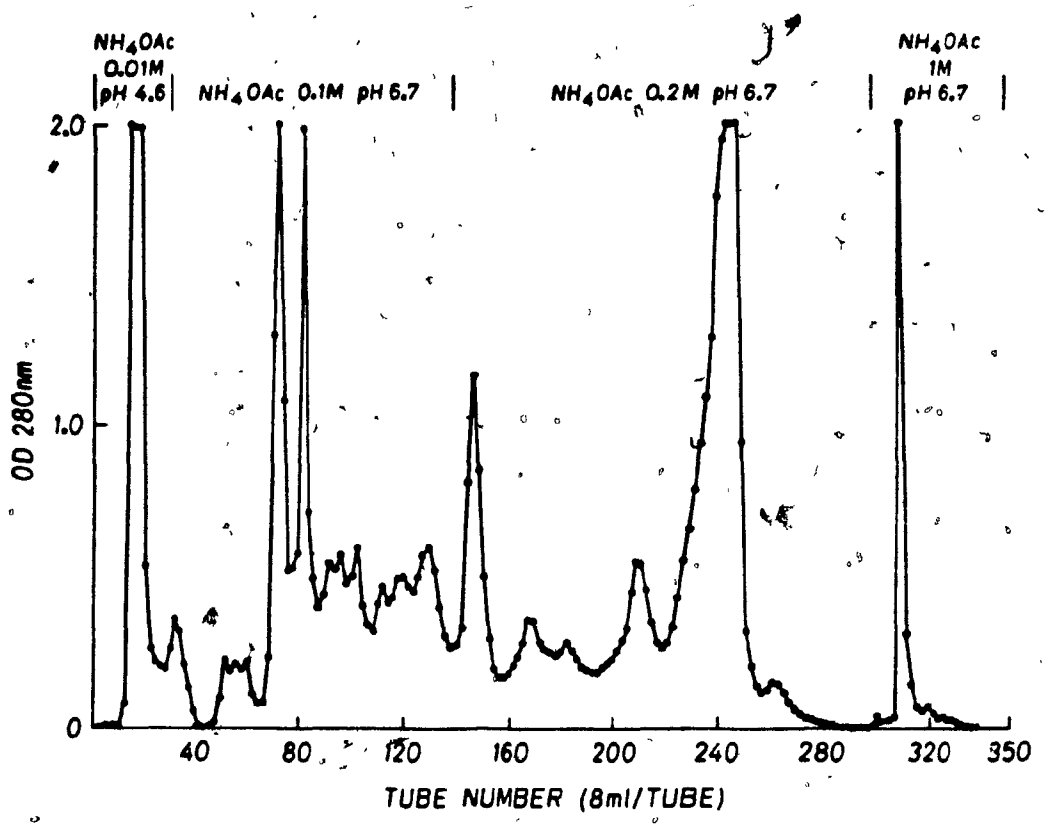


Figure 6. Carboxymethylcellulose chromatography of porcine pars distalis "fraction D". The total bed volume is 100 mL.

the purification procedure. From the amino acid sequence of the bovine POMC (18) it can be deduced that the amino-terminal segment of the porcine POMC should contain cysteine residues at positions 2, 8, 20, 24 of the peptide chain. Peptides present in "peak A" were labeled with ^{14}C-iodoacetamide after reduction with β-mercaptoethanol. The labeled material was desalted on a G-15 Sephadex column and eluted with 1 M acetic acid. The elution was performed at room temperature, the column being completely covered with aluminium foil. A sample of each fraction collected was added to a liquid scintillation medium and counted in a beta liquid scintillation counter for 1 minute. Results of this experiment are presented in figure 7. Microsequencing of ^{14}C-iodoacetamide labeled porcine material from "peak A" was performed. Thiazolinones from each sequanator cycle were counted directly. The results of the first 30 cycles of this sequence are presented in figure 8. These results clearly indicate that peptide(s) with cysteine residues at cycle 2, 8, 20, 24 is/are present and only slightly contaminated at least with peptides containing cysteine residues in their first 30 cycles. In order to study these peptides, several approaches, including high performance liquid chromatography (HPLC), gel permeation, and electrophoresis have been attempted.

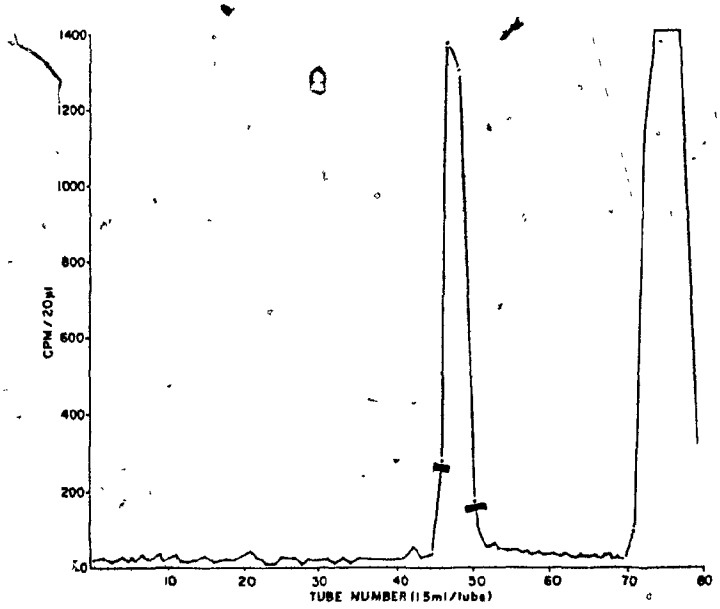


Figure 7. Elution profile of (^{14}C) iodoacetamide labeled porcine material from flow through material of CMC chromatography, on Sephadex G-25 fine column (160 X 1.25 cm) eluted with 2 M acetic acid at 22 C.

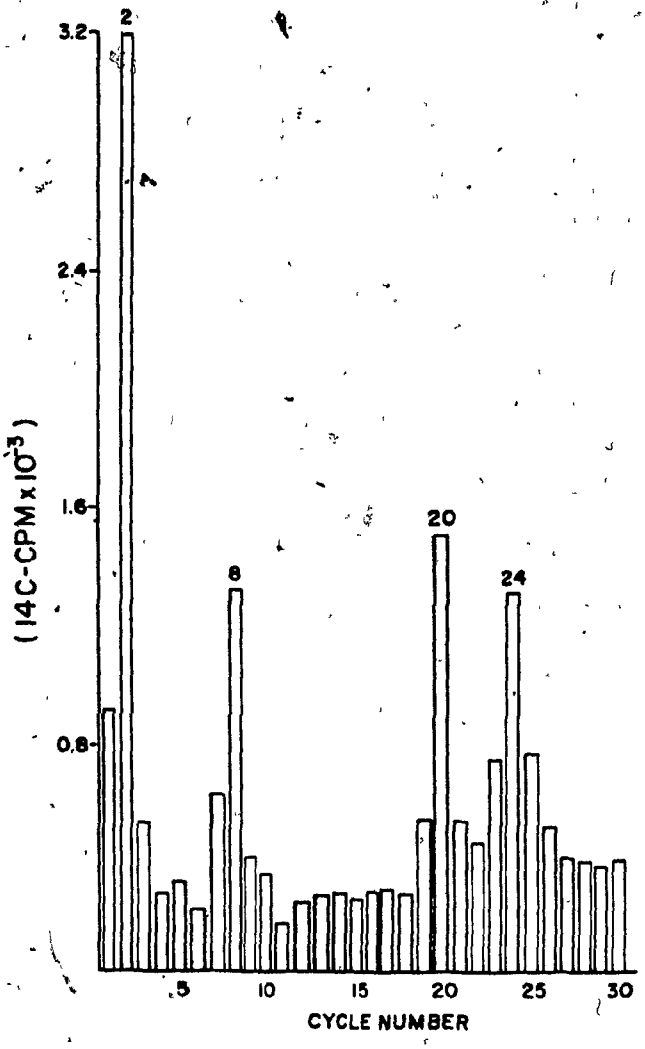


Figure 8. PTH amino acid yield of (^{14}C) labeled porcine material in CPM X 10^{-3} as a function of the sequanator cycle number.

Purification of peptides from "peak A" by HPLC

Crude peptides, obtained after ultrafiltration of "peak A", with molecular weights higher than 10 000 Daltons, were resolved by HPLC on a semi-preparative column (C18 column Waters Scientific). The TEAP system was used with 2-propanol as the organic phase. A chromatographic profile is presented in figure 9. Of the fifteen peaks collected and repurified under the same conditions peaks 5, 6, 6a, 7, and 8 incorporated ^{14}C-iodoacetamide. Microsequencing of each labeled peak was performed. Results indicated that peak 7 shows a great enhancement of a peptide with ^{14}C labeled cysteine residues at cycles 2, 8, 20, 24. This peptide was repurified by HPLC on an analytical column with the same solvents but with a slower gradient of the organic phase. We represent in figure 10 the final purification on HPLC of a peptide we considered to be potentially the amino terminal segment of the porcine POMC. The material collected was lyophilized, desalted on a G-15 Sephadex column, eluted with 1 M acetic acid, lyophilized a second and a third time and kept as a powder in a vacuum jar.

Gel permeation chromatography

The material from peak 7 (fig. 9) on a preliminary run on HPLC was chromatographed on an analytical column (fig. 10) and then separated by gel permeation chromatography. This chromatography was performed on a 180 mL (160 x 1,25 cm) column packed with G-75 Sephadex superfine from Pharmacia, eluted with 2 M

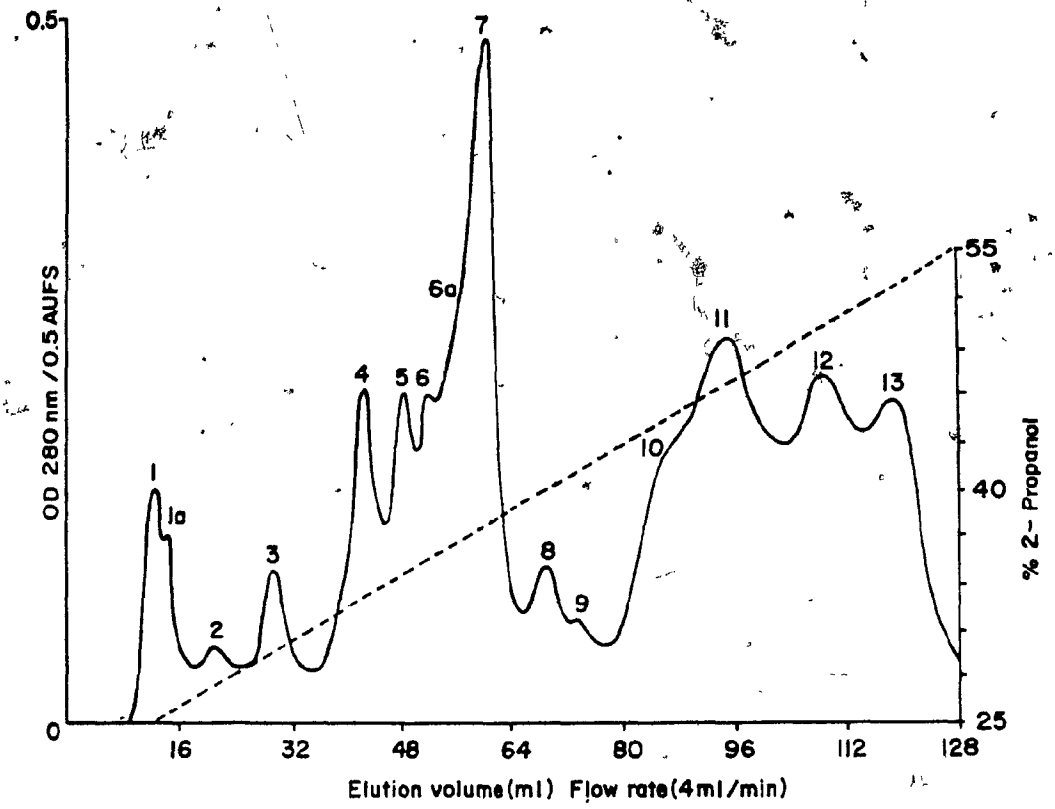


Figure 9. Chromatographic profile of unretained material of CMC chromatography after removal of 10 K material by ultrafiltration on a C_{18} semi-preparative column.

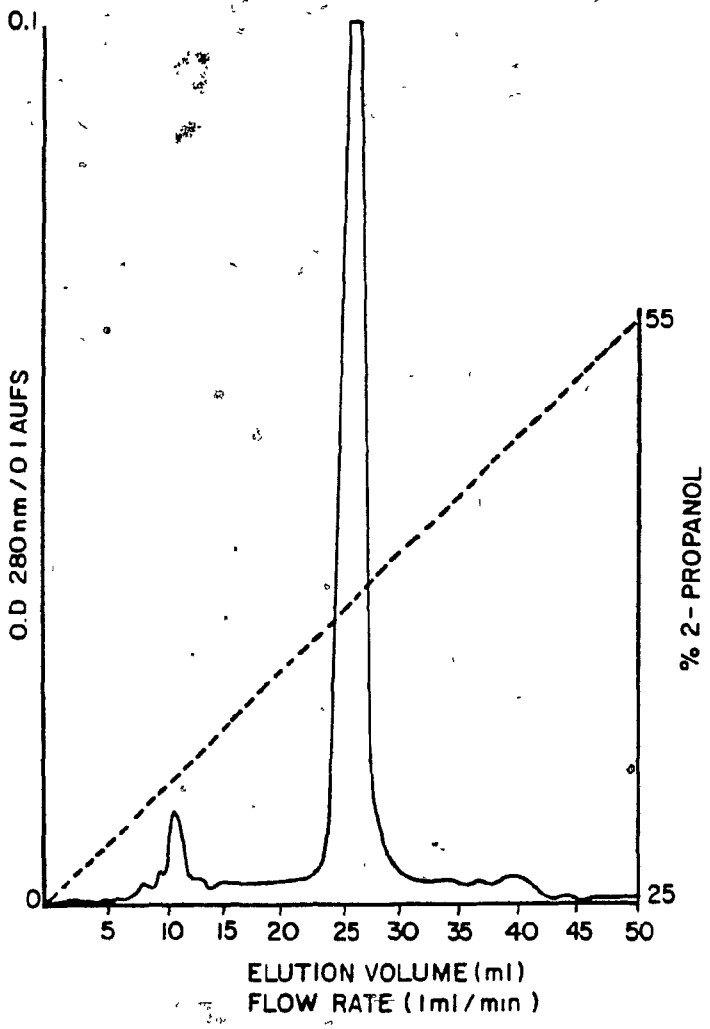


Figure 10. Chromatographic profile of fraction 7, previously purified by HPLC, on a C_{18} analytical column.

acetic acid at 4°C. Ten milligrams of material was poured on the column. The size of the fractions was 1 mL and the column had already been calibrated with standard peptides for a range of 14,5 K to 65 K. Results obtained are presented in figure 11. It is shown that the main product consisted of a peptide with an estimated 17 K molecular weight slightly containing on SDS-PAGE two contaminants of 20-21 K and 12-13 K estimated molecular weight. The 17 K peptide was used for further characterization and will therefore be named porcine N-terminal (pNT).

Amino acid analysis of pNT

Reduced and alkylated pNT was submitted to acid hydrolysis in 5,6 N HCl at 105° for 24, 48 and 72 hours. Results of this experiment are presented in table 1. For each period of time the value for each amino acid represents the mean of three analytical runs. From these results we concluded that the material was composed of 80 amino acid residues and was glycosylated. This peptide was named pNT 1-80.

Tryptic digest of pNT 1-80

One milligram of reduced and alkylated pNT 1-80 (fraction 98-116 from G-75 superfine, fig. 11) was submitted to tryptic digestion for 16 hours at 37°C. Then the solution was directly injected into an HPLC column and each peak was collected and repurified before hydrolysis and amino acid analysis. The chromato-

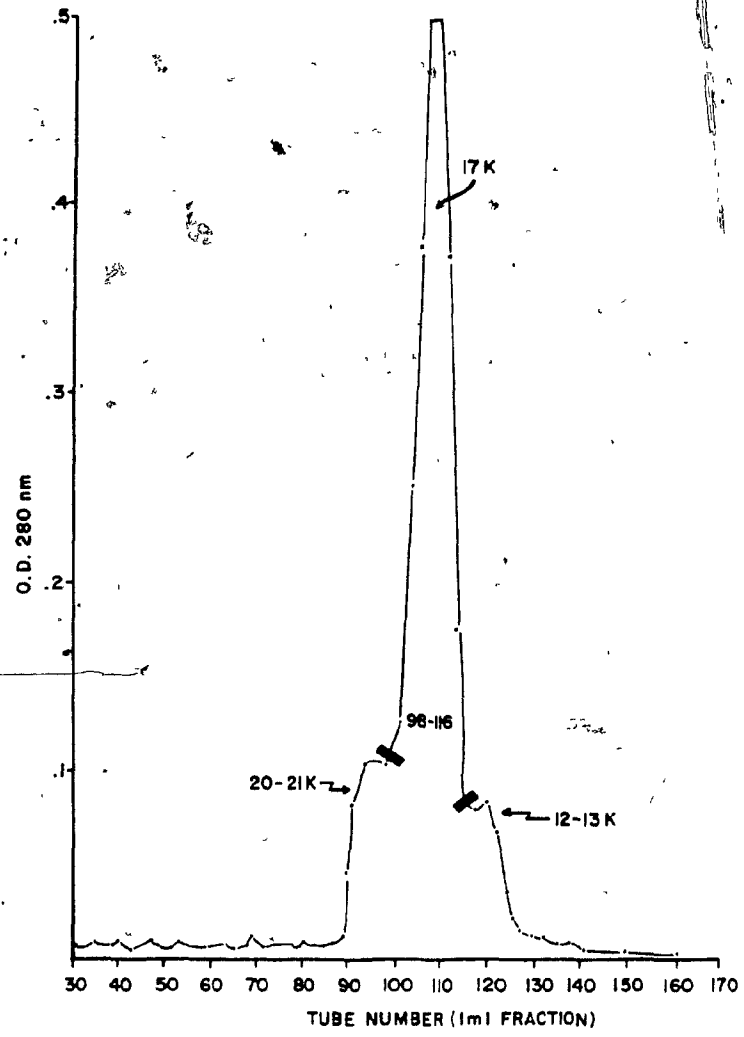


Figure 11. Elution profile of the re-chromatographed fraction 7 (fig. 10 and 11) on a Sephadex G-75 superfine column (160 x 1,25 cm) eluted with 2 M acetic acid at 4°C.

Table 1

	24 hrs	48 hrs	72 hrs	Mean	Integer
Lys	2.13	2.40	2.43	2.32	2
His	0.89	1.30	1.50	1.23	1
Arg	5.90	6.02	5.78	5.90	6
SCMC	3.27	3.14	2.53	2.98	(4)[1]
Asx	7.83	8.12	7.77	7.94	8
Thr	3.00	2.98	2.93	2.97	3
Ser	8.20	7.91	7.38	7.83	8
Glx	7.97	8.05	8.07	8.03	8
Pro	5.19	5.15	5.11	5.15	5
Gly	13.70	13.90	13.20	13.60	14
Ala	5.02	5.15	5.88	5.35	5
Val	2.04	2.35	2.39	2.26	2
HSL	+	+	+		
Met	0.39	0.25	0.53	0.39	1[2]
Ile	0.83	1.02	1.06	0.97	1
Leu	5.87	6.23	6.32	6.14	6
Tyr	0.87	0.93	0.87	0.89	1
Phe	3.21	3.09	3.45	3.25	3
Trp					(2)[3]
GlcN	++	++	++		
GalN	+++	+++	+++		
Total					80

AMINO ACID COMPOSITION OF the PNT 1-80 as determined after 24, 48 and 72 HOURS HYDROLYSIS IN 5,6 N HCl at 105°C.

1. Confirmed by sequence of ^{14}C-iodoacetamide treated PNT 1-80
2. Represent value for methionine plus homoserine lactone
3. Determined by sequence

SCM: S-carboxymethyl-cysteine
GalN: galactosamine
GlcN: glucosamine
HSL: homoserine lactone

graphic profile of this tryptic digest is presented in figure 12. Of the numerous tryptic fragments isolated, nine were identified as components of pNT 1-80. Amino acid composition of each fragment (T_1 to T_9) is presented in table 2. These preliminary results gave two new indications of the structure of the pNT 1-80. Firstly, based on sequence homology with the reported bovine c-DNA structure of the POMC (18) we observed a definite homology between the structure of the N-terminals in the two species (fig. 13). Secondly, we noticed that three tryptic fragments, T_3, T_4 and T_9 are glycosylated. Because T_3 and T_4 represent almost the same segment of pNT 1-80, T_3 having one less residue than T_4, there must exist at least two glycosylation sites on pNT 1-80. Based on sequence homology with bovine POMC the possible sites of glycosylation are on serine 29, threonine 32, 45 for T_3 and T_4, and on asparagine 65, serine 67, 68, 69 for T_9. On any of these tryptic fragments the possibility of more than one glycosylation site exists. The ratio of glucosamine to galactosamine in fragment T_9 could indicate that the glycosylation is of the N-type and therefore asparagine 65 should be the site of sugar attachment.

Cyanogen bromide cleavage

The presence of a single methionine in the pNT 1-80 probably at position 53 (figure 13) made possible the cleavage of the molecule into two fragments. Chromatographic profile of this

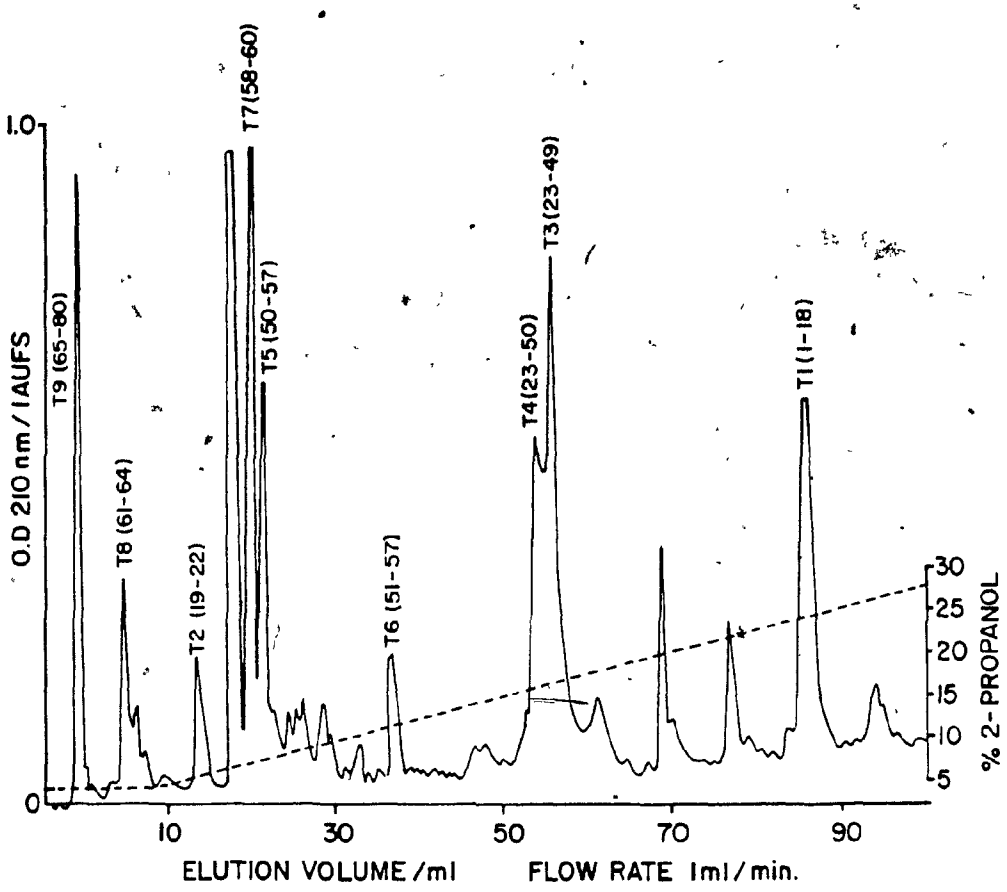


Figure 12. Chromatographic profile on HPLC of an 18 hour tryptic digest of pNT 1-80.

Table 2

	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9
Lys			1.10(1)	1.77(2)	0.75(1)				
His					0.75(1)	0.94(1)			
Arg		1.21(1)	1.00(1)	0.70(1)	0.89(1)	1.00(1)	1.00(1)	1.67(2)	
SOMC	2.34(2)	0.58(1)	0.90(1)	1.22(1)					
Asx	2.34(2)		4.10(4)	4.26(4)			0.92(1)		0.79(1)
Thr	1.09(1)		2.00(2)	1.91(2)					
Ser	3.61(4)		1.10(1)	1.22(1)					2.66(3)
Glx	4.36(4)		2.70(3)	3.05(3)					0.88(1)
Pro			5.00(5)	4.70(5)					
Gly			2.10(2)	2.09(2)	1.29(1)	1.39(1)		1.23(1)	10.00(10)
Ala		1.30(1)	3.10(3)	2.53(3)					
Val			0.90(1)	0.96(1)	1.25(1)	1.39(1)			1.03(1)
HSL									
Met					0.32(1)	0.85(1)			
Ile		1.15(1)							
Leu	4.30(4)		2.10(2)	2.09(2)					
Tyr					0.71(1)	0.94(1)			
Phe			1.01(1)	1.04(1)	0.75(1)	1.09(1)		1.00(1)	
Trp	(1)[1]						(1)[1]		
GlcN			+	+					+++++
GalN			+++	++					+
	1-18	19-22	23-49	23-50	50-57	51-57	58-60	61-64	65-80

AMINO ACID COMPOSITION OF PNT 1-80 AND ITS TRYPTIC FRAGMENTS

[1] Determined by sequence

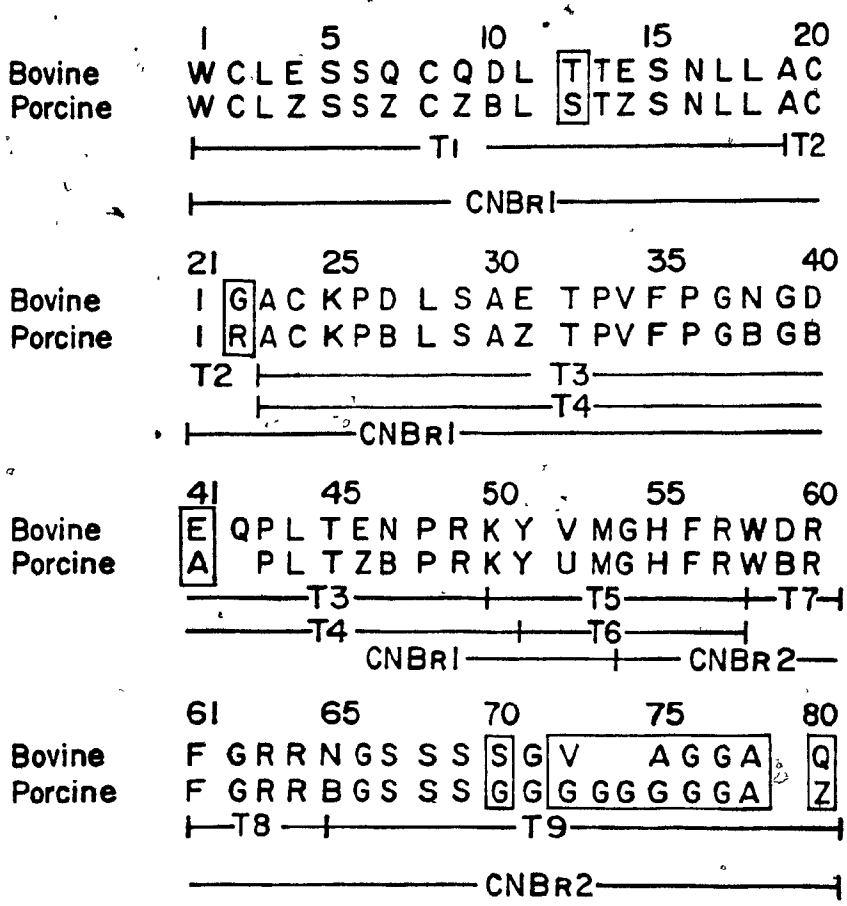


Figure 13. Homology between the predicted bovine first 77 residues NT and the alignment of pNT 1-80.

cleavage is presented in figure 14. As predicted two major fragments were obtained and were identified as Cyanogen bromide 1 (CNBr 1) and CNBr 2. Amino acid composition of these fragments is presented in table 3. The presence and ratio of sugars in each fragments also indicated the possibility of an O-glycosylation in the CNBr 1 fragment and an N-glycosylation in the CNBr 2 fragment.

During the repurification procedure of CNBr 2 a new fragment was isolated (fig. 15). This fragment named CNBr 3 is homologous with the bovine 54-61 sequence of the amino-terminal segment of POMC (18). Composition of CNBr 3 is also presented in table 3. This fragment is not glycosylated.

Proposed sequence of pNT 1-80

Tryptic fragments T_1 to T_9 and cyanogen bromide fragments were aligned with the reported sequence of the first 77 residues of the bovine amino-terminal segment of POMC (18) after removal of the 26-residue signal peptide (179). The proposed alignment of these molecules based only on sequence homology is presented in figures 13 and 16. This preliminary structure of PNT 1-80 showed that there could be as much as 88% homology between the porcine and bovine homologues. Most of the differences between the two species exist at the C-terminal of the molecule. This observation is deduced from the great heterogeneity between tryptic fragment 9, corresponding to PNT 65-80, and the bovine 65-77homologous segment (18). The sum of fragments T_5, T_7, T_8 has an identical

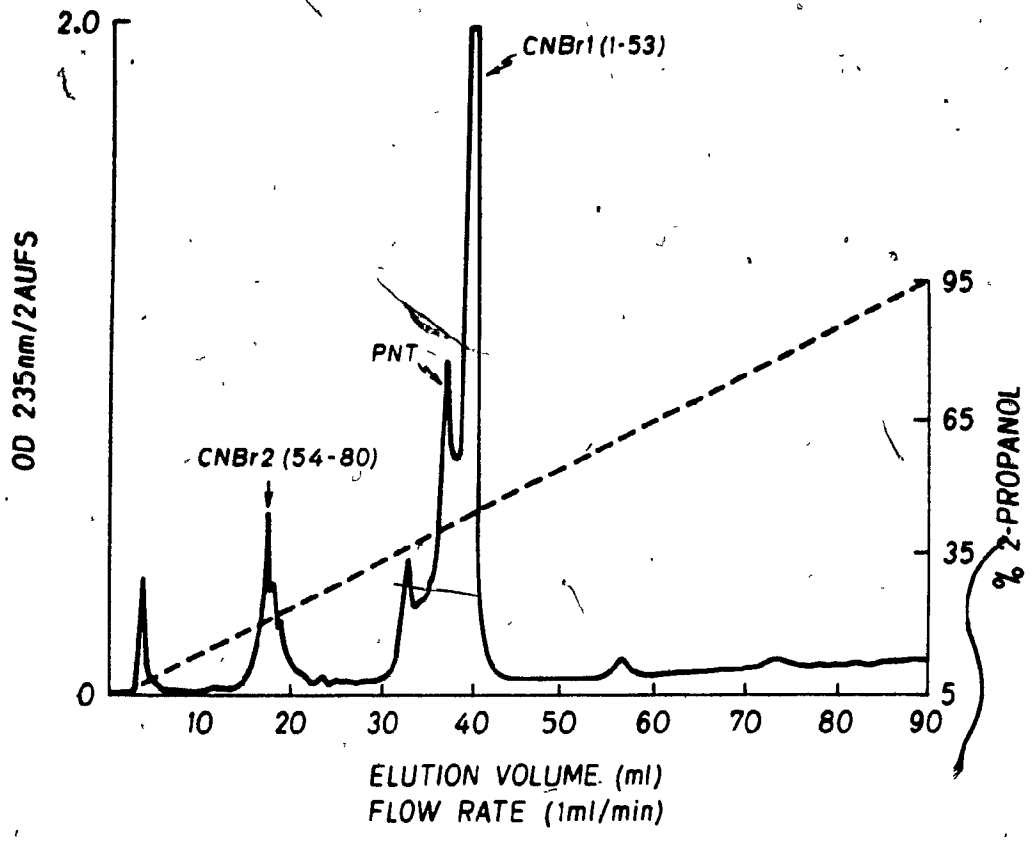


Figure 14. Chromatographic profile of pNT 1-80 cyanogen bromide fragments on a u-Bondapak C-18.

Table 3

	CNBr1	CNBr2	CNBr3
Lys	2.23(2)		
His		0.85(1)	0.82(1)
Arg	1.74(2)	3.95(4)	1.70(2)
SCMC	(4)[1]		
Asx	5.61(6)	2.04(2)	1.03(1)
Thr	2.87(3)		
Ser	4.84(5)	2.46(3)	
Glx	6.57(7)	1.45(1)	
Pro	5.30(5)		
Gly	2.45(2)	11.55(12)	0.97(1)
Ala	4.47(4)	1.03(1)	
Val	2.45(2)		
HSL	+++		
Met			
Ile	0.85(1)		
Leu	5.53(6)		
Tyr	0.71(1)		
Phe	0.92(1)	2.03(2)	1.83(2)
Trp	(1)[1]	(1)[1]	(1)[1]
GlcN		+++++	
GalN	+++	+	
Fragment	1-53	54-80	54-61

AMINO ACID COMPOSITION OF CYANOGEN BROMIDE FRAGMENTS OF PNT 1-80

[1] Determined by sequence

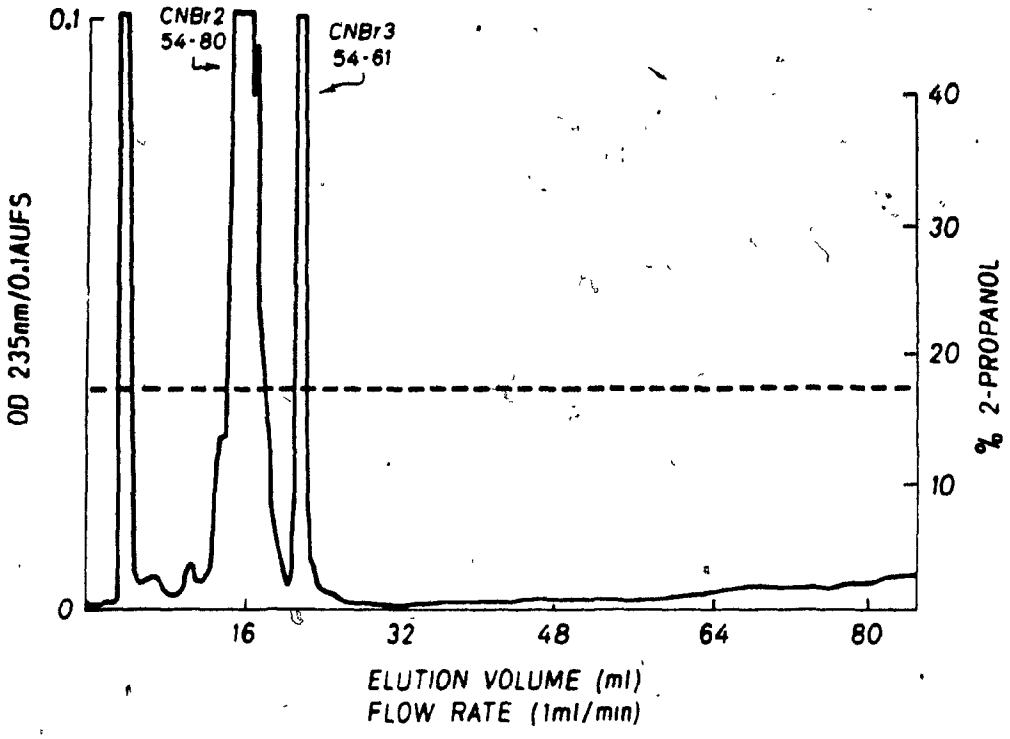


Figure 15. Chromatographic profile on HPLC u-Bondapak C-18 analytical column (Waters Scientific) of the repurification of CNBr2 under isopratic conditions.

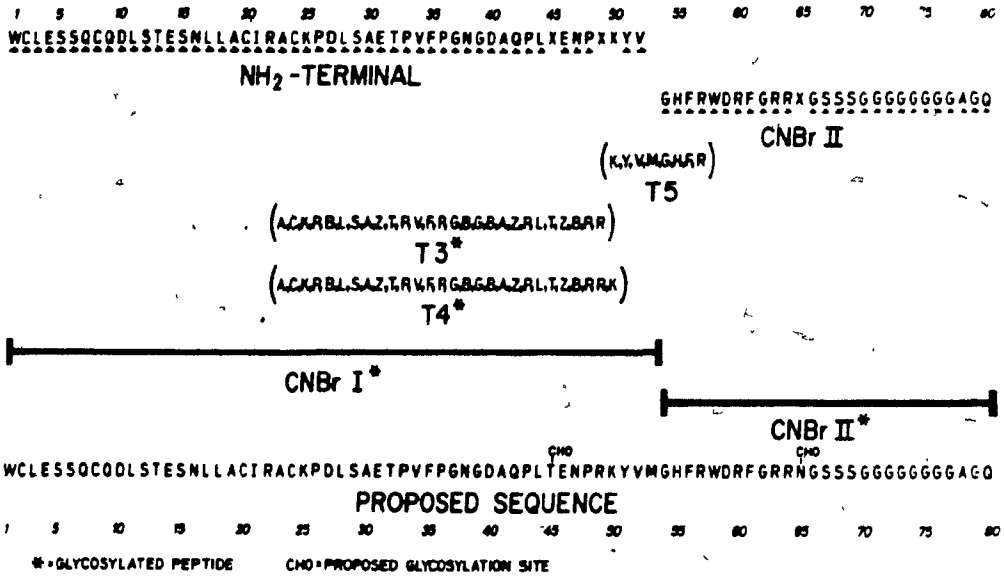


Figure 16. Alignment of the pNT 1-80 first 52 cycles sequence, CNBr II sequence, proposed sequence from amino acid analysis of T_3, T_4, T_5, CNBr I, CNBr II.

composition to the reported γ-melanotropin segment in bovine (18), human (185), and rat (104) species (table 2). However, a number of amino acid substitutions were also observed in fragments T_1, T_2, T_3, T_4. In agreement with the reported sequence of the porcine N-terminal 1-103 first 35 cycles (203) we deduced that Ser 12 replaces Thr 12 in the bovine homologue. We also observed that Arg 22 replaces Gly 22. In fragments T_3 and T_4 a single mutation exists. It is located at cycle 41 where an Arg replaces a Glu. All other mutations are located in T_9 where because of less than 60% homology between the ox and the pig, alignment of the fragments was difficult.

Based on the same alignment between the ox and pig homologous peptides, CNBr 1 showed 94% homology with bovine 1-53 and CNBr 2 78% with bovine 54-77. The third CNBr fragment showed 100% homology with bovine 54-61 homologue.

Proposed glycosylation sites

The ratio of glucosamine to galactosamine in T_9 and $CNBr_2$ clearly indicates that the glycosylation in these fragments is an N-type (244, 245). Based on the sequence homology between the ox and the pig N-terminal the Asn 65 was proposed as a possible site of N-glycosylation in the pNT 1-80. The other site of glycosylation located in fragments T_3 and T_4, i.e. between residues 22 and 51, should be of the O-type (245) according to the

ratio of glucosamine to galactosamine. This type of glycosylation occurs only on serine or threonine. In fragments T_3 and T_4 these residues appear as follows: Ser 20, Thr 32 and another threonine possibly located at site 45. So it is possible that one to three site(s) of glycosylation exists within this segment of the pNT 1-80. Based on the already published sequence of the first 35 residues of the pNT 1-103 (203) we proposed that the glycosylation site should be on the unlocalized threonine probably at site 45; this will be confirmed later in the sequencing results.

Sequence determination of pNT 1-80

Two milligrams of reduced and alkylated pNT 1-80 was submitted to direct sequencing. This first run allowed the identification of 49 out of the first 52 residues of pNT 1-80. Quantitative determination in micromoles of each amino acid for each cycle is presented in appendix I. The positive identification of residues 1-44, 46-48, 51, 52 was possible leaving blanks for residues 45, 49 and 50. This first sequence confirmed the great homology between the bovine and porcine homologues (18, 203). In the first 44 residues only three mutations exist between the ox and the pig. In this latter species Ser 12 replaces Thr 12, Arg 22 replaces Gly 22 and Ala 41 replaces Glu 41. Based on sequence homology between the bovine 22-57 sequence and the amino acid composition of the fragments T_3, T_4 and T_5, the three unidentified

residues could be, Thr 45, Arg 50 and Lys 51. This latter residue is indirectly confirmed by the fact that the difference between T_3 and T_4 is one residue and the amino acid composition of these two fragments (table 2) indicated the presence of one more Lys in T_4. So this Lys could only be located at site number 50.

To complete the sequence of pNT 1-80 we sequenced the fragment CNBr II corresponding to pNT 54-80. Out of the 27 residues present in this fragment 26 were positively identified. Yields in nanomoles of PTH amino acid as a function of sequanator cycle number of CNBr II fragment is presented in figure 17. The number above each peak represents the assigned sequence position of that particular amino acid residue along the CNBr II sequence. The only unidentified residue was at site 12, corresponding to site 65 in the whole pNT 1-80. No PTH was detected at this cycle. The only amino acid left based on the composition of CNBr II is an asparagine or an aspartic acid. Because the only N-glycosylation site in this fragment could be on an asparagine, we assumed that site 12 was occupied by a glycosylated asparagine.

Out of the 80 residues of the pNT only 4 residues were left unidentified. They were residues 45, 49, 50 and 53. Residue 53 was identified as methionine because the site of cleavage with cyanogen bromide two fragments: 1-53 and 54-80, thus placing methionine at site 53. Lysine 50 was already identified based on the amino acid composition of T_3, T_4, T_5 with homologous segment of bovine N-terminal. With this latter amino acid placed,

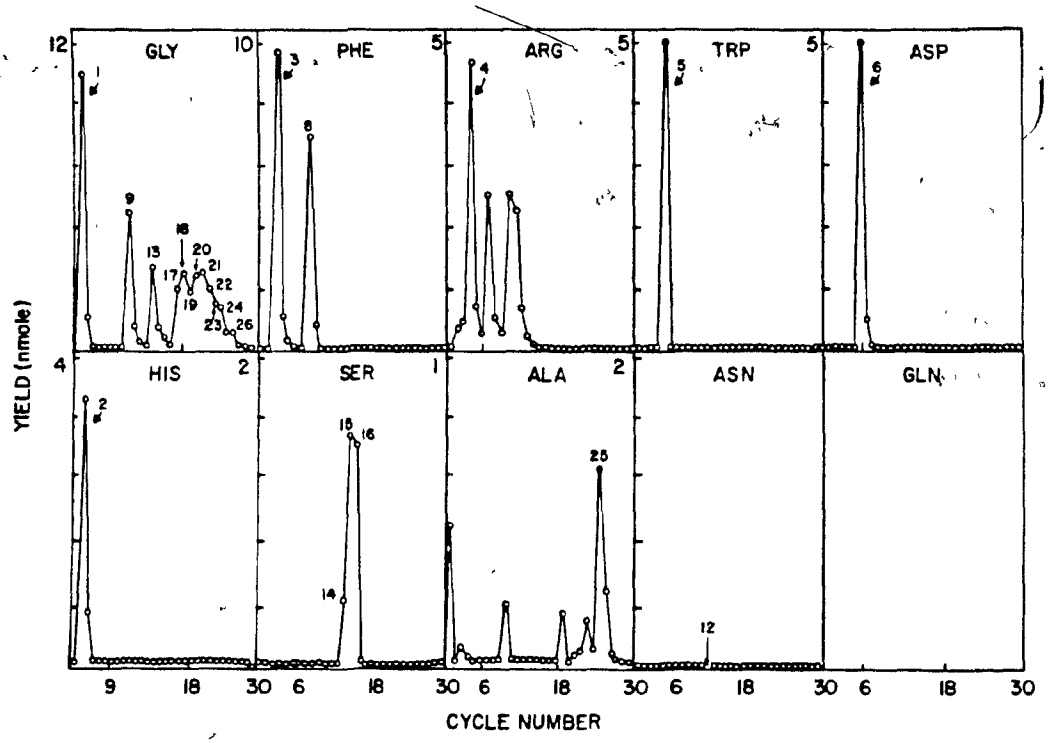


Figure 17. Yields in nanomoles of PTH-amino-acid as a function of sequanator cycle number of CNBr II fragment.

methionine in fragment T_5 could only be located at site 53. The two residues left were placed as follows: threonine at site 45 and arginine at site 49, based on sequence homology with bovine (18), human (185) and rat (104) homologues. Alignment of the first 52 residues sequence, CNBr II sequence, amino acid composition of T_3, T_4, T_5, CNBr I and CNBr II is presented in figure 16. The proposed sequence of pNT 1-80 is presented in figure 18.

Purification and characterization of the porcine higher molecular weight N-terminal

Chromatography of the preparation of porcine N-terminal 1-80 glycopeptide on Sephadex G-75 superfine (fig. 11) resulted in the purification of pNT 1-80 with an apparent Mr of 17 K and in the isolation of a fraction enriched with Mr 20-21 K peptide. We investigated the possibility that the high molecular weight (HMr) form corresponds to an elongated form of the pNT.

Tryptic digest of the high molecular weight pNT

Five hundred micrograms of reduced and alkylated high molecular weight pNT (HMrpNT) fraction 88-97 from Sephadex G-75 superfine (fig. 11) was submitted to tryptic enzymatic cleavage. Digest was chromatographed on HPLC in the TEAP/acetonitrile system (fig. 19). All previous characterized fragments T_1 and T_9 (table 2, fig. 12) were isolated plus another minor fragment, T_{10}. Amino acid composition of this last fragment showed enough homology with the reported bovine 78-92 amino terminal homologue (18) to consider it an extension of the pNT 1-80 (table 4).

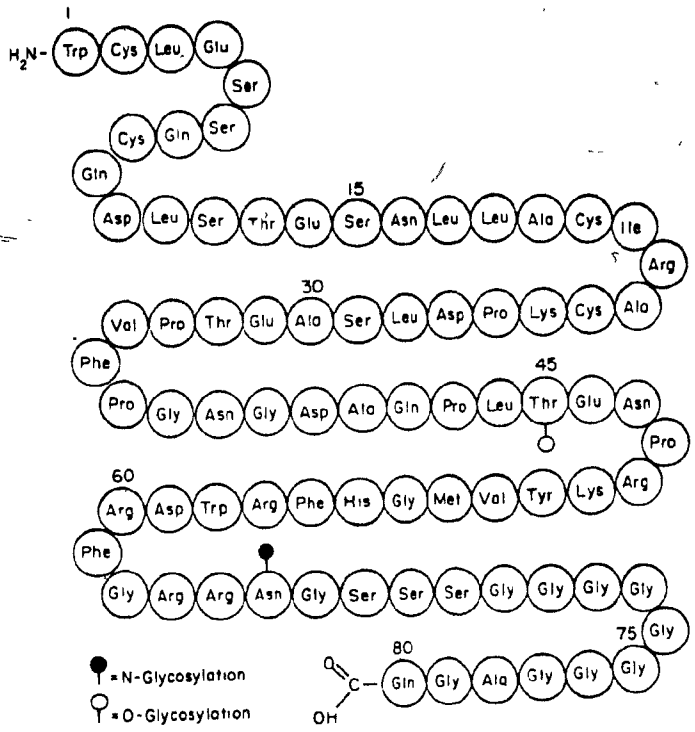


Figure 18. Proposed sequence of pNT 1-80 including two possible glycosylation sites.

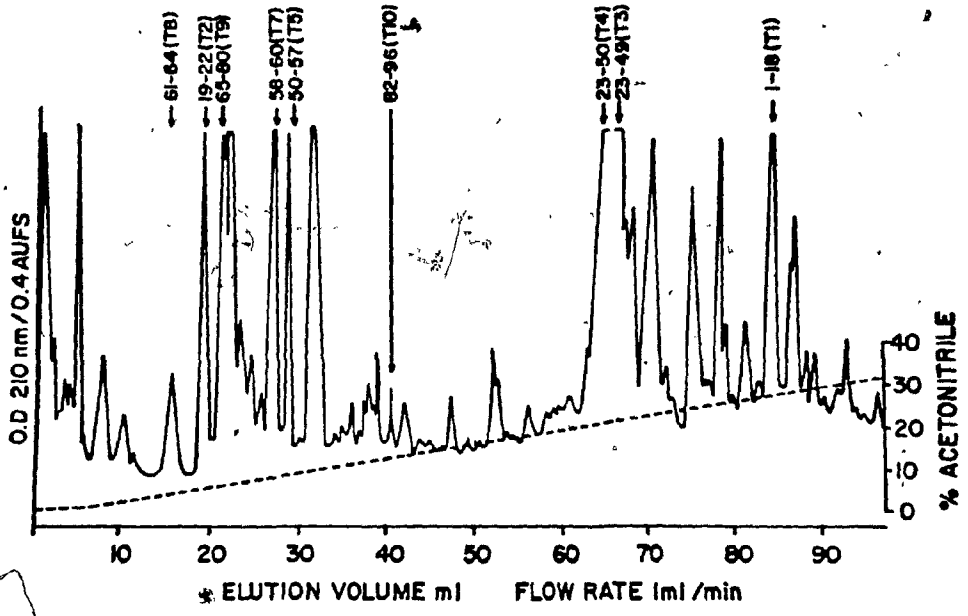


Figure 19. Chromatographic profile on HPLC u-Bondapak C-18 analytical column (Waters Scientific) of the tryptic digest of the 20-21 K apparent Mr peptide isolated by gel permeation chromatography.

Adrenocorticotropic hormone radioimmunoassay

An ACTH radioimmunoassay was used to determine whether or not the HMrpNT included the ACTH sequence. We characterized an anti-ACTH antibody, produced in our laboratory from synthetic pig ACTH 1-24. The specificity of the antibody is directed against the amino-terminal portion of the ACTH. The cross-reactivity of that antibody against some ACTH related peptides is presented in Figure 20. No ACTH structure was detected in the HMrpNT (fig. 20).

Digestion with cyanogen bromide of HMrpNT

The presence of a single methionine as far as the first 96 amino acid residues of the non determined length HMrpNT gave us the opportunity to cleave the molecule in a limited number of fragments with CNBr. The chromatographic profile of the result of this reaction is presented in figure 21. Three major peptides were collected and their amino acid composition established. These analyses revealed that the HMrpNT was around 110 residues long (pNT 1-107) and was cleaved in two fragments, the first one (CNBr 1) contains 53 residues and corresponds to the already characterized CNBr 1 fragment of pNT 1-80, the second contains 54 residues (CNBr 4) and showed a high degree of homology with the bovine 54-103 amino-terminal segment of POMC (18). We assumed that the HMrpNT was 107 residues long based on the addition of residues in fragments CNBr 1 and CNBr 4. We were unable to give at the time, the exact composition of the HMrpNT based only on the

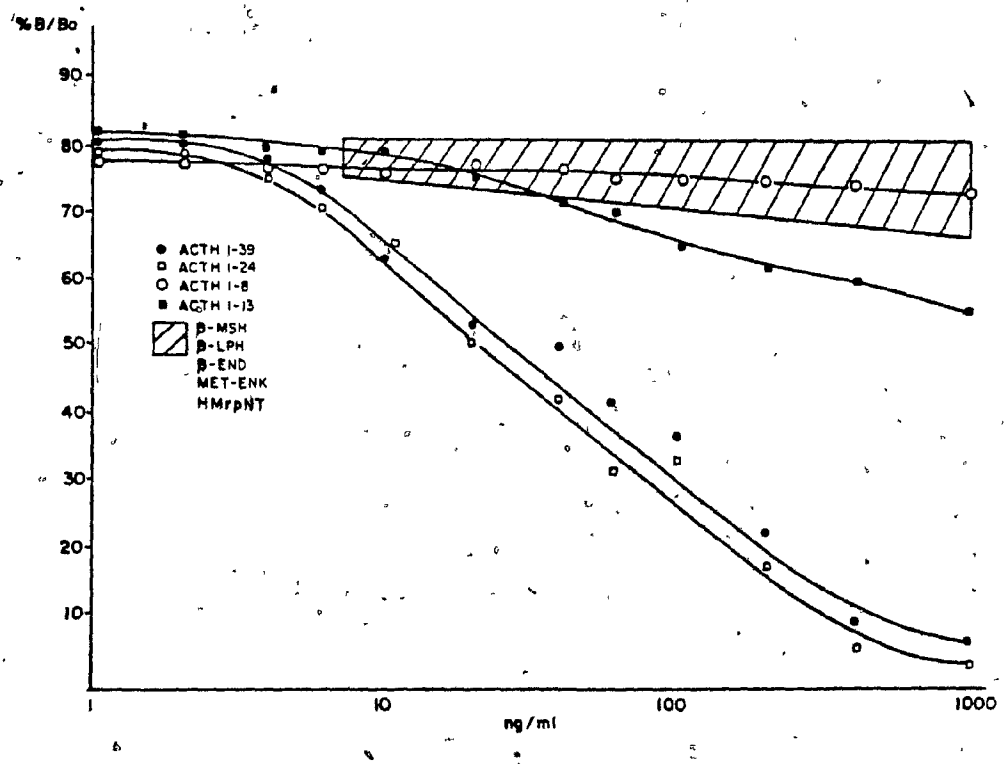


Figure 20. Cross reactivity of the anti-porcine-ACTH 1-24 antibody against some pituitary peptides.

Table 4

	Porcine 82-96 (T10)	Bovine 78-92
Lys		
His		
Arg	2.05(2)	2
SCMC		
Asx		
Thr		
Ser		
Glx	5.00(5)	4
Pro	1.86(2)	2
Gly	3.29(3)	4
Ala	1.81(2)	1
Val	1.00(1)	2
HSL		
Met		
Ile		
Leu		
Tyr		
Phe		
Trp		
GlcN		
GalN		

AMINO ACID COMPOSITION OF TRYPTIC FRAGMENT T10 AND BOVINE PREDICTED 78-92 AMINO-TERMINAL SEGMENT OF POMC HOMOLOGUE

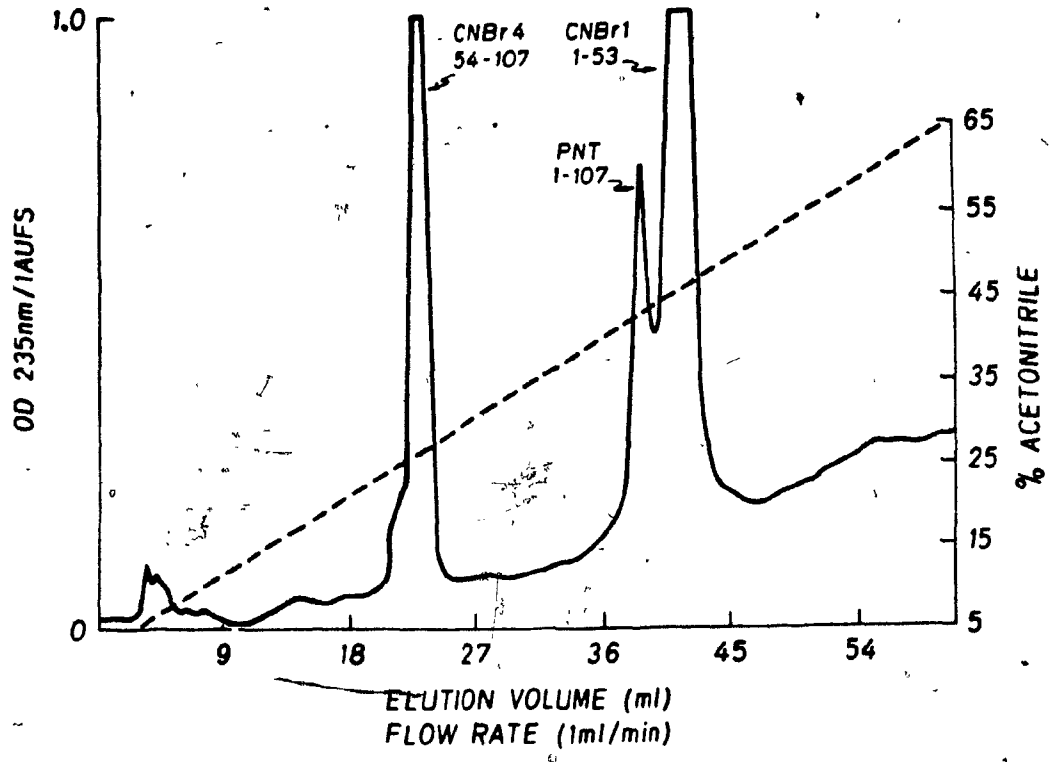


Figure 21. Chromatographic profile on HPLC u-Bondapak C-18 analytical column (Waters Scientific) of the cyanogen bromide fragment of the HMrpNT.

analysis of the hydrolysis of this repurified peptide. Amino acid composition of the CNBr 4 fragment is presented in table 6. The HMrpNT will now be called pNT 1-107.

Sequence determination of CNBr 4

Direct sequencing of CNBr 4 allowed the unambiguous identification of the first 51 residues with the exception of the residues in position 44 and 46 (corresponding to residues 97 and 99 of pNT 1-107). No residue could be identified at position 12 (corresponding to position 65 of pNT 1-80) which we believe to be an N-glycosylated asparagine residue. This sequence confirmed the identity of pNT 1-80 and the presence of an extension at its C-terminal. It was also possible with this sequence to fit the tryptic fragment T_{10} as an extension of pNT 1-80. Based on the amino acid composition of CNBr 4, 6 positions of the CNBr 4 sequence had to be filled. At position 65 we assigned an Asn. The five residues left had to be occupied by the following amino acids: 2 glycines, 1 arginine, 1 glutamine (or glutamic acid), 1 asparagine (or aspartic acid). Yields of each phenylthiohydantoin amino acid as a function of sequanator cycle number in the sequence of CNBr 4 are presented in figure 22. We represent in figure 23 the alignment of residues 54-80 from pNT 1-80, the sequence of CNBr4 and homology of fragment T_{10} to these two sequenced peptides.

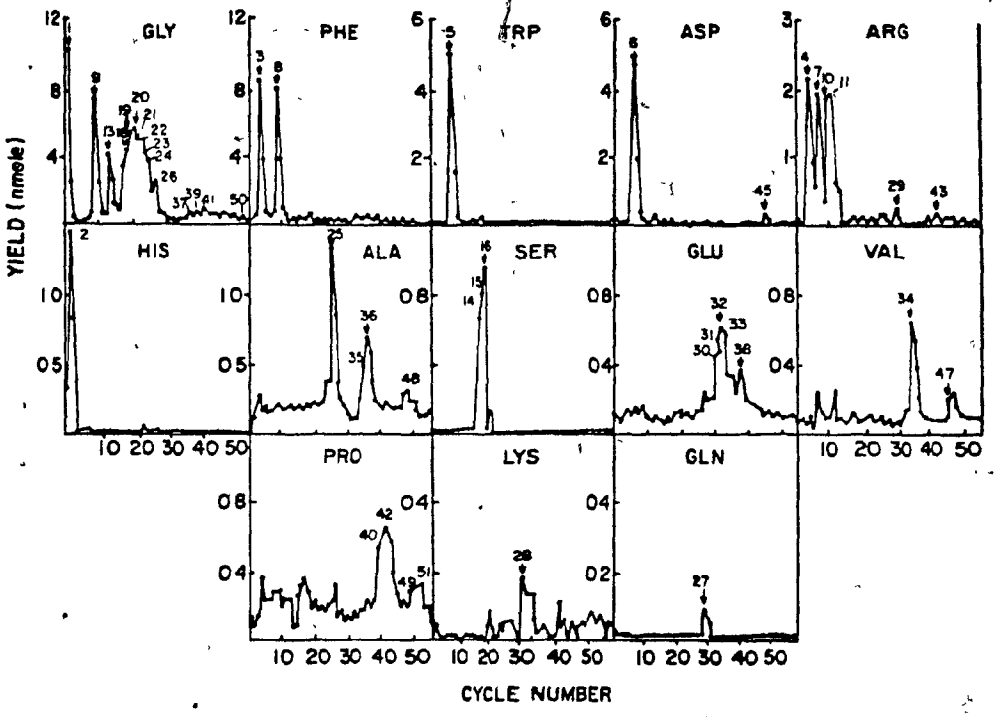


Figure 22. Yield of PTH-amino acid as a function of the sequanator cycle number in the sequence of CNBr 4 fragment.

Cleavage with Myxobacter Lys-C protease

The enzyme Myxobacter Lys-C protease, which is specific for lysine, was used to cleave three peptides: pNT 1-80, pNT 1-107 and CNBr4. PNT 1-80 was digested in order to have further indication that the indirectly assigned position 50 is a lysine residue. In the whole molecule only two lysines exist, one at position 25 and another possibly at position 50 (fig. 18). The lysine at position 25 is followed by a proline which should prevent the action of the enzyme. For these reasons we expected pNT 1-80 to be cleaved into two fragments. Results of this reaction are presented in the HPLC chromatographic profile in figure 24. Amino acid composition of the two major peptides collected, MB3 and MB4, are presented in table 5. It is clear that the enzyme specifically acted at the lysine residue on the N-terminal side. Fragment MB3 (pNT 1-49) and fragment MB4 (pNT 50-80) gave another clear indication that position 50 is occupied by a lysine.

PNT 1-107 was also cleaved. The lysine residues in this molecule are located at positions 25, 50 and 81. As in pNT 1-80, the peptide was not cleaved at lysine 25. Three peptides were collected: MB2 (pNT 82-107), MB3 (pNT 1-49), and MB5 (pNT 50-81). The chromatographic profile is presented in figure 25 and the amino acid composition of each peptide presented in table 5. We noticed that at position 81 the enzyme also cleaved at the amino terminal side of the lysine.

Alignment of MB1, MB2, and MB4 did not allow the identification of the amino acid residues at positions 97, 105, 106, 107.

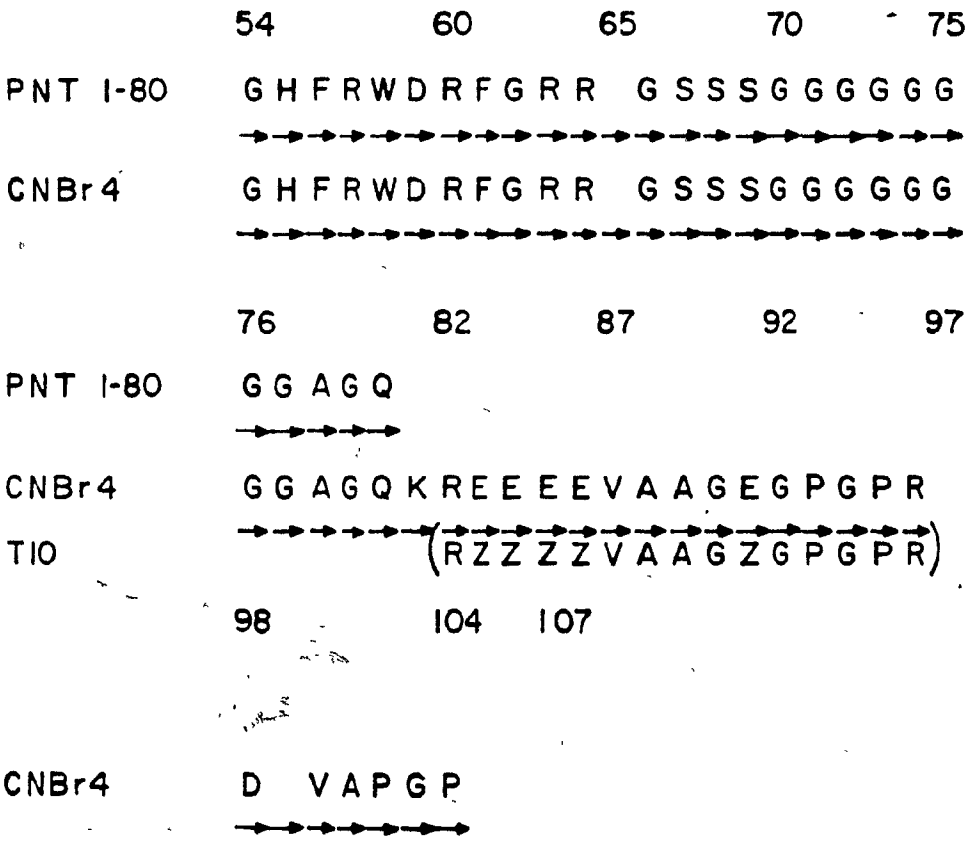


Figure 23. Alignment of fragment 54-80 of pNT 1-80 with sequence of CNBr 4 and amino acid composition of T_{10}.

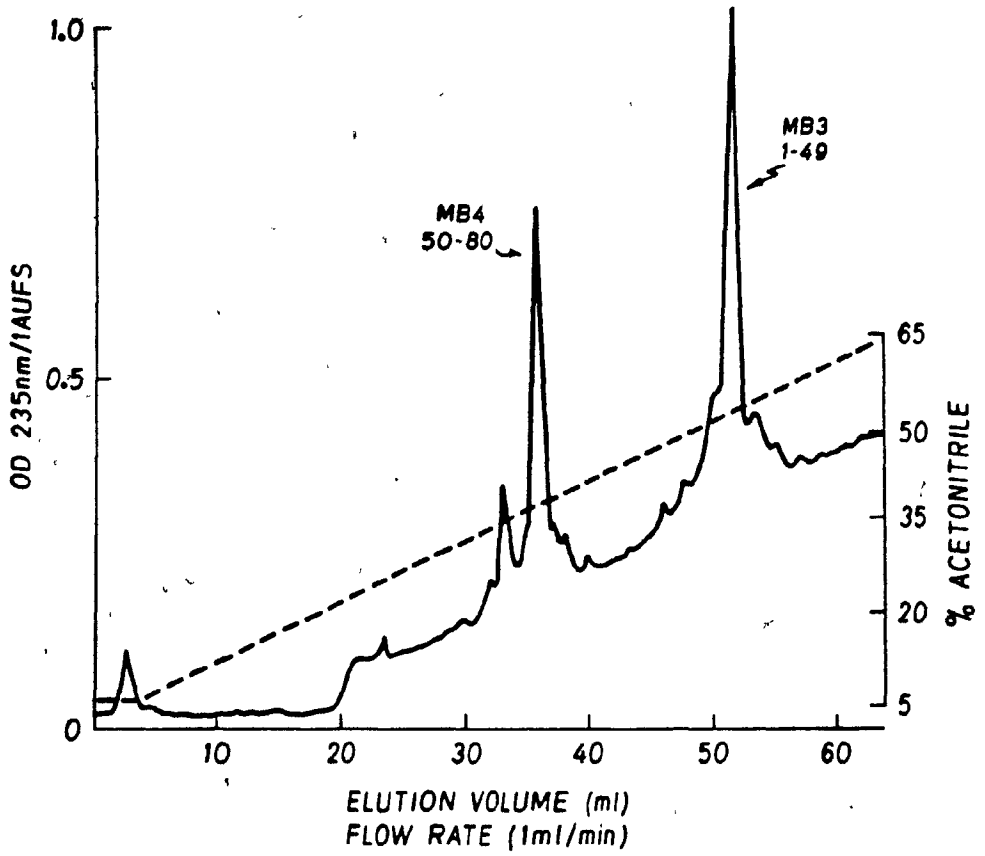


Figure 24. Chromatographic profile on HPLC u-Bondapak C-18 analytical column of pNT 1-80 digested with Myxobacter Lys-C protease.

Table 5

	T_{10}	$CNBr_4$	MB1	MB2	MB3	MB4	MB5
Lys		1.18(1)	0.83(1)		0.85(1)	0.87(1)	1.70(2)
His		1.18(1)	0.97(1)			0.63(1)	0.54(1)
Arg	2.05(2)	7.19(7)	3.87(4)	2.93(3)	0.90(1)	3.90(4)	3.57(4)
SCMC					(4)[1]		
Asx		4.07(4)	1.89(2)	1.87(2)	5.57(6)	2.17(2)	1.67(2)
Thr					2.63(3)		
Ser		3.32(3)	2.60(3)		4.52(5)	2.52(3)	2.51(3)
Glx	5.00(5)	6.67(7)	1.25(1)	5.77(6)	6.73(7)	0.92(1)	0.93(1)
Pro	1.86(2)	3.55(4)		3.80(4)	4.11(4)		
Gly	3.29(3)	17.63(18)	11.69(12)	6.33(6)	1.97(2)	11.87(12)	12.15(12)
Ala	1.81(2)	4.35(4)	1.37(1)	3.40(3)	4.39(4)	1.24(1)	1.07(1)
Val	1.00(1)	2.30(2)		1.69(2)	2.18(2)	1.14(1)	1.19(1)
HSL							
Met							
Ile					0.74(1)		
Leu					6.17(6)		
Tyr					0.62(1)	0.70(1)	0.63(1)
Phe		1.98(2)	1.85(2)		0.89(1)	1.77(2)	1.82(2)
Trp		(1)[1]	(1)[1]		(1)[1]	(1)[1]	(1)[1]
GlcN		++++				++++	++++
GalN							
Segment	82-96	54-107	54-81	82-107	1-49	50-80	50-81

AMINO ACID COMPOSITION OF MYXOBACTER LYS-C PROTEASE FRAGMENTS, TRYPTIC FRAGMENT T_{10} AND CYANOGEN BROMIDE FRAGMENT $CNBR_4$

[1] Determined by sequence

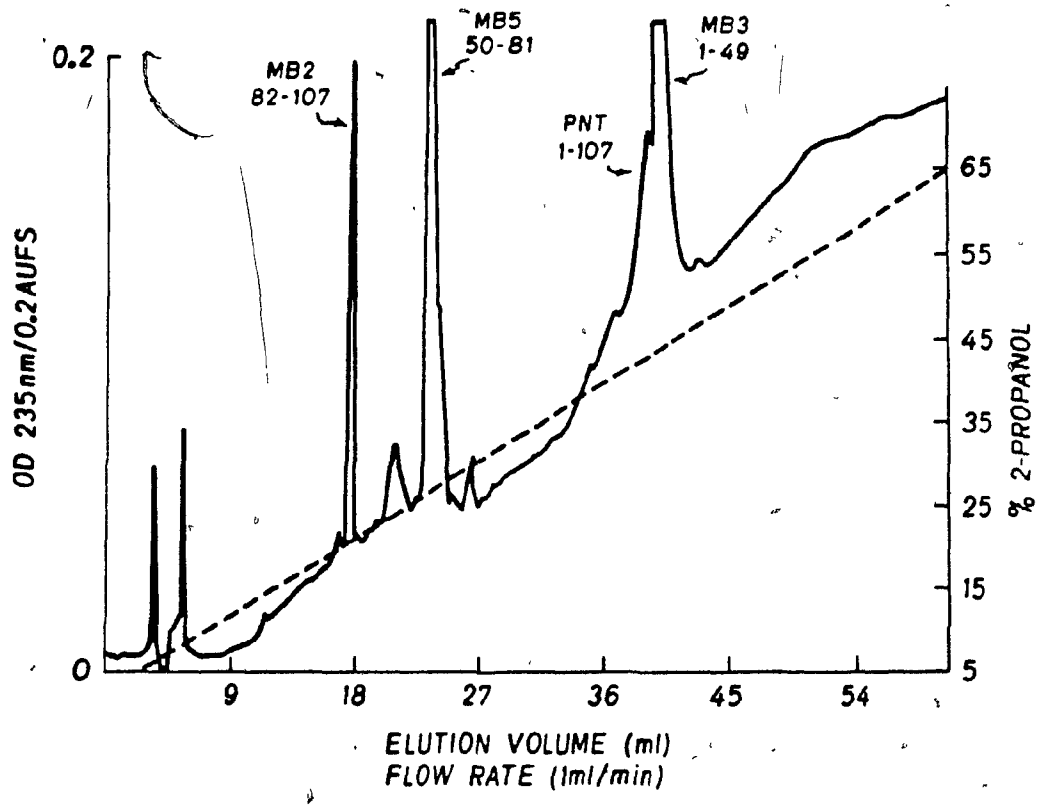


Figure 25. Chromatographic profile on HPLC u-Bondapak C-18 analytical column of pNT 1-107 digested with Myxobacter Lys-C protease.

Sequencing of fragment MB2 (pNT 82-107) should allow the identification of these residues.

To prepare enough material of MB2, fragment CNBr 4 (pNT 54-107) was cleaved with Myxobacter Lys-C protease. This reaction gave two fragments: MB1 (54-81) and MB2 (82-107). A chromatographic profile of the results of the reaction is presented in figure 26. All MB2 peptide collected so far was repurified using HPLC under the same conditions. When 500 µg of lyophilized MB2 was prepared, it was submitted to automatic Edman degradation in the sequanator. Yields of each phenylthiohydantoin amino acid as a function of sequanator cycle number on the sequence of MB2 are presented in figure 27. This sequence allowed the identification of all 26 residues of the molecule. Alignment of sequenced MB2 with the porcine 82-107 segment of pNT 1-107 is presented in figure 28. Alignment of all fragments isolated and submitted to amino acid analysis and, in some cases to sequencing, are presented in figure 29. The primary structure of pNT 1-107 is presented in figure 30.

Purification and characterization of the missing fragment between pNT 1-80 and ACTH

"Fraction D" from porcine pars distalis was chromatoraphed on carboxymethylcellulose resin. The unretained material (peak A) was lyophilized and resolved using HPLC without being ultrafiltered through a dialysis membrane. This approach allowed the recovery of small molecular weight peptides. The isolation of two

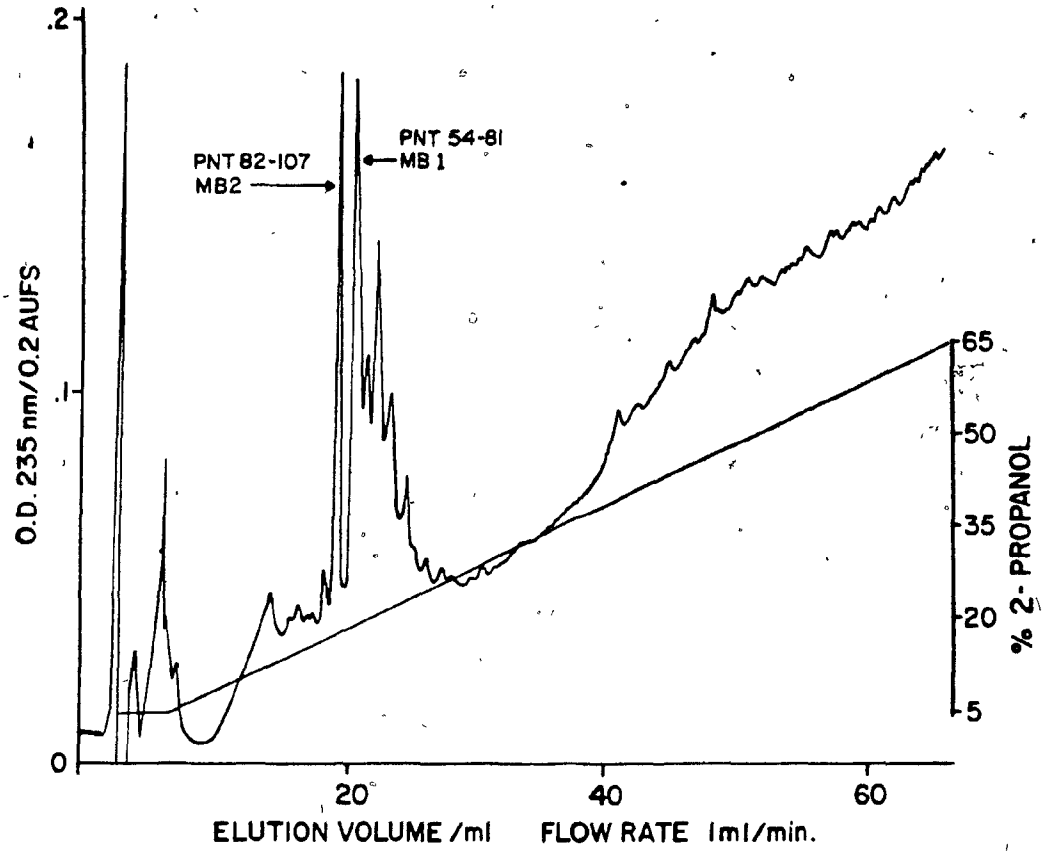


Figure 26. Chromatographic profile on HPLC u-Bondapak C-18 analytical column of CNBR 4 fragment digested with Myxobacter Lys-C protease.

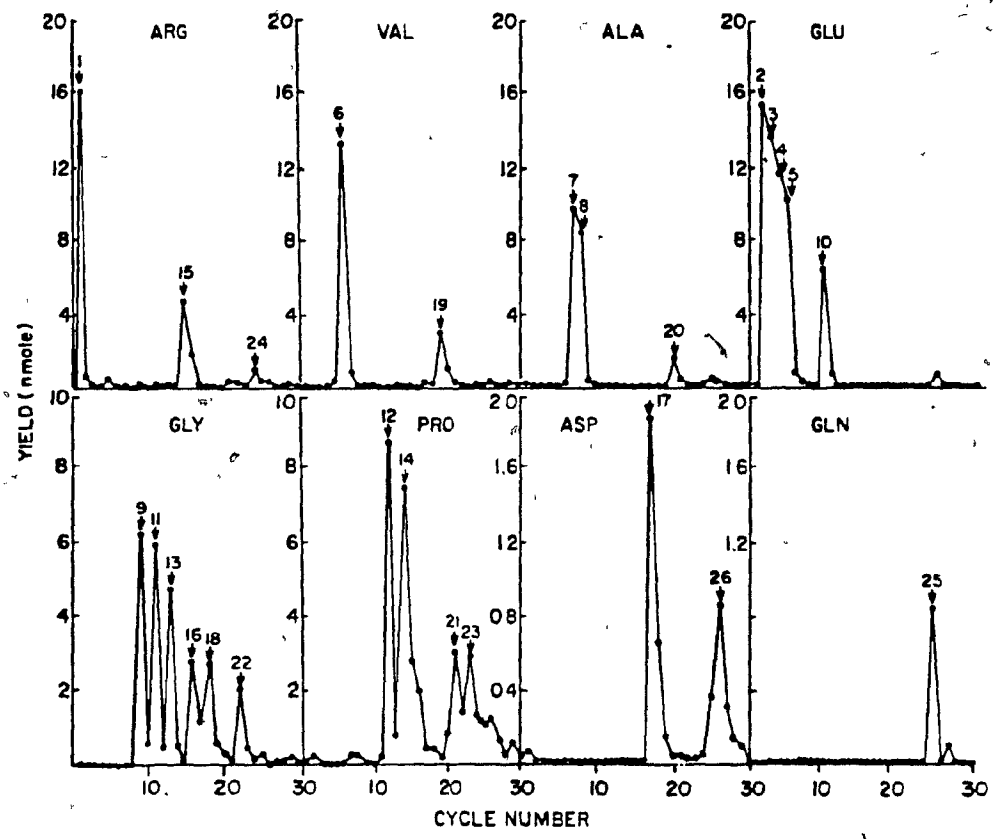


Figure 27. PTH amino-acid yield of MB2 as a function of the sequanator cycle.

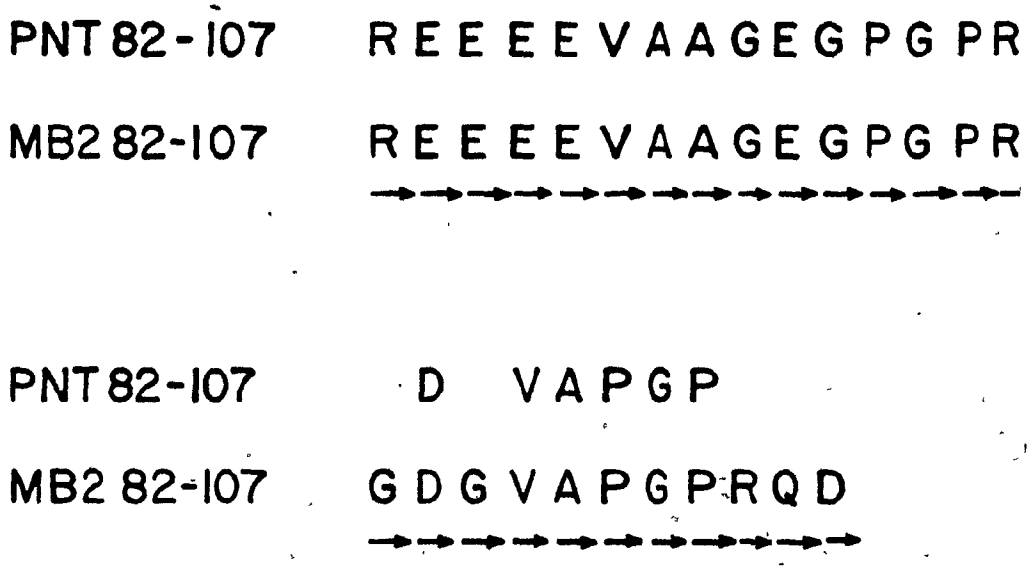


Figure 28. Alignment of sequenced MB2 fragment with porcine 82-107 segment of pNT 1-107.

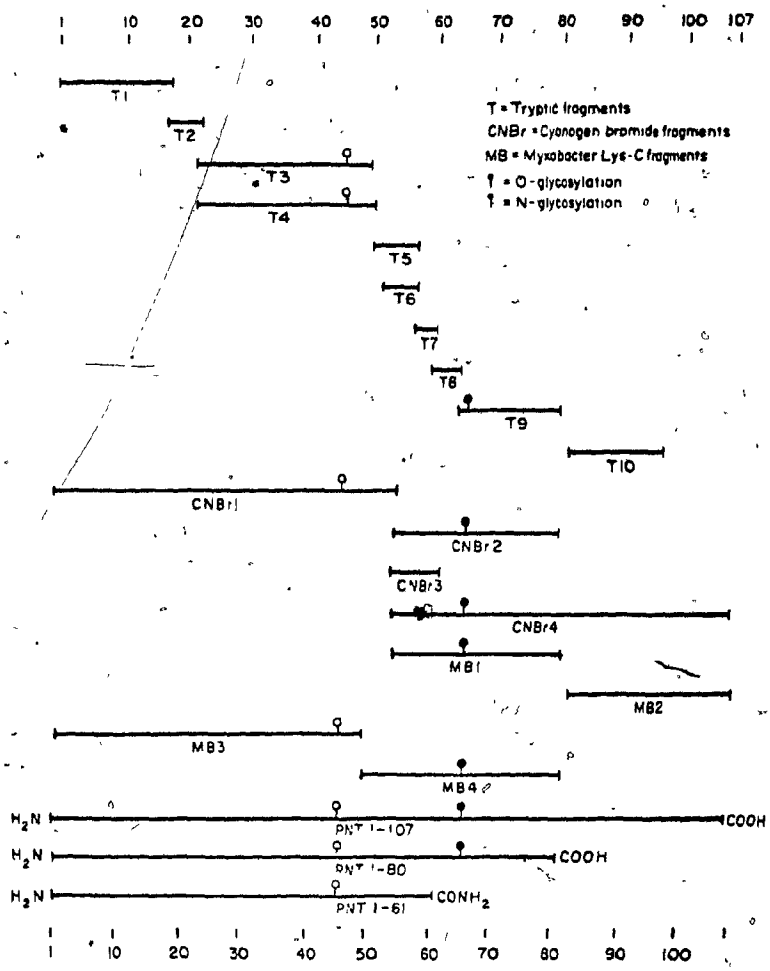


Figure 29. Schematic representation of all tryptic, Cyanogen bromide and Myxobacter Lys-C protease fragments used to propose the sequence of pNT 1-80, pNT 1-107 and pNT 1-61.

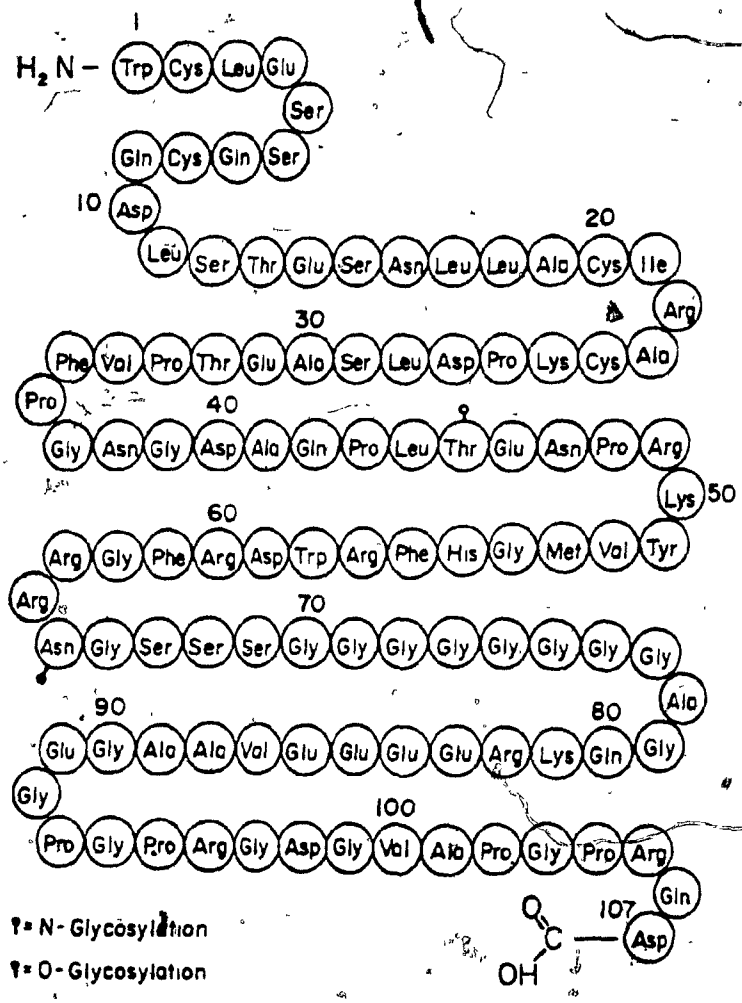


Figure 30. Proposed sequence of pNT 1-107.

forms of the amino terminal segment of POMC, one with 107 amino acid residues and the other with 80, indicated the possibility that the high Mr form might be the precursor for the low Mr N-terminal. If this be the case, 27 residues of the pNT 1-107 would have to be removed to give the 80-residue pNT glycopeptide. If this hypothesis was correct and if the extension from residue 80 up to carboxy-terminal of the precursor were removed as an entity, one should be able to detect a 27 amino acid residue non-glycosylated peptide, corresponding somewhat to the MB2 fragment (pNT 82-107). Because of the localization of such a putative peptide within POMC, it was named the porcine joining peptide (pJP) explaining the fact that it is the link between the pNT 1-80 glycopeptide and the adrenocorticotropic hormone.

Identification and purification of the porcine joining peptide using HPLC

Two milligrams of undialysed "peak A" from CMC chromatography was resolved by HPLC under the same elution parameters used for purification the of MB2 fragment. A peptide with the same retention time as the MB2 fragment (18,35 min) was collected (fig. 31) and repurified under the same conditions (fig. 32). Amino acid composition of the pJP after 24 hours hydrolysis in quadruplicate is presented in table 6. This table included the amino acid composition of the sequenced MB2 fragment. Because of the great similarity between pJP and the MB2 fragment it is possible that pJP corresponds to the pNT 83-107 sequence of pNT 1-107.

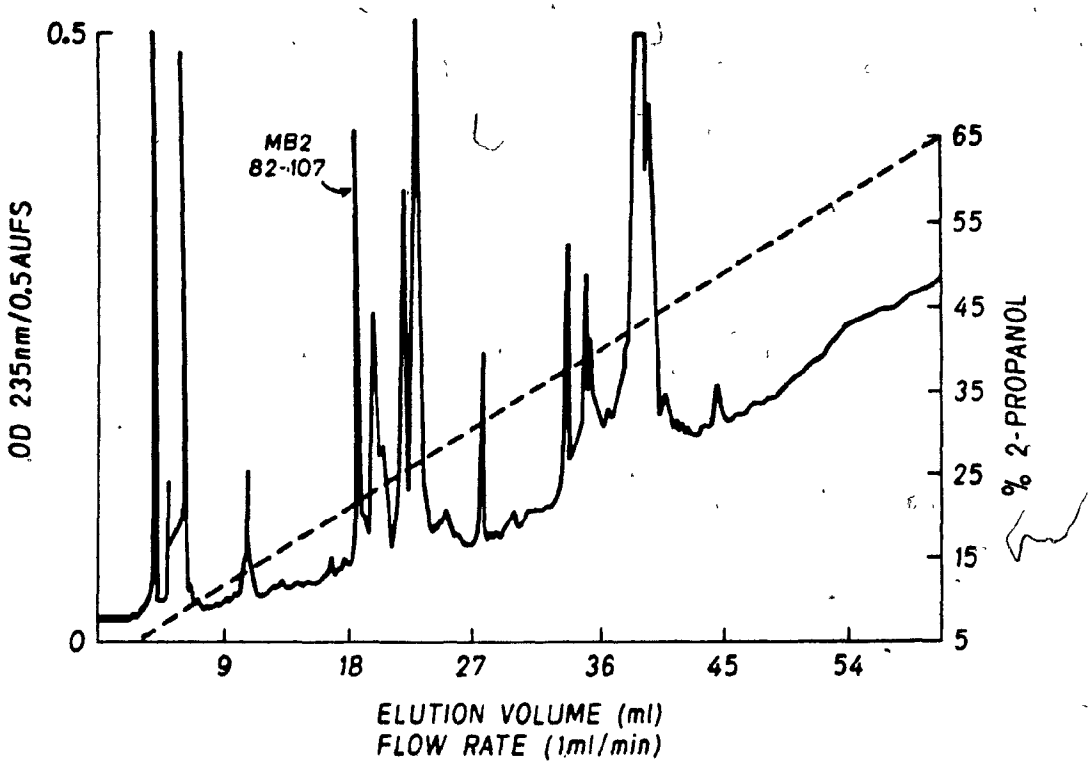


Figure 31. Chromatographic profile on HPLC u-Bondapak C-18 analytical column of undialysed flow through material from CMC of pig pars distalis extract.

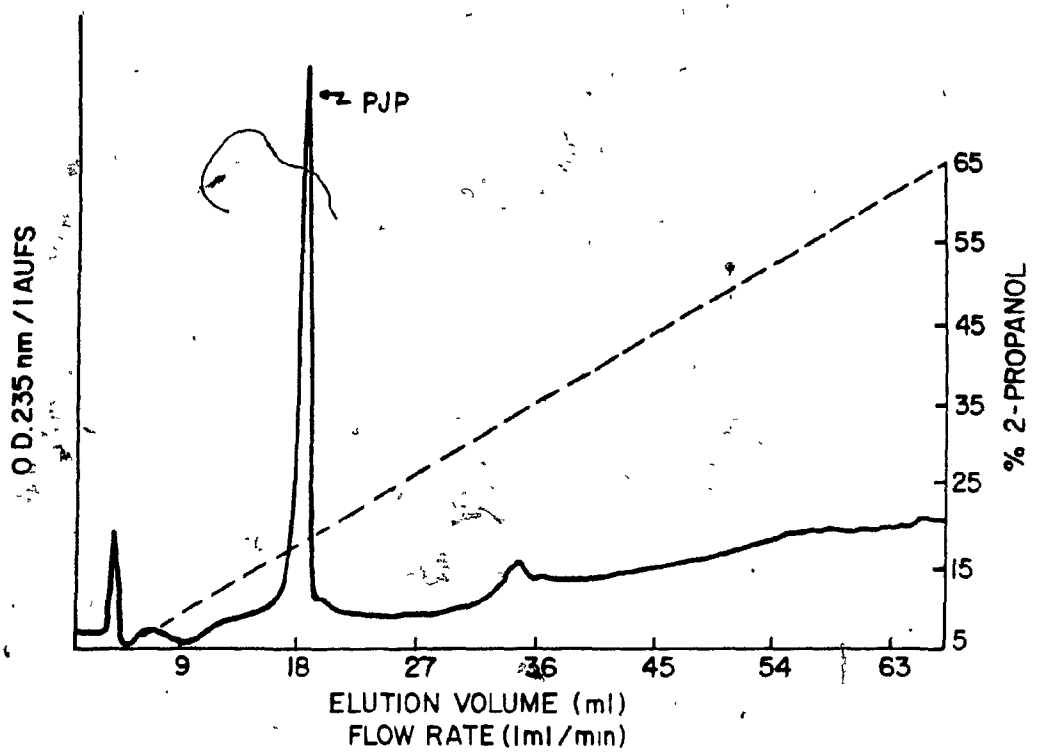


Figure 32. Chromatographic profile on HPLC u-Bondapak C-18 analytical column of the final purification of the porcine joining peptide.

Proposed alignment of pJP against the sequenced MB2 fragment is presented in figure 33.

Sequence of pJP

Direct sequencing of 500 ug of repurified pJP allowed the identification of all 25 residues. The quantitative determination in nanomoles of the phenylthiohydantoin amino acid as a function of sequanator cycle number is presented in figure 34. The proposed sequence of pJP is presented in figure 35.

Characterization of the pNT 1-61 amide

Determination of the amino acid composition of the pNT cyanogen bromide fragments indicated that the CNBr 3 fragment (table 3) contains only seven amino acid residues. These seven residues match exactly the composition of sequence 54-61 of pNT except for the tryptophan residue which was destroyed during acid hydrolysis of the peptide. An aliquot of the fraction 117-130 of the G-75 superfine chromatography of pNT (fig. 12) was digested with pronase and the resulting digest was dansylated. Chromatography of the dansylated digest on thin layer polyamide plates revealed the presence of that residue at position 61 of a lower molecular weight form of the amino-terminal of POMC. Results of the chromatography experiments on thin layer polyamide plates are presented in appendix III.

Table 6

	PJP	MB2	PNT 83-107
Lys			
His			
Arg	2.00(2)	3	2
SCMC			
Asx	1.89(2)	2	2
Thr			
Ser			
Glx	6.00(6)	6	6
Pro	3.93(4)	4	4
Gly	6.05(6)	6	6
Ala	3.00(3)	3	3
Val	1.75(2)	2	2
HSL			
Met			
Ile			
Leu			
Tyr			
Phe			
Trp			
GlcN			
GalN			

AMINO ACID COMPOSITION OF THE PORCINE JOINING PEPTIDE, THE MB_2 FRAGMENT AND THE SEGMENT 83-107 of PNT 1-107.

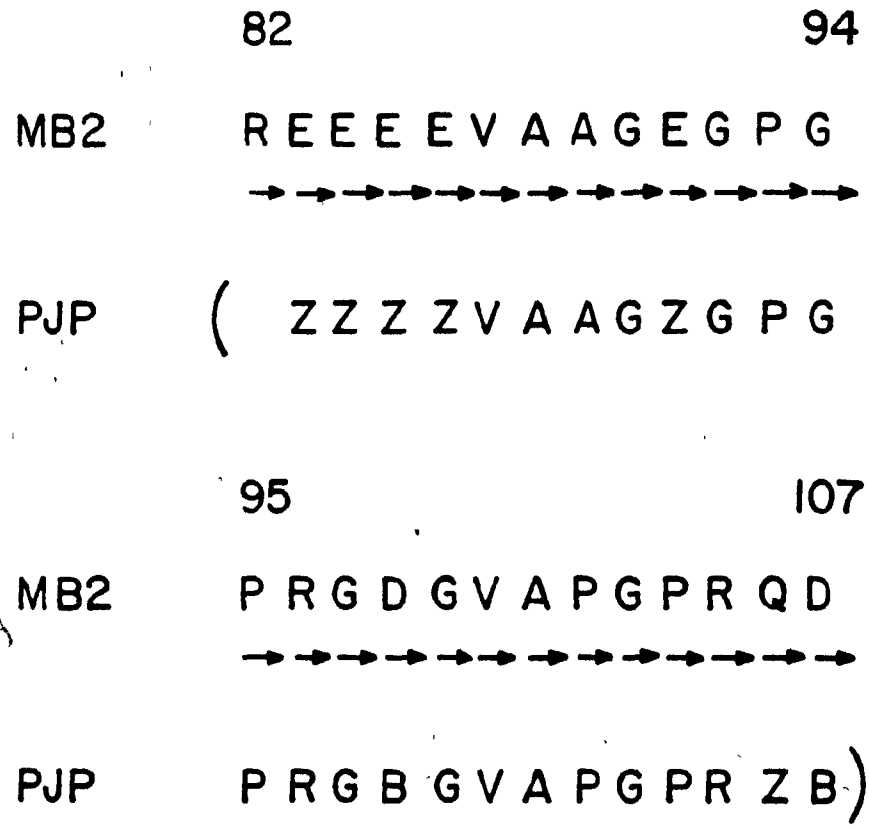


Figure 33. Proposed alignment of the PJP against the sequenced MB2 fragment (residues 82-107 of pNT 1-107).

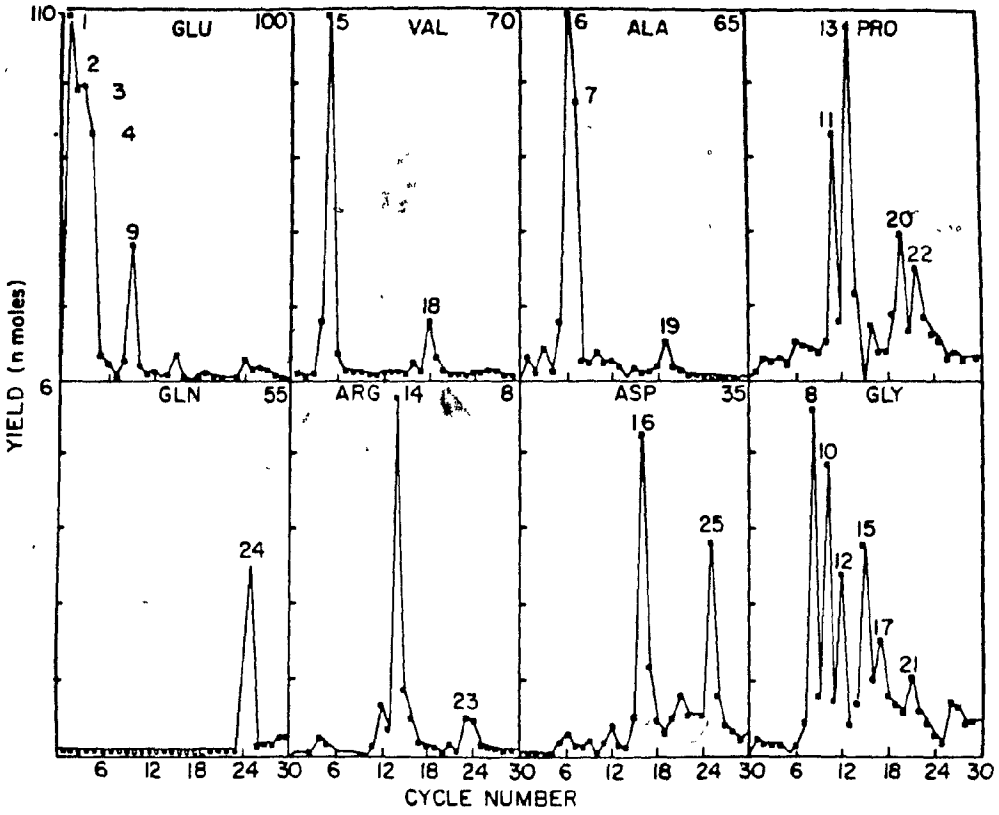


Figure 34. Yield in nanomoles of the phenylthiohydantoin amino acid as a function of the sequanator cycle number for the PJP.

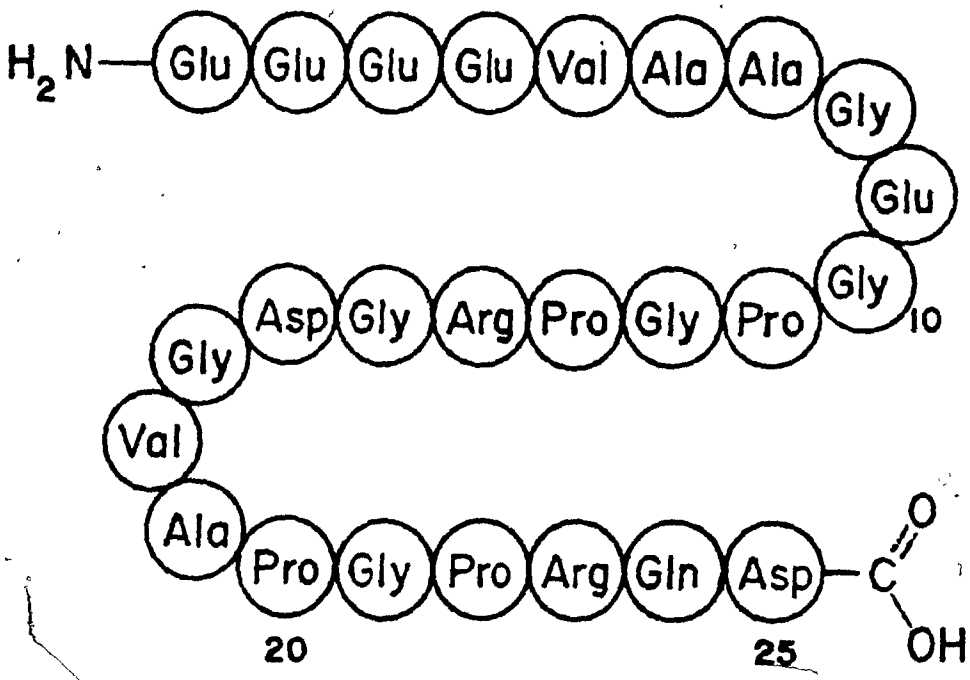


Figure 35. Proposed sequence of the porcine joining peptide.

Radioimmunoassays for ACTH and pNT on extracts of carboxymethyl cellulose chromatography of porcine pars distalis

Fifty microliters of each tube from CMC chromatography of pars distalis presented in figure 6 were assayed with antibodies raised against pNT 1-80 and ACTH 1-24 to detect the presence of these peptides throughout the CMC. This experiment was also set up to detect a peptide which contained both the ACTH and the pNT linked together. Such a peptide appeared in the first step of the maturation process of POMC immediately after cleavage of β-LPH from the common precursor. Distribution of pNT throughout the CMC is concentrated in tubes 10 to 20 and also in tubes 65 to 120. However, ACTH is found in tubes 220 to 250. There is no peak throughout the CMC where both ACTH and pNT together have been detected. Results of the radioimmunoassay for quantitative determination of pNT and ACTH throughout the CMC are presented in figure 36.

A summary of the purification procedure and of the enzymatic and non-enzymatic cleavage of the pNT-related material is presented in figure 37. A diagram of the proposed structure of the four peptides related to the amino-terminal segment of the porcine POMC is presented in figure 38.

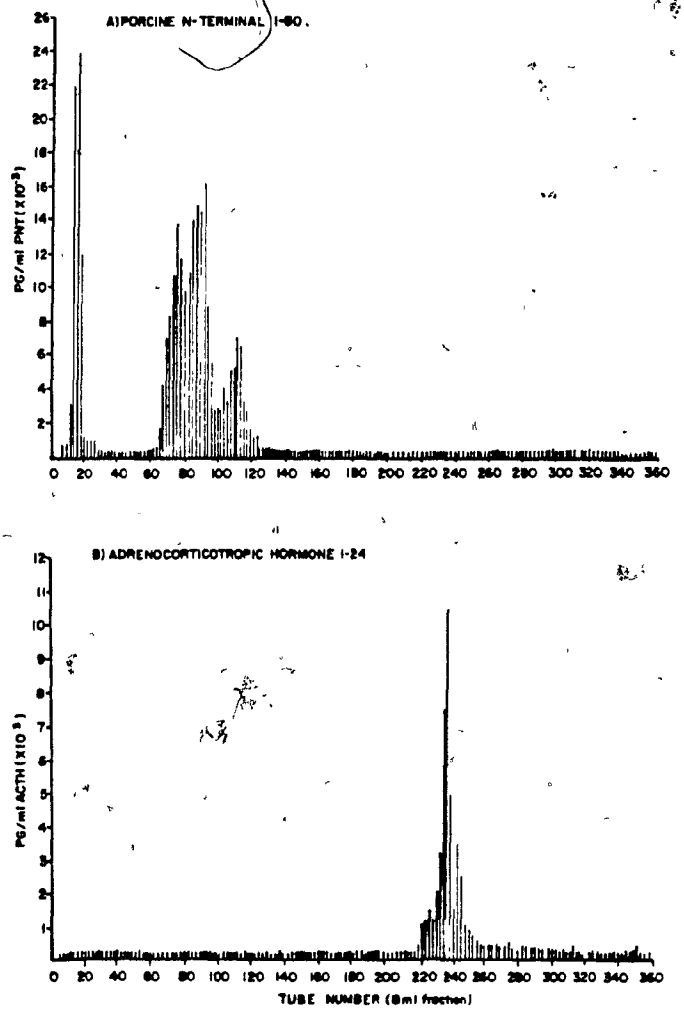


Figure 36. Quantitative radioimmunoassay in pg/mL of pNT related material and ACTH through a CMC chromatography of porcine pars distalis extract.

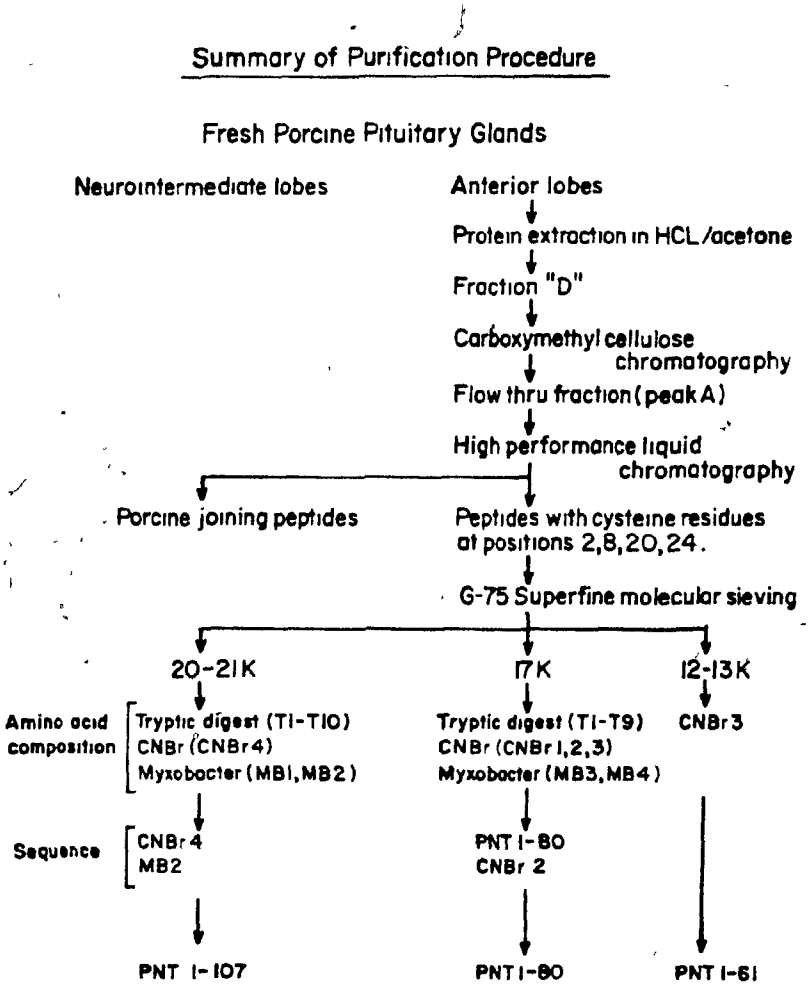


Figure 37. Summary of the purification procedure.

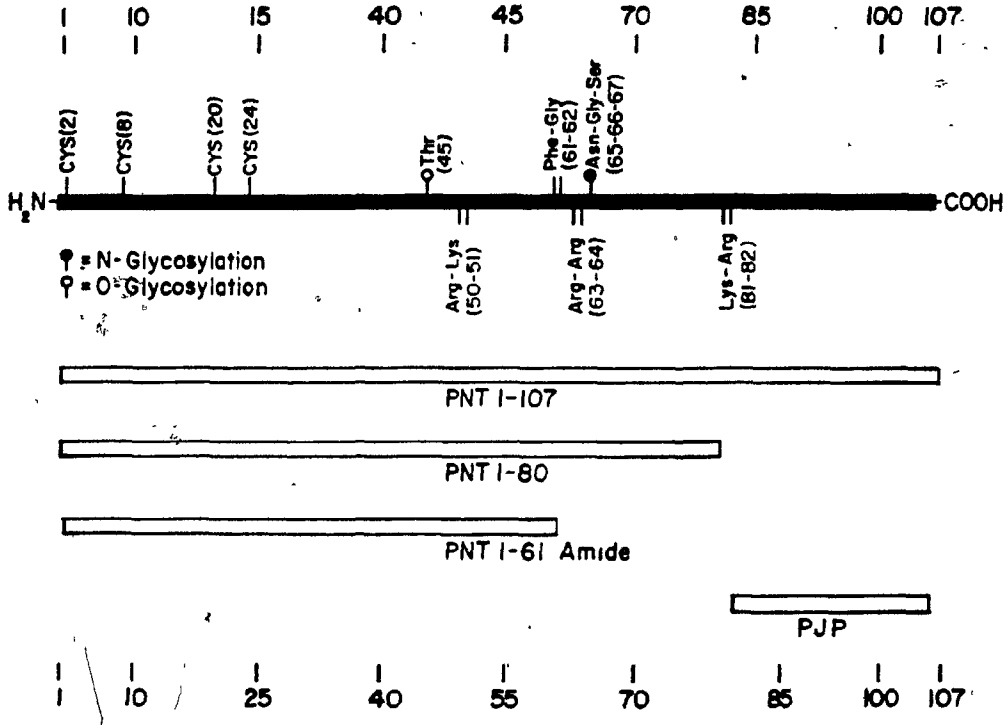


Figure 38. Schematic representation of four peptides isolated and related to the amino terminal segment of the porcine POMC.

DISCUSSION

Results presented allow the identification of new pituitary peptides of the ACTH and β-LPH family. Preliminary indications that ACTH and β-LPH could be issued from a common parent molecule are based on immunochemical and immuno-histochemical studies (132-133). The use of antibodies was also a starting point in the discovery by Orth et al. (50) of high molecular weight forms of ACTH. Immunoprecipitation techniques used by Herbert's group (143, 144, 152, 167, 168), Eipper and Mains (24, 56-60, 142, 148-151, 166) and Chrétien et al. (73, 100, 102, 103, 159, 163-165, 176, 177, 192, 200) are key elements which allowed descriptive identification of the common precursor and of its maturation process.

This procedure must be considered a good criterion for the establishment of a precursor-product relationship. The most important point in this view is to consider the chemical characterization of a peptide's family, including peptide mapping and sequence determination to give the final proof to clarify its maturation process. Such an observation was first made by Chrétien and Li (12) when they proposed that β-LPH could be the precursor of Y-LPH.

Many research groups have clearly determined that during the maturation of ACTH from its precursor molecule, an important additional fragment is produced. Depending on the technique used, this molecule was established to have an apparent molecular weight of 16 000 (148-149) or 17 000 and 19 000 (200) Daltons.

Complete purification and chemical characterization of this molecule would have been extremely difficult without the development of genetic engineering. In fact even if this pituitary peptide was inadvertently isolated and purified in the early seventies by Hakanson et al. (196-198) its relation with the ACTH family of peptides was not established.

Nucleotide sequencing predicts the amino acid sequence of the common precursor. The complete structure of the POMC was first deduced and established for the bovine species by Nakanishi et al. (18). The approach they used could not however specify the exact length of the signal peptide. Once this last problem was solved by comparing the amino acid incorporation and sequence determination of the purified product with its predicted sequence (179, 202), it was clear that in many species the cryptic portion of POMC contains cysteine residues at its amino-terminal (18, 104, 185). Reduction and alkylation of these cysteines with tritiated iodoacetic acid would be a simple way of labeling pituitary peptides and then using microsequencing techniques to find those with cysteines at these precise cycles. From bovine predicted sequence, those residues should be at cycle 2, 8, 20 and 24 (18). Assuming a sequence homology between species, we purified using HPLC a fraction enriched with labeled cysteines at position 2, 8, 20, 24. This approach was the best compromise considering that no specific antibody against this molecule was then available and that no biological activity had been described to use a bio-assay.

Purification of the native 16 K fragment was carried out in the mouse pituitary tumor by Keutmann, Eipper and Mains (202). The material was chromatographed on carboxymethylcellulose and as in our results most of the material was found in the flow-through fraction. We must add that material related to the so called 16 K fragment was also found during the first salt gradient which corresponds to a 0,04 M ammonium acetate pH 4,8, and in the second salt gradient, 0,1 M ammonium acetate pH 4,8 (202). In our hands, using the same type of cation exchanger and the same counter-ion salt, most of the material eluted in 0,01 M ammonium acetate pH 4,6 and an important quantity of immunochemical related pNT material eluted in 0,1 M ammonium acetate pH 6,7 (fig. 36). No other immunochemical-related material was found at higher salt concentration and higher pH through the CMC both in our experiments and those of Keutmann (202). These results indicate that there must be some charge heterogeneity within amino terminal POMC related peptides. This heterogeneity was also observed by Hakanson et al. (202) when they chromatographed what they called the Trp-peptides on a DEAE-Sephadex A-25 column. They further characterized only one fraction enriched in Trp-peptide by chromatography on a Sephadex G-100 superfine column and by SDS-PAGE according to the method of King and Laemmli (246). This latter experiment gave an apparent Mr weight of the purified peptide of about 15 000 Daltons compared to the 11 000 Daltons determined by Sephadex G-100. It was however demonstrated that

SDS-PAGE tends to overestimate the Mr of glycosylated peptide (244).

The presence of sugars in the amino-terminal segment of POMC was demonstrated earlier by Eipper and Mains (148-149). Hakanson et al. (203) clearly showed the presence of glucosamine and galactosamine, in the so-called 11 K trp-peptide, by detection of these carbohydrates on the amino acid analyser. The presence of these sugars explains, at least in part, the heterogeneity observed in the amino-terminal and even in the whole POMC. Experiments which allowed the synthesis of such a peptide albeit without the incorporation of a carbohydrate side chain into the nascent peptide backbone were an essential step in discriminating between the two most probable areas of heterogeneity: the carbohydrate side chain and/or the peptide backbone. Such experiments were carried out by Crine et al. (73, 165) who concluded that the two common precursor forms, 34 K and 36 K, are two similar or identical polypeptides differing mainly in the number of asparagine-linked carbohydrate side chains. According to our results such a hypothesis cannot be retained for the porcine N-terminal. Within pNT 1-80 there is only one position where an N-glycosylation can occur, Asparagine 65. The following amino acid sequence was determined Asparagine^{65}, Glycine^{66}, Serine^{67}, corresponding to the general model proposed by Li, Tabas and Kornfeld (244, 245) for N-glycosylation. So within pNT 1-80 there is only one N-glycosylation site. There is however some belief that micro-

heterogeneity is an inherent characteristic of the carbohydrate moieties of glycoproteins (247). Although studies of the biosynthesis of asparagine-linked sugar side chains have revealed that the core portion of these sugar chains is transfered "en bloc" as a lipid-bound intermediate and transfered to the nascent polypeptide chain (248-250) the elongation step that follows can produce a more or less complete sugar chain. Such heterogeneity is reported in two peptides where there is only one N-glycosylation, hen egg albumin (250) and bovine pancreatic ribonuclease (251). In each of these molecules asparagine side-chains were shown to be a mixture of a series of biosynthetic intermediates. A more detailed study of the asparagine-linked sugar chains in a complex molecule was carried out by Kobata et al. in the human ceruloplasmin (252) and they demonstrated that within the same molecule there is a difference between these chains. In fact some sites are occupied by biantennary and others by triantennary complex type, asparagine-linked sugar chains. On the human chorionic gonadotropin the four asparagine loci link sugar chains that are of different and definite structure (253-254).

These factors considered together should explain some charge or possibly molecular weight heterogeneity within the amino-terminal of POMC. However according to the predicted amino acid sequence for the bovine (18), rat (104) and human (185) there is only one possible asparagine-linked glycosylation site within the whole amino-terminal segment of POMC. In general, the aspara-

gine residue within the amino acid sequence Asn-X-Ser (or Thr) is the site to which a carbohydrate chain is linked through an N-acetylglucosamine (255). Such a sequence is present at only one location within these predicted peptides and the sequence is Asn-Gly-Ser or Asn-Ser-Ser. It is obvious that some heterogeneity might exist within this linked sugar structure but we do not take it into consideration in explaining the molecular weight differences reported for the amino-terminal (165, 200, 202-204, 214).

Considering this glycosylation, which in the porcine N-terminal is possibly located at Asn^{65} (208), we have never isolated a fragment including the sequence Asn^{65}-Gly^{66}-Ser^{67} that was not glycosylated. According to our results fragments T9, CNBr 2, CNBr 4, MB1, MB4 are glycosylated with a predominance of glucosamine over glucosamine. So there must be only one N-glycosylation site within the porcine N-terminal 1-80 and according to our results the Asn^{65} is the most probable locus where it occurs and it is always glycosylated. We have another indirect evidence that the Asn^{65} is glycosylated. During automatic Edman degradation of fragments CNBr 4 we observed a blank at cycle 12 which corresponds to Asn^{65} in pNT 1-80 (fig. 22). Based on the amino acid composition of this fragment we assume that this site is occupied by an aspartic or asparagine. Because of homology with the predicted bovine sequence (18) we assigned Asn as the amino acid residue at cycle 12 of CNBr 4.

Seidah et al. (209) reported the complete primary sequence of the human homologue amino-terminal with an N-glycosylation at Asn^{65}. Hakanson et al. (203) reported the presence of glucosamine and galactosamine within their 103-residue porcine amino terminal but did not assign any site for this glycosylation (s) to occur. In the bovine species, all immunologically related Y-MSH peptides of both the anterior and intermediate lobes were shown to be glycopeptides because they were retarded in the concanavalin A (Con A) affinity chromatography (206). Although this criterion is somewhat irrefutable the conformational difference in the sugar prosthetic groups play an important role to strengthen or weaken binding to the column (256). So even if a peptide is not retained by Con A it is not a proof that this peptide is not glycosylated. In fact a minimum of two interacting mannose residues is required for binding to Con A (257). Considering the binding of γ-MSH related bovine pituitary peptides and the proposed sequence for these peptides (18), Guillemin (206) suggested that the Asn^{65} can be glycosylated in the bovine POMC and that other glycosylation site (s) might exist, namely an O-glycosylation type on the serine residue that follows Asn^{65}.

Our results seem to be more exact and suggest the presence of only one N-glycosylation site within pNT 1-80 and assign this glycosylation to occur at the asparagine 65 residue. We therefore consider that this type of glycosylation cannot by itself explain

all the heterogeneity observed in the amino-terminal segment of POMC.

While analysing tryptic fragments of the pNT 1-80 (fig. 12) on the amino acid analyser, in tryptic fragments T3 and T4 (which, based on sequence homology with the bovine homologue correspond respectively to fragments 23-49 and 23-50 of the N-terminal) we detect the presence of sugars. Galactosamine was predominent over glucosamine in both of them. Further cleavage with cyanogen bromide and Myxobacter Lys-C protease of the pNT 1-80 gave two other fragments, CNBr 1 and MB3, which also contain sugars in the same ratio as T3 and T4 (tables 2 and 3). Both the presence of large amounts of galactosamine as compared to glucosamine and the amino acid sequence of the porcine N-terminal 1-80 point to an argument against an Asn glycosylation site in these fragments (258).

Tabas and Kornfeld (245) proposed that O-glycosylation occurs on either serine or threonine in a proline-rich region and contains galactosamine as the major hexosamine. According to this hypothesis, we observed that within amino acid residues 23 and 49 there is one serine and two threonine with five proline in their immediate surrounding. So between one to three O-glycosylation site(s) may be present. Data obtained from Hakanson (259) for the amino-terminal sequence determination of the first 35 residues of a similar peptide extracted from whole porcine pituitaries clearly revealed the presence of Ser 29 and Thr 32 without loss in the PTH

determination. From these quantitative results and our amino acid composition of fragments T3 and T4, we suggest that the Thr 45 is an O-glycosylation site (209). This hypothesis was reinforced when we completely sequenced the first 52 residues of pNT 1-80 (211). During this sequence study, we observed a net loss of PTH yield at cycle 45 which could have been caused by the presence of sugars on this amino acid. Nevertheless, this is still only an indirect evidence.

In the human N-terminal 1-76 homologous glycopeptide, methionine is substituted for valine at residue 33 (209). The presence of a methionine residue in the human N-terminal homologus peptide allowed Seidah et al. (210) to chemically cut the human N-terminal 1-76 with cyanogen bromide and to obtain three fragments one of which, CNBr II, includes residues 33 to 52. According to the ratio of glucosamine/galactosamine, they concluded that CNBr II contains an O-glycosylation and that the only possible site is threonine 45. No sugars were detected in fragment CNBr I (residues 1 to 33) which contains one serine at position 29 and one threonine at position 32. So based on homology between these peptides in the human and porcine species (220-230) we assumed that there is an O-glycosylation on Thr 45 of pNT 1-80. Based on sugar detection on the amino acid analyser, fragments T3, T4, CNBr 1, MB3, all of which contain the Thr 45, are always glycosylated. We therefore conclude that pNT 1-80 is a

glycopeptide with at least two glycosylation sites, an O-glycosylation on Thr 45 and an N-glycosylation on Asn 65.

Based on the amino acid sequence of these peptides, we concluded that there are no other possible sites for N-glycosylation whereas other O-glycosylation sites may potentially exist. Guillemin (206) proposed that a likely site is the multiple serine that follows asparagine in the γ3-MSH sequence. In the porcine pNT 1-80 asparagine 65 is followed by a glycine then three serines. Within residues 65 to 80 there is no proline which leave some doubt about the presence of O-glycosidic linked oligosaccharides at one or more of these serines (245). We have nevertheless no direct evidence of the presence or absence of other glycosylation site(s) within fragment 65-80 of pNT 1-80.

Based on the amino acid analysis of the tryptic fragments of pNT 1-80 there is no sugar present between residues 1 and 22 and 50 and 64. So it is only between residues 23 and 49 that O-glycosylation could possibly occur. The heterogeneity in the relative masses observed for the common precursor and even in the non-ACTH non-LPH segment of POMC is, therefore, not essentially due to differential glycosylation of very similar peptides as proposed by Crine (73) and Roberts (167). We must however consider heterogeneity between the murine species and the porcine and human homologous peptides. In fact even with the genomic nucleotide sequence (104) and the amino acid sequence of the rat amino-terminal segment of the POMC first 20 residues (179) there

is still the possibility that residue number 10 could be an asparagine instead of an aspartic acid as in the porcine, human and bovine homologues. Since this would give the sequence Asn10-Leu11-Thr12, there is possibly an N-glycosylation on Asn-10. Such an observation was made by Eipper and Mains (60) for the ACTH molecule where it was later demonstrated that Asp 29 found in most mammalian species is replaced by an Asn in the rat (104) and that this Asn is sometimes glycosylated.

Primary structure of the pNT 1-80

Nucleotide sequence of the genetic material coding for POMC predicted great homology among mammalian species (18, 104, 185). Detailed analysis of whole and fragments of the purified amino-terminal from pig and man pituitaries revealed some heterogeneity among these molecules (209, 214). As mentioned in these two species, glycosylation can be one of the causes of this heterogeneity but the possibility of a peptide backbone mutation cannot be excluded. We therefore attempted to verify the possibility of elongation or shortening of the peptidic backbone as a cause for the different apparent Mr of the N-terminal. Analysis of tryptic fragments, cyanogen bromide fragments and two-dimensional polyacrylamide gel electrophoresis of our purified pNT 1-80 isolated from anterior pituitaries revealed heterogeneity in molecular weight and isoelectric points of this molecule (214).

Such differences were observed to a lesser degree in a homologous peptide prepared from human pituitaries (209).

The major isolated form of pNT was established to be 80 amino acid residues (211) with a least two glycosylation sites (208). Based on amino acid analysis and peptide mapping we, in a preliminary attempt, predicted this peptide to be 103 residues (208). Hakanson (203) isolated a very similar glycopeptide from pig whole pituitaries also with an estimated length of 103 residues. The amino acid sequence of the first 35 residues of this peptide was also reported (203).

We therefore completely sequenced our 17 K apparent Mr glycopeptide and found that it is composed of 80 residues. From these experiments we also concluded that position 4 is occupied by a Glu instead of an Asp as published by Hakanson (203) and filled the blanks at positions 15 and 25 where we assigned a serine and a lysine respectively. From our complete sequence it is apparent that the γ-MSH sequence (residues 51-62) is identical to the predicted bovine (18) human (155, 185, 204, 209, 212) and rat (104) homologues and that this peptide segment is flanked on both sides by glycosylation sites (208-211). The role of these prosthetic groups is not yet understood except that their presence seems to increase the half-life in circulation (260), and, as reported for other secretory proteins, they protect the molecule from random intracellular degradation (244, 260-263) or are involved in guiding the maturation of pro-secretory proteins (168, 274).

In our preliminary report (208) our predicted 103 amino acid residue amino-terminal was based on amino acid analysis of tryptic fragments and homology with bovine cDNA sequence (18). It is now clear that tryptic fragment T10 represented only a minor constituent of the tryptic digest of the purified 17K pNT. Further analysis by two-dimensional PAGE (appendix 2) also revealed three forms of the N-terminal glycopeptide with respective apparent Mr of 21 K, 17,5 K and 13,5 K previously isolated by chromatography on Sephadex G-75 superfine. As described earlier, ~~the~~ 17 K is an 80 amino acid residue glycopeptide and was completely sequenced (211). We also investigated the high molecular weight (H Mr pNT) and the low molecular weight (L Mr pNT) peptides and found them to be respectively longer and shorter forms of the porcine amino terminal segment of POMC.

Determination of the amino acid composition and amino acid sequence of fragments CNBr 4 and MB2 after cleavage of the H Mr pNT with cyanogen bromide or with myxobacter Lys-C protease (Mannheim-Boehringer) has shown that the H Mr pNT is a longer form of the pNT 1-80 containing an extension of 27 amino acid residues at its C-terminal. The extension is connected to the pNT 1-80 through a pair of basic residues LYS-ARG at positions 81 and 82 respectively. This pair of basic residues is also present in bovine (18), rat (104) and human (155) homologues. In this latter species the major secretory form of the amino-terminal segment of the POMC contains 76 residues (210) and is the homologue of pNT

1-80. Preliminary results from whole human pituitaries has revealed that the major secretory form, hNT 1-76, is slightly contaminated by two extra peptides of higher apparent Mr as shown by SDS-PAGE and their isoelectric points of 5,0 and 4,9 as compared to 5,3 for the hNT 1-76 (210, 265).

The presence of the pair of basic amino acid residues at positions 81-82 suggests that the extension peptide can be readily removed by the same maturation enzyme(s) that released ACTH and β-LPH since the LYS-ARG sequence is also present at the cleavage sites of these peptides (18, 104, 155). Such a processing of the N-terminal would yield in the pig the pNT 1-80 and a 25 amino acid residue after excission of the LYS-ARG sequence at position 81-82, and a putative peptide between the pNT-terminal 1-80 and the ACTH. Such a peptide was previously isolated from human pituitaries and was named the "joining peptide" (212). However in man, this peptide is 31 residues long as compared to 25 in the pig. This explains the results of our preliminary reports (208) in which tryptic digest had revealed the presence in minor amounts of peptides such as fragment T10, related to the C-terminal portion of the HMr pNT.

Such an observation was made with human material where two minor tryptic fragments, T15 and T16, represented only minor constituents (205) and where T16 was not part of the hNT 1-76 (210). From these results we assumed that the 21 K apparent Mr form of the amino-terminal segment of POMC in the anterior lobe of

the pig pituitary consists of 107 amino acid residues and may represent the whole amino-terminal from the TRP 1 residue (203, 211) to the putative pair of basic residues preceding ACTH.

Chemical characterization of the LMr pNT with an apparent Mr of 13,5 K on SDS-PAGE was performed on a fraction enriched with the peptide obtained from a Sephadex G-75 superfine chromatography of pNT 1-80. The fraction was submitted to cyanogen bromide cleavage and HPLC revealed the presence of three fragments and showed them to correspond respectively to the amino acid composition of fragments 1-53, 54-80 and 54-61. So we believe that the 13,5 K Mr pNT represents the first 61 residues of pNT 1-80. The loss of 19 amino acid residues including one site of glycosylation at Asn65, is consistent with the loss of around 4 K on SDS-PAGE. Shibasaki et al. (266) already suggested the presence of an amidated phenylalanine residue at the C-terminal of Y_1-MSH (residues 51-61 of the bovine N-terminal homologous peptide). This observation is based on immunochemical data considering the specificity of an antibody. The presence of a phenylalanine amide at position 61 as revealed by dansylation (214), suggests that LMr pNt although found in low amounts, may be maturation product of the N-terminal. Amidation of the penultimate residue of a peptide often occurs when a glycine residue is at its C-terminal (267-270). Examination of the sequence of pNT 1-80 (fig. 18) indicates that the phenylalanine residue at position 61 is followed by the sequence -Gly^{62}-Arg^{63}-Arg^{63}. It is proposed that the cleavage of

the peptide first occurs at the position of the paired basic residues generating a peptide with a glycine residue at its C-terminal. Removal of this glycine residue by specific enzyme(s) results in the amidation of the phenylalanine residue at position 61. Even if in our preparation the LMr pNT is present in small amounts, recent studies by Hsi, K.L. et al. (271) found large amounts of such a peptide in the "prolactin fraction" extracted from anterior lobes of porcine pituitaries.

These results suggest that the N-terminal is partially processed further than the 80 residues glycopeptide and that amidation reaction does occur in the anterior lobe of the pituitary.

In the rat pars intermedia, Crine et al. (165, 200) showed that the N-terminal glycopeptide of POMC accumulates during maturation as two peptides with apparent Mr of 19 K and 17 K. Based on the predicted sequence deduced from rat genomic DNA library the whole rat amino terminal should be 95 amino acid residues long with the presence of a LYS-ARG sequence at position 75-76 (106). However in the rat species the sequence -ARG-ARG- found at position 63-64 as in most mammalian species is replaced by -PRO-ARG-. By analogy with the porcine species it can be speculated that these two rat N-terminal glycopeptides conceivably represent the 1-95 and 1-74 forms of the predicted sequence (106). A 21 amino acid residue loss can logically account for a 2 K diminution on

SDS-PAGE. However further chemical chracterization of both 17 K and 19 K is needed to confirm this assumption.

Chemical characterization of peptides related to the amino terminal segment of POMC from the anterior lobe of pig pituitaries has revealed that the smallest peptide isolated so far is 61 amino acid residues long. We must however keep in consideration that all these peptides were isolated from the flow-through fraction on carboxymethylcellulose chromatography followed by ultrafiltration through a 10 000 Mr cutoff dialysis membrane. Radioimmunoassay with an antibody raised against the pNT 1-80 has revealed the presence of an N-terminal related material in the second elution step of the CMC. We, however, did not characterize this material. Crine (165) has isolated the 17 K and 19 K fragments from rat pars intermedia cells chased for up to 4 hours with the same yield as after 2 hours chase. Considering this metabolic stability, he proposed that the entire glycosylated N-terminal fragment, and not the γ-melanotropin heptapeptide (18), might be the true biologically active species (165, 200).

Shibasaki (206) working on anterior and intermediate lobes of bovine pituitaries proposed a different maturation process of the γ-melanotropin related peptides in these two tissues. In the anterior lobe the γ-MSH like peptides have apparent Mr of 13 K and 8,8 K whereas in the intermediate lobe a third peptide with an apparent Mr of 4,5 K was found. All these peptides cross-react with an anti γ_3-MSH antiserum. He proposed that the N-terminal is

further processed to smaller peptides and even to γ-MSH. We have, to date, no evidence of such a process and have never found the native peptide in the pig anterior pituitary.

Working in the same tissue as Crine, Browne et al. (272) chemically characterized a peptide related to the N-terminal which extends from the first residue of γ-MSH (106) to beyond the site of amidation at Phe^{61} (214). These data, in addition to those from the bovine pituitary and ours from the pig pituitary are sufficient to consider a more detailed characterization of the maturation products of the N-terminal. We proposed that a number of peptides and glycopeptides are produced from the whole N-terminal POMC and that differences may be due to composition of the peptide chain, glycosylation and even some post-translational modification such as glycosylation and phosphorylation as reported for the CLIP in the rat pars intermedia (74).

Even if primary structure alone cannot be used to determine the maturation process of a peptide, our finding of the HMr pNT and of what we consider its maturation product, pNT 1-80, LMr pNT and the "joining peptide", gave further indication of the role of the pair of basic residues -LYS-ARG- in the enzymatic processing of some precursor molecules. As was proposed by Chrétien (12), based on the LPH model, all precursor peptides contain paired amino acid basic residues at the site of cleavage. Steiner later observed that arginine rather than lysine seems to be preferred on the carboxyl side of the pair (13). Such a sequence is present at

only one place in the whole N-terminal and in all species studied so far. Our finding of a 107 residues pNT (HMr pNT) and of the pNT 1-80 plus a 25-residue "joining peptide" indicates a step in the maturation process of the N-terminal. We proposed that such a step occurs in the anterior lobe and that the -LYS^{81}-ARG^{82}- is the site of cleavage. The pair of basic residues -ARG^{63}-ARG^{64}- is also cleaved, to further yield the pNT 1-61 amide but our results did not allowed quantitative evaluation of this reaction. However this pair of basic residues is not present in the rat (106). There is still some controversy about the role of the -ARG^{49}-LYS^{50}- found in all species studied so far. Browne (272) has some clear evidence of a further processing of the rat N-terminal including a cleavage at this precise site. We however did not isolate such a peptide from the anterior lobe of the pig pituitary. Based on our sequence data, we proposed a tentative model (shown in fig. 39) for the maturation of the N-terminal peptide of the porcine POMC in the anterior lobe.

It was proposed for the processing of the POMC in the human (209-210) that the maturation enzyme responsible for the generation of all ACTH-related peptides is very selective, since it cleaves exclusively at the pair of basic residues-Lys-ARG. This pair of basic residues occurs in both the human (209) and porcine species (214) after the N-terminal of respectively 76 and 80 amino acid residues and furnishes the opportunity for the release of the "joining peptide". Such a peptide has been

isolated in the human species (212) and now in swine but in small amount. Explanations for such a low recovery include a loss during the extraction and purification procedure, the destruction of the peptide by protease(s), or its existence in other forms such as found in ACTH. Such other forms have not been isolated as yet. It is clear from nucleotide sequencing in other species that this "joining peptide" shows more heterogeneity than other segments of the common precursor, making more unlikely the possibility of a biological function.

No higher molecular weight peptide (above 21 K) was isolated from material related to pNT. We believe this is due to the fact that we dissected pituitary gland into anterior and neurointermediate lobes approximately one hour after death of the animal. Pulse-chase experiments in rat pars intermedia have shown that about 30 minutes are necessary to process the common precursor into a 25 or 27 K fragment and β-LPH and that within two hours the 17 K and 19 K fragments are released (165). If the synthesis of POMC is arrested at the time of death, it is conceivable that after 60 minutes there is no common precursor and a very little amount of the peptide containing HMrpNT and ACTH.

Since no labelling of cysteine residues occurred upon (^{14}C) iodoacetamide derivatization in the absence of a reducing agent,

the four cysteine residues at positions 2, 8, 20 and 24 must be involved in disulfide bridging. Theoretically only three possible linkages exist. In order to differentiate between the three possibilities we used the staphylococcal V8 protease (273), a glutamic acid specific endopeptidase theoretically capable of cleaving between the Cys bridges at Glu 4 and 14. Unfortunately, even if such a cleavage occurred (unpublished data), we were unable to characterize by amino acid analysis the fragments obtained and therefore could not assign a definite location for the cysteine bridges. However in the human (210) homologous peptide the two disulfide bridges were assigned to a position between the cysteine residues 2-8 and 20-24. The first bridge contributes further to the homology between the sequence of the known N-terminals and all the known calcitonins (274). This type of homology raises the possibility that the immunochemical approach used to detect the presence of calcitonins within the anterior and intermediate lobes of the rat pituitary (267) might cause cross reactivity with the amino terminal segment of the POMC.

Most of the conclusions drawn from this work are based on chemical characterization of native peptides extracted from fresh porcine pituitaries. The quality of the tissue we used is, we believe, the best available if we consider that the amount of time spent between the slaughtering of the animal and the preservation of its pituitary in liquid nitrogen was less than one hour. Before collecting pituitaries on a large scale we perform the follow-

ing experiment on rat to evaluate the viability of the gland after death. We killed rats by decapitation and wait 15, 30, 60 and 120 minutes before dissecting the pituitary. At each time period, we carried a typical pulse experiment in the intermediate lobe and we observed that even 120 minutes after death this tissue is still able to synthesize ACTH related peptides (unpublished results). So we believe that the peptides we characterized should not represent artifacts or post-mortem degradation products. As published in results the cyto-architectural organization of the gland is well preserved one hour after death and no cell lysis was observed.

Most of our knowledge about the POMC model is based on pulse-chase experiments in the rat intermediate lobe or in a tumor type cell line, mouse ATt-20/D16v, from nucleotide sequence determination and from whole pituitary extract. We concentrated our effort on an homogenous tissue, the porcine anterior pituitary, and were able to isolate by chemical approach, multiple forms of the amino terminal segment of the POMC. From our results we concluded that the whole amino-terminal segment of POMC in the porcine anterior pituitary is 107 amino acid residues and it contains within its primary structure three pairs of basic residues, -ARG^{49}-LYS^{50}-, -ARG^{63}-ARG^{64}-, and -LYS^{81}-ARG^{82}. Of these three pairs the latter is enzymatically cleaved to give pNT 1-80 and pJP. Both of these peptides were isolated and their complete structures determined. PNT 1-80 is a glycopeptide with at

least two sites of glycosylation, one N-glycosylation on ASN^{65} and at least one O-glycosylation on THR^{45}; we cannot exclude the possibility that other O-glycosylation sites could be present. There is no glycosylation within pJP and this peptide shows great heterogeneity with homologues from other species. In the rat, considered a model for studying the maturation of POMC, there is some mutation in the dibasic residues. These dibasic residues are $-ARG^{49}-LYS^{50}-$; and $-ARG^{75}-ARG^{76}-$. According to Crine's work where the 19 K and 17 K represent the maturation products of the rat POMC (200), it is conceivable that the whole 95-residue amino-terminal does not mature further as in the porcine homologue.

Based on amino acid composition and two dimensional gel electrophoresis (appendix II), we isolated a third form of the amino terminal composed of only 61 amino-acid residues with an amidation of the phenylalanine residue at position 61. We believe that such post-translational modification could not occur in the rat because of the lack of the dibasic residues $-ARG^{63}-ARG^{64}-$ which is replaced by $-PRO^{63}-ARG^{64}$ (104). This 61 residue amidated glycopeptide is the smallest form of the N-terminal we have isolated so far. We did not succeed in our efforts to isolate γ-MSH as such (unpublished results) even if a pair of basic residues $-ARG^{49}-LYS^{50}$ immediately preceded it in all species studied (18, 104, 185, 210).

Other post-translational and/or co-translational modifications of the POMC family of peptides have been reported. Among

them, Browne et al. (74, 276, 277) reported that about half of the ACTH 1-39 in the rat anterior pituitary and two thirds of the CLIP in the rat intermediate pituitary are phosphorylated on Ser_{31}. Eipper and Mains (278) reported that about 5% of the 16 K fragment molecules in rat pituitary contain phosphoserine and that Ser_{29} present in all known 16 K fragment (18, 104, 204, 211) is part of the sequence Ser-X-Glu found in most phosphorylated proteins (279-280). The incorporation of [32P] in glycosylated and nonglycosylated forms of ACTH and CLIP argues against the suggestion that phosphorylation at Ser_{31} prevents glycosylation (276-277). However the physiological role of phosphorylation of ACTH and CLIP and 16 K remains to be elucidated (279).

The role of sugars in synthesis, processing and secretion of POMC related peptides was studied by Budarf and Herbert (281) and they concluded that glycosylation is not an essential step for correct cleavage of secretion of POMC or its products. A more essential role was assigned to the arginine residues when Crine and Lemieux (282) using canavanine, an analog of arginine, found that its incorporation into rat's neurointermediate lobe considerably slows down the conversion of POMC into its different end products.

It is believed that nascent POMC molecule follow the general pattern of most secretory proteins studied so far in its travel through the cell's compartments. In a first step the POMC is inserted into the lumen of the RER where removal of the 26

residues signal peptide and addition of high mannose oligosaccharide side chains occur (5, 6, 25-29, 30-36, 38, 39). Then all the subsequent cleavages presumably occur while the molecule travels from the RER to the Golgi and into the secretion granules (283). Trimming of the high mannose and addition of terminal sugars occurred during this journey especially at the Golgi level where alpha-1,2-mannosidase and glycosyl transferases activites are reported (40, 284, 285). In the secretion granules, where protease(s) involved in the processing of the POMC are located some maturation enzymes have been partially purified from rat intermediate and anterior lobes (286-287). Crine et al. (288) using Monensin which has been shown to slow down the intracellar transport of newly synthesized proteins demonstrated that the POMC leaves the Golgi intact and is then packaged into secretory granules where the first cleavage occurs. It has been suggested that the enzymes involved into the maturation should have trypsinand carboxypeptidase B-like specificities (289, 290) but very little is known about whether the processing of the POMC is catalysed by a single enzyme or a group of enzymes. However the primary structure of the arginine vasopressin-neurophyrin II precursor as defined by cDNA sequence (291) which is also processed into several functional polypeptides is cut at a double basic residues LYS_{11}-ARG_{12} whereas the ARG 108 is cut instead of the pair of basic residues ARG_{105}-ARG_{106}. In the case of the pro-enkephalin a 40-50 K Mr precursor synthesized in the adrenal medulla which

contains several copies of a biologically active peptide, five of the enkephalins are immediately bounded on the C-terminal side by a pair of basic amino-acid residues and all the seven enkephalins in the precursor is preceded immediately by this processing signal (292). Other hormones and active peptides have been found to be contained within the sequence of a larger precursor protein and are released by limited proteolytic cleavage (13-21). In the majority of these structures, the cleavage mechanism is clearly directed towards pairs of basic amino acids which act truly as "markers" directing the release of sometimes biologically active products (290). However, the presence of such "markers" cannot be thought as a prerequisite for maturation since for propressophysin (291), prosomatostatin (293-294), chicken proalbumin (21, 295) and preprorelaxin it has been proposed that conformation around the cleavage site could alter the normal processing (297). It also has been found that basic pairs, which are recognized, are located in structureless region whereas most unrecognized ones are localized in highly structured regions (298). Nevertheless, it seems reasonable to consider taht the basic pair is clearly not the sole requirement for recognition during the maturation process and that factors such as glycosylation, phosphorylation, sulfation and factors influencing the conformation around the recognition site are participating.

We present in figure 39 a tentative model for the maturation of the N-terminal peptide of porcine POMC in the anterior lobe of the

pituitary gland. The N-terminal portion of POMC is first released as a glycopeptide of 107 residues by enzymatic cleavage at the site of the putative pair of basic residues 80-residue glycopeptide which was identified as a major form of the N-terminal base on extraction recovery. This peptide can further be processed into a 61-residue glycopeptide by enzymatic cleavage at the -ARG^{63}-ARG^{64} site with concomitant removal of the glycine residue at position 62 and amidation of the phenylalanine at position 61. We have no evidence so far for further processing of the N-terminal in the anterior lobe.

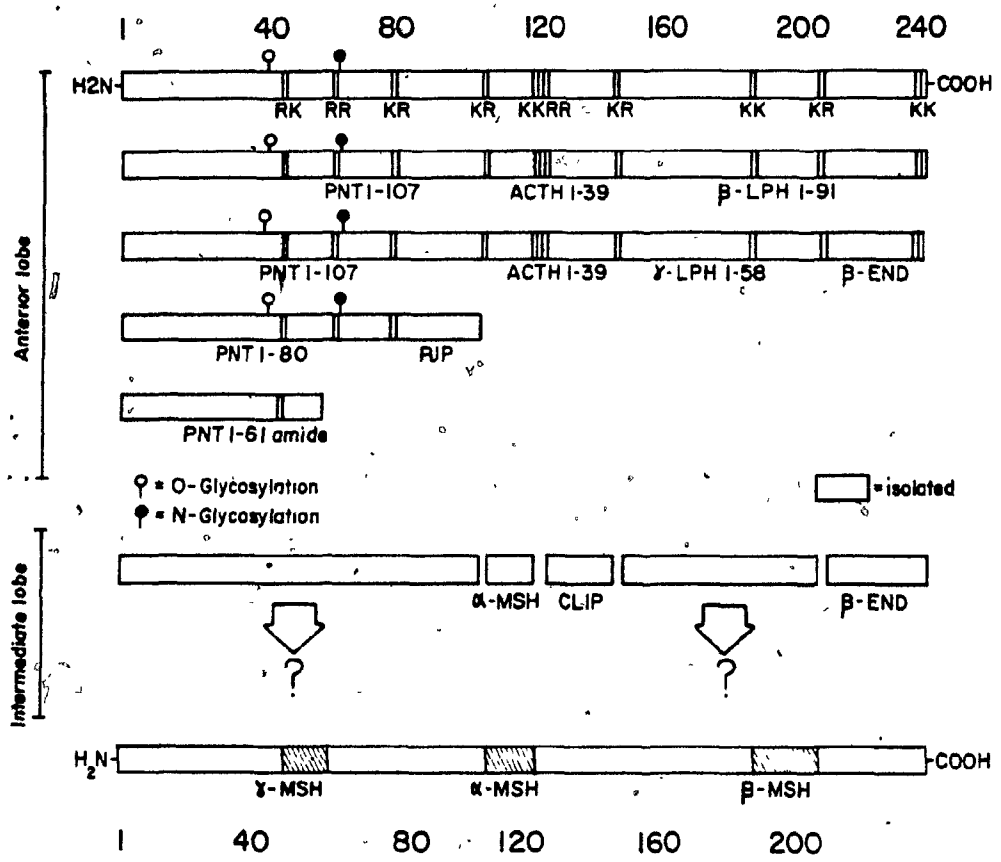


Figure 39. Proposed maturation pathway of the aminno terminal segment of the porcine POMC in the anterior and intermediate lobes of the pituitary.

Claims to originality

1- In all domestic mammals the pituitary complex is characterized by the presence of the anterior, intermediate and posterior lobes. In our experimental procedure we separated the anterior from the neurointermediate lobe to study the amino-terminal segment of the POMC in an homogeneous tissue. Because both the intermediate and anterior lobes are able to synthesize ACTH and its related family of peptides we were interested in the fate of the amino-terminal segment of the POMC in a tissue where pulse-chase experiments are difficult to carry out. This is why we have chosen to isolate from the anterior lobe multiple forms of an homologous peptide which may represent different steps in the maturation of the amino-terminal of the POMC.

2- Using a chemical approach we presented further evidence of the glycopeptidic nature of the pNT. We were the first to propose that at least two glycosylation sites are present within pNT, an N-glycosylation on Asparagine 65 and an O-glycosylation probably on threonine 45. There is the possibility of other O-glycosylation sites within the molecule but the primary structure of HMrpNT does not allow for the presence of other N-glycosylation sites.

3- Our results give some information about the role of pair basic residues in the processing of POMC itself which is also found in most of the peptide models.

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APPENDIX I

	CYCLE 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Aspartic																	
S-CMC		.1	2 9						1.5			.7					
Asparagine					2.2			.2					1.1		1.0	[6 1]	4.2
Glutamic		.3		[1 1]									.45				
Serine		.3			[2.2]		.24	.6	.3			[1.4]	1.3		[1 2]		
Glutamine		.5		.5			[5 5]	3 8	[4 9]			.8	1 1		.9		.4
Threonine		.3						1.2	.6	1.3	.4	3.0	[6 2]		1.3		1.1
Glycine	2.8	9 5	12 2	5 1	1.9		5 0	3.3	3.5		2.9	4.2	5 3		5.7	8.7	10.2
Histidine												3					
Alanine	14 1	5 5	16 1	4 7	16.6		7 0	8 0	6.9	6 4	5.3	10 6	7.1	5.2	9.2	10 0	14 6
Asp-O-Me	9	9	1 9	2 1	1 7			.8	3.4	[4 5]	4.5	3.4	2 5		1 2	4.6	
Arginine		.8									1.2	.7					
Glu-O-Me	6 8	3.9	6.9	[17 1]	6.5		10 9	11 0	7.5		4.4	3.5	6.6	[12 9]	8.0	6 9	4 9
Thyrosine	10 6	1.3	.5												7	3.2	
S-CMC-O-Me	5 9	[13 3]	2 4		6.5		3 4	[12 5]	3.7			9	1 3				
Proline	5 5		3 5				85	5.8			1.0	5.5	7.8		5.1	7.6	5 3
Methionine															4.8		
Valine	5 6	6.2	5 9	2 0	1.9		6 6	3.2			2.3	3.7	2.9		1.0	4 4	5 1
Tryptophan	[28 8]	1.9													1 0		
Phenylalanine	6 4	1 0	1.4	8 3	1.8							1.4	1.5		1 9		
Isoleucine	.8	9						1.6			1.2	1.6	.9				
Lysine		.7															
Leucine	3 3	2	[41 0]	5 9	7 5		4 4	4.8	5.4	4.8	[18 1]	9.9	4.9	7 1	5 5	13 8	[34 2]
	TRP	CYS	LEU	GLU	SER	( )	GLN	CYS	GLN	ASP	LEU	SER	THR	GLU	SER	ASN	LEU

CONFIRMED RESIDUES

	CYCLE																
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Aspartic																	
S-CMC						1.9											
Asparagine	2 3		9	1 2		4.2									4		
Glutamic													6	1.7			
Serine	.2		.6	2	.8							[2]		5	.3		
Glutamine	1.4		7	7		5	3	8	.7	6	.7	5	.1	8	[.5]	1.1	
Threonine	6		.7	5	.3		1					4	.6	1.5	1 9		
Glycine	6.4		4.9	5 2	5 1	4 7	4 3	4.3	3 7	2 5	3 0	6 4	5 8	7 3	4 0	5.0	4 3
Histidine																	
Alanine	12 4		15.2	14 2	14 1	[20 8]	13 8	8 9	6 2	4 6	4.7	7 5	[13 9]	10.9	6 6	5 7	6 1
Asp-O-Me	1.4		1 3	2	1 2	1.5	1 02	1 2	1 9	[3 9]	2.7	2 4	2.4	1 9	1 1	1.1	.8
Arginine			.7	1 4	[1.5]	1 1		.6	3		.4	4	.4	.9	6	.5	
Glu-O-Me	4.2		3.7	4.4	3 2	3.9	3 8	3.4	3 5	3 1	3 5	3 1	4 7	[9 3]	4 1	3 8	2 8
Tyrosine	.5		.4	6	.4	.4					1.7	1 5	8	1 4	.6		
S-CMC-O-Me	1 1		[8 2]	4.2	1 7	2 6	[6 1]	[2.6]	1 3	1.2		1.2			.7		
Proline	3 0		3.6	3.3	3 2	4 1	4 7	4.7	[7 1]	5 1	.9	3 6	5 6	7 2	3 9	[6 8]	4 7
Methionine																	
Valine	2.3		3 2	2.9		3.0	3.5	2 6	2.5	1 7	2.1	3 4	3 0	6.5	3 1	4 5	[9]
Tryptophan	2 4		1 6	2 0	.8		.4	3				.6		5	.5		
Phenylalanine	2 4		1 5	1 9	2 8	2.2	1 5	1 4	1.3	1.2	1.2	1 9	2 2	2 8	1.6	1 9	6 9
Isoleucine	1 3		4 0	[13 0]	5.5	2.6	1.5	.7	1.4	.8	.7	.9	1.0	1.1	7	1 0	9
Lysine	5		.5	.3			.1	4	.1			2			.3	.4	5
Leucine	[25 1]		6.6	5.9	4.3	5.4	4 5	4.2	4 6	5.0	[7 9]	7.7	7 3	6.2	3 9	3 8	-3.5
	LEU	( )	CYS	ILE	ARG	ALA	CYS	CYS	PRO	ASP	LEU	SER	ALA	GLU	THR	PRO	VAL

CONFIRMED RESIDUES

CYCLE

	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
Aspartic		3 3				[2 3]			3 5			3 6	.8		3 8		
S-CMC		2 2										2 2			2 4		
Asparagine		1.0	1 3	[1 4]	2.0		.9	1 5	.8		.7	1.4	[1.6]	.8	2.0	1 2	1.6
Glutamic																	
Serine		.1	.2	.3	.3	.5	.4	1	.2		.4	.3	.7	.5	3	2	.6
Glutamine		6	.2	3	.6	.5	.5	[9]	.8		6	.8	.5	.3	.2	.6	
Threonine		4	5	6	.7		.6	3	.4		.8	.8	.7	.5	.9	.5	.5
Glycine	4 6	5 7	[8 9]	8 2	[8 6]	6 6	5 8	4 5	4.9	3 6	5 2	5 3	5.6	4.4	5 2	6 1	4 0
Histidine																	
Alanine	5.3	5 8	4 7	5 3	5.0	4 6	[6 2]	5 0	3.7	4 1	4.0	3 7	4 3	3 8	4 3	4 3	2 9
Asp-O-Me	.8	8	.8	1 4	1 6	[1 8]	1 7	1.5	.9	1 0	1.1	1.1	1.9	1 1	1.5	1 3	9
Arginine	.5	7	.8	7	.8	.2	5		3		.7	.5	7	.4	.9	.9	
Glu-O-Me	2 9	3 0	2 8	2.9	3 0	2 5	2 1	2 7	2.6	2 5	2 8	[4 5]	5 1	3.9	4.3	4.6	3 0
Tyrosine	.8	.6		.7	.9	.8	.5										[9]
S-CMC-O Me	.9	9		1 0	1 1	9	8		.6		1 1	1.0	1 3	9	1 0	1.1	
Proline	4 2	[6 3]	7 6	3 7	3 5	2 7	2 8	3 0	[3 3]	5 2	4 9	3 6	4 3	[3 9]	4 3	4.2	2 9
Methionine	5													.5	.5	4	
Valine	5 9	3 8		2 4	2 4	2 0	2 0	2 5	2 4	2 5	2 9	2 0	2 4	1.8	2 0	2 1	2 3
Tryptophan																	
Phenylalanine	[6 5]	4 8	3.3	2 7	2 0	1.7	1.5	1 6	1.5	1.9	1 9	1 7	2 2	1.8	2 0	2 8	1.9
Isoleucine	8	.8	9	.7	.7	.7	6	.2	.2	.8	.8	6		.6	6	.6	.4
Lysine	.4	4	4	1	.4	.4	.2			.2	2	3	4	.2	.3	.6	.1
Leucine	3 1	3.0	2 7	3 2	3.3	2 9	2 9	2.2	3 5	[4 5]	4.5	3.6	2 9	3 1	3 2	3 5	2 4
	PHE	PRO	GLY	ASN	GLY	ASP	ALA	GLN	PRO	LEU	( )	GLU	ASN	PRO	( )	( )	TYR

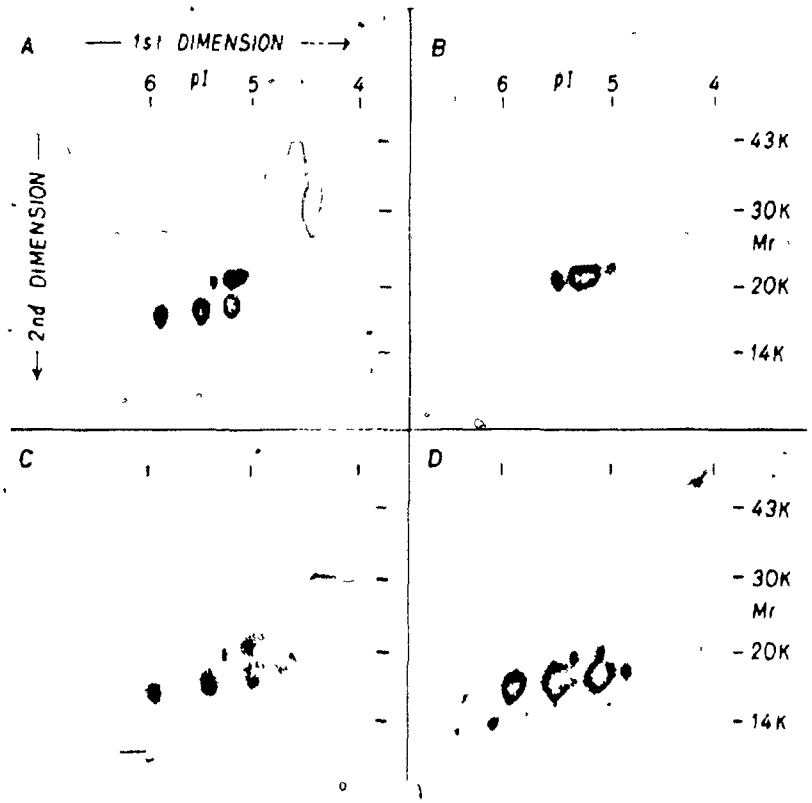
CONFIRMED RESIDUES

CYCLE

	52	53	54
Aspartic			
S-CMC			
Asparagine			
Glutamic			
Serine			
Glutamine			
Threonine			
Glycine	3.7	7.3	6.1
Histidine			
Alanine	3.9	3.1	3 7
Asp-O-Me	1.1	1.3	1.5
Arginine			
Glu-O-Me	3.5	3.3	3.5
Tyrosine			
S-CMC-O-Me	3.4	3.5	3.0
Proline			
Methionine		.8	1.1
Valine	3 4	3 1	2 8
Tryptophan			
Phenylalanine	1 9	1.9	1.9
Isoleucine	.5	.5	.5
Lysine	.2	.2	.3
Leucine	2.7	2.4	2.8
	VAL	( )	( )

CONFIRMED RESIDUES

APPENDIX II



TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS OF A PREPARATION OF PORCINE N-TERMINAL RELATED PEPTIDES

A- HPLC PURIFICATION OF PORCINE N-TERMINAL RELATED PEPTIDES (FIG 10)

B- G-75SF HMRPNT (20-21K) (FIG 11)

C- G-75SF FRACTION 98-116 OF PNT (FIG 11)

D- G-75SF LMRPNT (12-13K) (FIG 11)

Appendix Table 3.4.3. Analysis of variance for primary:secondary seed weight ratio, Macdonald.

Source	df	F value
Model	38	6.87**
rep	3	0.27
genotype	35	7.43**
error	105	
corrected total	143	

R-square 0.7131
C.V. 2.0402

* PR>0.05
** PR>0.01

Appendix Table 3.4.4. Analysis of variance for primary:secondary seed weight ratio, F_2 and F_3 generation at Joliette and Macdonald.

Joliette

	F_2 generation		F_3 generation	
Source	df	F value	df	F value
Model	17	5.97**	17	4.36**
rep	3	5.10**	3	1.66
genotype	14	6.16**	14	4.94**
error	42		42	
corrected total	59		59	

Macdonald

	F_2 generation		F_3 generation	
Source	df	F value	df	F value
Model	17	5.36**	17	5.91**
rep	3	0.27	3	0.62
genotype	14	6.45**	14	7.04**
error	42		42	
corrected total	59		59	

* PR>0.01
** PR>0.01

Appendix Table 3.4.5. Analysis of variance for gca and sca effects in the F_2 generation at Macdonald, primary:secondary seed weight ratio.

Source	df	SS	MS	F value
gca	5	1.68×10^{-2}	3.40×10^{-3}	15.47**
sca	9	6.00×10^{-4}	6.67×10^{-5}	0.30
error	42		2.20×10^{-4}	

* PR>0.05
** PR>0.01

δ^2_{gca}	8.33×10^{-4}
δ^2_{sca}	4.47×10^{-4}
gca SS / total genetic SS	0.97
N.S. heritability	0.71
B.S. heritability	0.91

Appendix Table 3.4.6. Analysis of variance for gca and sca effects in the F_3 generation at Macdonald, primary:secondary seed weight ratio.

Source	df	SS	MS	F value
gca	5	2.34×10^{-2}	4.68×10^{-3}	17.02**
sca	9	5.54×10^{-3}	6.16×10^{-4}	2.24*
error	42		2.75×10^{-4}	

* PR>0.05
** PR>0.01

δ^2_{gca}	1.02×10^{-3}
δ^2_{sca}	3.41×10^{-4}
gca SS / total genetic SS	0.81
N.S. heritability	0.77
B.S. heritability	0.90

Appendix Table 3.4.7. Analysis of variance for gca and sca effects in the F_2 generation at Joliette, primary:secondary seed weight ratio.

Source	df	SS	MS	F value
gca	5	2.49×10^{-2}	4.98×10^{-3}	15.31**
sca	9	2.05×10^{-3}	2.28×10^{-4}	0.70
error	42		3.25×10^{-3}	

* PR>0.05
** PR>0.01

$\hat{\sigma}^2_{gca}$	1.19×10^{-3}
$\hat{\sigma}^2_{sca}$	0.00
$\frac{\text{gca SS}}{\text{total genetic SS}}$	0.92
N.S. heritability	0.88
B.S. heritability	0.88

Appendix Table 3.4.8. Analysis of variance for gca and sca effects in the F_3 generation at Joliette, primary:secondary seed weight ratio.

Source	df	SS	MS	F value
gca	5	2.40×10^{-2}	4.80×10^{-3}	10.69**
sca	9	7.00×10^{-3}	8.00×10^{-4}	1.73
error	42		4.50×10^{-4}	

* PR>0.05
** PR>0.01

$\hat{\sigma}^2_{gca}$	1.00×10^{-3}
$\hat{\sigma}^2_{sca}$	3.50×10^{-4}
$\frac{\text{gca SS}}{\text{total genetic SS}}$	0.77
N.S. heritability	0.71
B.S. heritability	0.84

Appendix Table 3.4.9. Analysis of variance for secondary seed weight, Joliette.

Source	df	F value
Model	38	10.73**
rep	3	1.90
genotype	35	11.49**
error	105	
corrected total	143	

R-square 0.7952
C.V. 5.0649

* PR>0.05
** PR>0.01

Appendix Table 3.4.10. Analysis of variance for secondary seed weight, Macdonald.

Source	df	F value
Model	38	13.53**
rep	3	1.65
genotype	35	14.55**
error	105	
corrected total	143	

R-square 0.8305
C.V. 4.7700

* PR>0.05
** PR>0.01

Appendix Table 3.4.11. Analysis of variance for secondary seed weight, pooled over locations.

Source	df	F value
Model	74	12.89**
rep	3	3.49*
genotype	35	24.68**
location	1	23.62**
genotype*location	35	1.59*
error	213	
corrected total	287	

R-square 0.8174
C.V. 4.8852

* PR>0.05
** PR>0.01

Appendix Table 3.4.12. Analysis of variance for secondary seed weight, F_2 and F_3 generation at Joliette and Macdonald.

Joliette

	F_2 generation		F_3 generation	
Source	df	F value	df	F value
Model	17	8.91**	17	7.23**
rep	3	1.15	3	0.18
genotype	14	10.57**	14	8.75**
error	42		42	
corrected total	59		59	

Macdonald

	F_2 generation		F_3 generation	
Source	df	F value	df	F value
Model	17	10.46**	17	8.65**
rep	3	1.42	3	0.29
genotype	14	12.39**	14	10.44**
error	42		42	
corrected total	59		59	

** PR>0.01

Appendix Table 3.4.13. Analysis of variance for gca and sca effects in the F_2 generation at Macdonald, secondary seed weight.

Source	df	SS	MS	F value
gca	5	98.84	19.77	32.58**
sca	9	6.52	0.72	1.19
error	42		0.61	

* PR≤0.05
** PR≤0.01

σ^2_{gca}	4.76
σ^2_{sca}	0.11
$\frac{\text{gca SS}}{\text{total genetic SS}}$	0.94
N.S. heritability	0.93
B.S. heritability	0.94

Appendix Table 3.3.14. Analysis of variance for gca and sca effects in the F_3 generation at Macdonald, secondary seed weight.

Source	df	SS	MS	F value
gca	5	62.38	12.48	23.40**
sca	9	15.54	1.73	3.24**
error	42		0.53	

* PR≤0.05
** PR 0.01

σ^2_{gca}	2.69
σ^2_{sca}	1.20
$\frac{\text{gca SS}}{\text{total genetic SS}}$	0.80
N.S. heritability	0.76
B.S. heritability	0.92

Appendix Table 3.4.15. Analysis of variance for gca and sca effects in the F_2 generation at Joliette, secondary seed weight.

Source	df	SS	MS	F value
gca	5	66.52	13.30	28.85**
sca	9	1.68	0.19	0.40
error	42		0.46	

* PR>0.05
** PR>0.01

δ^2_{gca}	3.28
δ^2_{sca}	0.00
gca SS / total genetic SS	0.93
N.S. heritability	0.93
B.S. heritability	0.93

Appendix Table 3.4.16. Analysis of variance for gca and sca effects in the F_3 generation at Joliette, secondary seed weight.

Source	df	SS	MS	F value
gca	5	74.99	15.00	20.95**
sca	9	12.90	1.43	2.00
error	42		0.72	

* PR>0.05
** PR>0.01

δ^2_{gca}	3.39
δ^2_{sca}	0.71
gca SS / total genetic SS	0.85
N.S. heritability	0.83
B.S. heritability	0.91