A STUDY OF LIPOLYTIC, STEROIDOGENIC AND OPIATE HORMONES FROM VARIOUS VERTEBRATE TISSUES

by

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B.Sc.(Hon.) CUHK

A thesis submitted in partial fulfilment of

the requirement for the degree of

Master of philosophy

in

Biochemistry

May 1985

Department of Biochemistry

The Chinese University of Hong Kong

We accept this thesis as conforming to the required standard for the degree of

Master of philosophy

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Tze Bun Ng for his untiring guidance and stimulating advice throughout my study. I am also grateful to Prof. T.B. Lo, Dr. W.N. Leung and Dr. H.W. Yeung to be my examiners.

I would like also to thank for the generous help of Mr. K.C. Chung, and Mr. K.W. Ho in preparing this thesis and the technical assistance of Mr. C.M. Wong.

At last, I would like to express my grateful thanks to my family, Mr. W.Y Chan and everybody in Biochemistry department for their advice and spiritual support during my study.

ABBREVIATION

The following abbreviations are used in this thesis :

ACTH	:	Adrenocorticotropin
AAP	:	Acid acetone powder
AP	:	Acetone powder
BSA	:	Bovine serum albumin
CM-cellulose	:	Carboxymethyl-cellulose
DADLE	:	D-ala^2-D-leu^5-enkephalin
KRB	:	Krebs-Ringer bicarbonate buffer
LEK	:	Leucine-enkephalin
LPH	:	Lipotropin
MSH	:	Melanocyte-stimulating hormone
VIP	:	Vasoactive intestinal peptide

ABSTRACT

Opioid and corticotropin-like activities were studied in equine pancreas, rat placentas and other rat tissue, bovine placentas, mouse testes, carp (*Cyprinus carpio*) pituitaries, sockeye salmon (*Oncorhynchus nerka*) pituitaries and brains of two sea snakes (*Hydrophis cyanocinctus* and *Lapemis hardwickii*). Opioid and corticotropin-like activities were extracted from the tissues with a mixture of acetone, hydrochloric acid and water, sometimes after heating to inactivate tissue enzymes. Opioid activity was monitored using rat brain membranes and enzyme-resistant ligands such as [^{3}H]-naloxone and [^{3}H]-D-ala^2-D-leu^5 enkephalin. Corticotropin-like activity was monitored by enhancement of corticosterone production in isolated adrenal decapsular cells and by augmentation of lipolysis in isolated hamster adipocytes.

Both opioid and corticotropin-like activities were demonstrated in the acid acetone powder of equine pancreas. In Sephadex G-25 gel filtration, the majority of opioid activity was retarded indicating that it has a molecular weight small than 5,000 daltons but the corticotropin-like activity was unretared and thus apparently of large molecular weight. Both types of activities were adsorbed on CM-cellulose indicating their basic character. In both rat and bovine placentas, opioid activity was detected: the opioid activity found in rat placentas was estimated to be less than 5,000 daltons in molecular weight while bovine placental opioid activity was shown to be larger as evidenced from their different chromatographic behaviour on

Sephadex G-25. Corticotropin-like activity was also detected in rat placentas.

In the carp pituitary, a large number of chromatographic fractions with opioid and corticotropin-like activities were detected. Since the majority of the carp pituitary opioid activities was found to be adsorbed on CM-cellulose, it appeared that carp pituitary opioid peptides were mainly basic in nature. On the other hand, the widespread distribution of steroidogenic and lipolytic activities among the CM-cellulose column effluents suggested the presence of more than one form of corticotropin-like activity. Some of the corticotropin-like activity might be due to melanotropins. Similar results were obtained for sockeye salmon pituitaries.

Among the other tissues investigated for opioid and corticotropin-like activities, positive results were obtained for acid acetone powders of mouse testes, rat brains and snake brains but not for those of livers, kidneys and lungs of rat.

Results of the present investigation reveal the presence of opioid and corticotropin-like activities in various tissues in the mammal as well as in brain and pituitaries of non-mammalian vertebrates. The activities were either synthesized or internalized from the blood by the tissues suggesting certain physiological role(s) of the activities in the tissues. It remains to be elucidated whether the apparent absence of the activities from livers, kidneys and lungs was due to a lower content or to a real inability of the tissues to synthesize the

pro-opiomelanocortin-related peptides.

CONTENT

INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1 BIOCHEMISTRY OF PROOPIOMELANOCORTIN RELATED PEPTIDES

The discovery of a large polypeptide possessing the immunoactivities of both corticotropin and β-lipotropin (Lowry et al, 1976) has led to the idea of their synthesis as parts of a single precursor polypeptide. This polypeptide was named proopiomelanocortin because of its subsequent processing into opioid peptides, melanotrophin and adrenocorticotropin. From studies on mRNA or cloned cDNA synthesized from the mRNA template (Nakanishi et al, 1977, 1979), this precursor was found to be a 31 K polypeptide containing one molecule of α-melanotrophin, one molecule of corticotropin, and one molecule of β-lipotropin linked to each other by a pair of dibasic amino acids.

This precursor molecule is processed into its biologically active fragments in the pituitary gland (Hope and Lowry, 1981), but in different regions of pituitary the processing is found to be quite different. In the pars anterior of the ovine pituitary, the final products are corticotropin (1-39), pro-γ-melanotropin (1-77) and β-lipotropin (1-91). However, in pars intermedia, corticotropin (1-39) is further processed into α-NSH and corticotropin-like intermediate lobe peptide (CLIP); β-lipotropin to β-melanotropin, β-endorphin and γ-lipotropin and pro-γ-melanotropin (1-77) to pro-γ-melanotropin (1-48) and pro-γ-melanotropin (51-77). Corticotropin, α-melanotropin, β-melanotropin, β-lipotropin and β-lipotropin share the common core sequence Met-Glu-His-Phe-Arg-Trp-Gly (Li et al,

1961) while γ-melanotropin possesses a similar sequence Met-Gly-His-Phe-Arg-Trp-Asp (Nakanishi et al, 1977, 1979). Corticotropin, melanotropins and lipotropins possess lipolytic activity while γ-melanotropin does not, apparently because of the difference in the heptapeptide core sequence.

1.1.1 Adrenocorticotropin

Adrenocorticotropin is cleaved from proopiomelanocortin in pars anterior as a 39 amino acid peptide. The steroidogenic N-terminal region (1-19) of corticotropin is conserved in all known mammalian corticotropins and variations are only found at the carboxyl-terminal regions (Lowry et al, 1977). Moreover , in the studies of adrenocroticotropin isolated from cartilaginous fish Squalus acanthias (Lowry et al, 1974) and α-MSH and CLIP from chum salmon (Kawauchi et al, 1979, 1982), their structures were found to be very similar to their mammalian counterparts. This finding indicated that the functional core of corticotropin may be conserved even in non-mammalian species.

Adrenocorticotropin, so called because of its action on the adrenal cortex to stimulate production of steroid hormones, is secreted from the pituitary under the control of a large number of factors : the hypothalamic corticotropin releasing factor (CRF), circadian rhythm, feedback suppression of the corticosteroid and some other neural and peripheral factors (Jones et al, 1981). By these controls, the adrenocorticotropin level in an organism can be carefully adjusted to meet the need

of the organism. The functions of this hormone are extensive. Primarily, adrenocorticotropin stimulates production of corticosteroids from the adrenal gland. However, apart from its steroidogenic activity, adrenocorticotropin was found to have lipolytic activity and weak melanocyte stimulating action which may be due to the amino acid sequence of α-MSH that it contains at its N-terminal. From the finding that adrenocorticotropin like activities were found in a large number of extrapituitary tissues (Saito et al, 1983) and the discovery of extra-adrenal functions of corticotropin (Juniewice et al, 1984, Pitzel et al, 1982), more roles are expected to be uncovered for this peptide.

1.1.2 Opioid peptides

Since the discovery of endogenous opiate peptides (Hughes et al, 1975a), the physiological roles and sites of production of these peptides have captured the attention of a large number of scientists. By now a large number of opioid peptides have been isolated from different tissues, characterized, and classified into categories according to their primary structures including the enkephalins, endorphins and dynorphins.

Met- and leu-enkephalins, the two pentapeptides discovered and characterized by Hughes et al (1975a) differ by a single amino acid replacement in position 5. Methionine-enkephalin has the structure Tyr-Gly-Gly-Phe-Met and leucine-enkephalin the structure Try-Gly-Gly-Phe-Leu. The enkephalins were found to have potent morphine-like activities in various

assay systems for morphine (Hughes et al, 1975a,b; Simantov and Snyder, 1976). The production of enkephalins by the brain and the adrenal medulla is well established (Kimura et al, 1980).

Thereafter, the isolation and characterization of a peptide, β-lipotropin, from sheep pituitary glands (Birk and Li, 1964a; Li and Chung, 1976) revealed the fact that the amino acid sequence of enkephalins was present in other peptides suggesting that β-lipotropin might be a precursor of opioid peptides. This suggestion was later confirmed by Li and Chung (1976) who isolated a peptide with opioid acitivity from camel pituitaries and identified it as the 61 to 91 carbonyl-terminal amino acid sequence of β-lipotropin. This peptide, together with other fragments of β-lipotropin with opioid activities (Graf et al, 1976; Cox et al, 1976) were termed as endorphins. It is now known that enkephalins and endorphins come from different precursors (Nakanishi et al, 1977, 1979).

Although opioid peptides are capable of inducing analgesia and other opiate-like effects, their physiological roles have not yet been completely elucidated. By some recent studies endorphins have been found to be involved in pain sensitivity (Terenius, 1981) and many other neural functions (Ree et al, 1981). Moreover, the discovery of more than one type of opiate receptor (Kosterlitz, 1979) and the discovery of opioid peptides in extra-pituitary tissues (Spampinato and Goldstein, 1983; Houck et al, 1981) further complicate the systems of endogenous opioid peptides. To obtain more informations on this

system, studies of opioid peptides in extra-pituitary tissues and non-mammalian species may be useful.

1.2 THE STUDY OF PROOPIOMELANOCORTIN-RELATED PEPTIDES IN EXTRAPITUITARY TISSUES

Adrenocorticotropin (ACTH) and β-endorphin, previously thought to be exclusively produced in pituitary glands, were recently found to be produced in extrapituitary tissues including placentas (Liotta et al, 1977), and pancreas (Stern et al, 1982; Houck et al, 1981). Studies on these peptide hormones of extrapituitary origins might be helpful in understanding the physiological roles of these peptides in mammalian systems.

1.2.1 Pancreas

The pancreas, as an endocrine gland, has been extensively studied for its secretion of insulin, glucagon and somatostatin which are extremely important for carbohydrate homeostasis in mammals. Recent immunocytochemistry studies revealed that the pancreas contained a large number of peptides hormones other than the well known insulin, glucagon and somatostatin (Alumets et al, 1983; Sjolund et al, 1983; Bruni et al, 1979). In these studies, adrenocorticotropin containing cells were located in porcine pancreas, and immunoreactivity of β-endorphin was found in the human pancreas (Bruni et al, 1979) and the D-cells of rat and guinea pig pancreas (Watkin et al, 1980). The detection of immunoreactive and biologically active opioid polypeptides in the extracts of pancreas from human (Bruni

et al, 1979), guinea pig (Stern et al, 1982) and porcine (Houck et al, 1981) strongly supported the proposal of opioid polypeptide secretion from pancreatic cells.

Although it is well known that opioid substances are capable of reducing the motility of the gastrointestenal tract and the secretions of some gastrointestinal hormones, the physiological functions of these pancreatic opioid polypeptides are still under research. However, in view of the finding that morphine can induce hyperglycemia (Feldbery et al, 1972) and the presence of opiate receptors in the membrane of guinea pig pancreatic cells (Barkey et al, 1981), a pancreatic regulatory role for endogenous opioid polypeptides was proposed. Recently, the effects of morphine and opioid peptides in pancreas were further investigated in a study using isolated perfused dog pancreas (Hermansen, 1983). The administration of opioid peptides into the perfused pancreas was found to inhibit somatostatin secretion and stimulate insulin secretion in a dose dependent manner. The finding that this regulatory effect of opioid peptides could be antagonized by naloxone, an opiate receptor antagonist, but not by adrenergic and cholinergic receptor blockers suggested that these effects were mediated by a direct interaction with the opiate receptors located in pancreas. Similar results obtained in dogs in an in vivo study (Schusdziarra et al, 1983) provided additional proof for this hypothesis. Although the physiological importance of these regulatory effects of opioid polypeptides in pancreas is not yet clear, the finding that β-endorphin was effective in raising

plasma insulin level and reducing plasma glucose progressively in diabetes mellitus Type II subjects suggested that further studies to elucidate the role of pancreatic opioid peptides on glucoregulation may have some clinical importance. In view of the lack of unanimity in opinions regarding the type of opiate present in the mammalian pancreas (Houck et al, 1981; Stern et al, 1982), we decided to undertake a study on the equine pancreas.

1.2.2 Placentas

It was established that, in human beings, placenta was a multi-functional endocrine gland secreting a large number of hormones including chorionic gonadotropin and chorionic somatomammotropin for the maintenance of pregnancy. However, the secretion of corticotropin or β-endorphin from placenta was not known until recently when the evidence collected suggested the presence of these peptide hormones in placentas. In human beings, it was noticed that the maternal plasma level of corticotropin increased progressively during pregnancy and that this increase was not affacted by glucocorticoids, the negative feedback control for pituitary corticotropin secretion (Rees et al, 1975). This observation suggested an extrapituitary origin of this corticotropin activity.

In a study to locate the origin of this corticotropin activity by Hodgen (1975), it was found that hypophysectomy and fetectomy of pregnant monkeys could only partially reduce the

maternal adrenal weight and cortisol output from adrenal glands but that the subsequent removal of placenta rapidly reduced the cortisol output to undetectable levels. This result indicated that placenta may play a role in the maintenance of maternal adrenal function, probably by secretion of corticotropin-like activity. Recently, immunoreactive and biologically active corticotropin have been detected found in placental extracts (Liotta et al, 1977; Rees et al, 1975). In vitro synthesis of immunoreactive and biologically active corticotropin was demonstrated in placental cells (liotta et al, 1980, 1982a). Hence it can be accepted that human placenta is a corticotropin secreting organ. Furthermore, the discovery of high molecular weight corticotrophin-like immunoreactivity in the study of Liotta (1977) suggested that placental corticotropin is synthesized through a high molecular weight precursor as in pituitary.

In the mammalian system, it has been established that corticotropin, β-lipotropin, and β-endorphin are synthesized in the same precursor, proopiomelanocortin. The synthesis of corticotropin through post-translational processing of a large precursor in placentas may also be an indication of the presence of these peptides in placentas. Their presence was recently confirmed by the detection of the immunoreactivities of these peptides in placental extracts (Odagiri et al, 1979; Houck et al, 1980). Although the presence of placental corticotropin and opioid polypeptides in human placentas was demonstrated, their physiological functions are still undetermined and their presence

in placentas of other mammals has not been reported. Hence, further studies of these hormones in rat and bovine placentas may be helpful in answering these questions.

1.2.3 Testes

Following the detection of immunoreactuve β-endorphin in human semen and rat testicular extracts (Sharp et al, 1980, 1981), the search of β-endorphin and its related peptides was conducted by several groups. Recently, immunoreactive β-endorphin and another proopiomelancortin-derived peptide, corticotropin, were detected in the male reproductive tract of rats (Tsong et al, 1982). By means of immunocytochemical methods, immunostainable β-endorphin-like material was found to localized in Leydig cells of rat testes and tubular structures of the male reproductive tract. In a follow-up study by the same group, β-endorphin-like and corticotropin-like immunoreactivities were also measured in the Leydig cells of mouse, hamster, guinea pig and rabbit (Tsong et al, 1982). In their study, the concentration of these immunoreactive peptides were shown to be 10 - 100 times higher than those in the blood. The finding of the persistence of the immunoreactivities of these peptides in hypophysectomized animals with very low blood level of corticosterone and β-endorphin suggests that these testicular peptides may be locally produced and not accumulated from blood. However, the low concentration of immunoreactive β-endorphin and corticotropin found in testes indicates it is unlikely that the testis contributes significantly to plasma levels of these peptides.

And so, a paracrine action of these peptides on neighbouring tissues seems to be a more reasonable suggestion.

The report that corticotropin stimulates the growth of Sertoli cell lines (Mather et al, 1980) and that vas deferens contains opiate receptors strengthens the suggestion for the paracrine action of these peptides. It has been hypothesized that testicular β-endorphin may be secreted into the lumen of the male reproductive tract and act on the opiate receptors along the tract. Another possible paracrine function for these testicular pro-opiomelanocortin-related peptides may be the media for the intratesticular communication of different cell types such as Leydig cells and Sertoli cells. The observations that elevation of plasma corticotropin exogenously (Juniwice et al, 1984) or endogenously (Pitzel et al, 1982) could stimulate the release of testosterone from testis and that opiate antagonist naloxone could reduce testicular testosterone output significantly (Gerendai et al, 1984), suggest that these peptides do have their physiological roles in the male reproductive organ.

Although the presence of β-endorphin in rat testes has been demonstrated by immunoreactivity eluting in the same position as authentic peptide in high performance liquid chromatography, no studies on the opiate activity of this testicular β-endorphin have been conducted (Tseng et al, 1982). In the investigation we showed that substance(s) with opiate receptor binding activity were present in an acid acetone powder of mouse testes.

1.3 PROOPIOMELANOCORTIN-RELATED PEPTIDES IN NON-MAMMALIAN SPECIES

After the discovery of β-endorphin and corticotropin, in mammalian pituitaries (Li et al, 1976, 1977, 1981), these peptides were detected in a large number of extra-pituitary tissues (Saito et al, 1983) and non-mammalian species such as teleost (Kawauchi et al, 1980), frog (Jegou et al, 1983), bird (Li et al, 1977) and unicellular organism, Tetrahymena pyriformis (Lakoith et al, 1982). So as to obtain a complete evolutionary history of these peptide hormones, the studies of these hormones in non-mammalian species would be essential.

1.3.1 Fish pituitaries

Biological activity corresponding to corticotropin has been detected in extracts of pituitaries from several fish species including elasmobranchs (DeRoos and DeRoos, 1967) but the molecular characteristics of these corticotropin-like activities remained largely unknown. A large number of proopiomelanocortin-related peptides have been isolated and characterized from the pituitaries of dogfish (Squalus acanthias) (Lowry et al, 1974) and salmon (Oncorhynchus keta) (Kawauchi et al, 1980). Immunoreactivities of proopiomelanotropin-related peptides have been detected in the pituitaries of the rainbow trout (Salmo gairdneri) (Rodrigues et al, 1982). Moreover, Kawauchi and his colleagues have now purified and sequenced many of the peptides derived from pro-opiomelanocortin in the chum salmon (Kawauchi

et al, 1980). From these results, a more complete picture of the pro-opiomelanocortin-related peptide system in fish was obtained.

Among these characterized peptides, only one α-MSH in salmon was found to be identical to its mammalian counterpart while most of the others showed differences in the amino acid sequences. For example, the endorphins isolated from salmon pituitary extract (Kawauchi et al, 1980) were shown to have different amino acid sequence from mammalian endorphins and the acetylation of their N-terminus rendered them inactive in a radioreceptor assay for opiates. The differences in structures and biological activities of these peptides created a lot of difficulties in their detection and quantitation using the mammalian assay system. As found by Takahashi et al (1984), salmon endorphin and human β-endorphin are immunologically completely different with the result that antibodies raised against one of them shows no cross-reactivity with the other. Moreover, the unique discovery of not just one but at least two sets of pro-opiomelanocortin-related peptides in the chum salmon (Oncorhynchus keta) pituitary indicative of the presence of two proopiomelanocortin molecules further complicated the fish system of pro-opiomelanocortin derived peptides (Kawauchi et al, 1981). In order to obtain more information about the functions and structures of the pro-opiomelanocortin-related peptides in fish, studies on more species are essential. For this purpose we have chosen to work on the pituitaries of the carp Cyprinus carpio and the sockeye salmon Oncorhynchus nerka.

1.3.2 Snake brain

β-Endorphin-like immunoreactivity has been demonstrated in mammalian brain tissues by immunocytochemistry and radioimmuno-assay (Rossier *et al*, 1977; Kreiger *et al*, 1977; Bloom *et al*, 1978; Matsukura *et al*, 1978) and β-endorphin-like peptides have actually been isolated and characterized from bovine cerebral hemispheres (Swann and Li, 1980; Ng *et al*, 1982). The brain β-endorphin level is not affected by hypophysectomy, indicating that the brain peptide is not derived from the pituitary (Rossier *et al*, 1977; Kendall and Orwoll, 1980). Evidence for a neurotransmitter function of β-endorphin in the adult rat brain has accumulated (Bloom *et al*, 1978; Osborne *et al*, 1979; Wolstencroft *et al*, 1978). The peptide has potent analgesic activity (Loh *et al*, 1976; Tseng *et al*, 1976). The brain β-endorphin-like immunoreactivity in rats has been found to change with development (Ng *et al*, 1984). Similarly, immunoreactive corticotropin has been found in the brain. It was shown, in a recent study, that immunologically active β-endorphin, adrenocorticotropin and melanotropins were present in the brain of the frog (Jegou *et al*, 1983). Immunoreactivities of pro-opiomelanocortin-derived peptides have also been demonstrated in the pituitary of the lizard *Anolis carolinensis* (Dores, 1982, 1984). Based on these observations, the presence of proopiomelanocortin derived peptides in the brains of another representative of the reptilian class, the snake, was expected. We have therefore set out to use bioassay to detect the presence of these materials in snake brains.

1.4 STRATEGY OF STUDY

In this project, the presence of corticotropin-like and opioid peptides in equine pancreas, carp and salmon pituitaries, bovine and rat placentas, mouse testes and snake brains was detected by a steroidogenesis assay and an opiate receptor binding assay respectively. The bioassay and radioreceptor assay were preferred to radioimmunoassays because immunoreactive materials do not necessarily possess bioactivity and also because sometimes apparent immunoreactivity arises from degradation of the labeled ligands by enzyme present in the sample. In this study the labeled ligand utilized in the radioreceptor assay for opioid peptides ^{3}H-naloxone and ^{3}H-DADLE, are resistant to the common proteases. The distribution of corticotropin-like and opioid peptides was also examined in other body tissues including the livers, the kidneys and the lungs to see if the occurrence of the peptides in a biologically active form was restricted to certain tissues.

MATERIALS AND METHODS

Chapter 2 MATERIALS AND METHODS

2.1 MATERIALS AND ANIMALS

2.1.1 ANIMALS

Male Sprague-Dawley rats, weighing 160 g - 190 g, originated from Charles River Laboratory (Japan) and maintained on Purina Chow and water, were used for hormone-induced steroidogenesis assay and opiate receptor binding assay.

Male golden hamsters, weighing 150 g - 200 g and obtained from the animal house of the Chinese University of Hong Kong, were used for hormone-induced lipolysis assay.

2.1.2 MATERIALS

All the reagents and chemicals used were of analytical grade or the best quality available. The suppliers of the chemicals used were listed in table 2-1 and table 2-2.

2.2 ESTABLISHMENT OF ASSAY SYSTEMS

2.2.1 HORMONE-INDUCED LIPOLYSIS ASSAY USING HAMSTER EPIDIDYMAL ADIPOCYTES

2.2.1a Isolation of adipocytes

Adipocytes were isolated from epididymal fat pads of hamsters by the method of Rodbell (1964) with minor modifications. Male golden hamsters, weighing 150 g-200 g were sacrificed by cervical dislocation. The epididymal fat pads

Table 2-1. Suppliers of chemicals

Chemicals	Supplier
Acetic acid	Merck
Bacitracin	Sigma
[1,2,6,7-^{3}H] Corticosterone	Amersham
Corticosterone	Sigma
CM-cellulose	Sigma
Dextran T-70	Pharmacia Fine Chemical
4,5-dihydroxynapthalene-2,7-disulfonic acid disodium salt	Sigma
D-glucose	BDH
Glycerol	Sigma
Lima bean trypsin inhibitor	Sigma
Norit A (charcoal)	Serva
POPOP (2,2'-phenylen-bis(5-phenyloxazole))	Merck
PPO (2,5-diphenyloxazole)	Sigma
Sephadex G-10	Sigma
Sephadex G-25	Sigma
Sephadex G-100	Sigma
Sodium pentobarbital	Serva
Sucrose	Merck
Tris [Tris(hydroxymethyl) amino-methane]	Sigma
Triton X-100 (Octylphenoxy poly-ethoxyethanol)	Sigma

Table 2-2. Peptides and protein suppliers

Chemical	Supplier
ACTH (porcine corticotropin)	Sigma
Anti-corticosterone	Miles
BSA (fraction V)	Sigma
BSA (fraction V, RIA grade)	Sigma
Collagenase (Type II)	Sigma
α-Chymotrypsin	Worthington
D-ala^2-D-leu^5[tyrosyl-3,5-^{3}H] enkephalin	Amersham
Dynorphin (1-13)	Sigma
Glucagon	Sigma
Leucine-enkephalin	Sigma
Methionine-enkephalin	Sigma
α-MSH	Sigma
β-MSH	Sigma
Neurotensin	Sigma
Somatostatin	Sigma
Trypsin	Worthington
β-endorphin	Gift from Dr. C.H. Li
Salmon pituitaries	Gift from Dr. D.R. Idler
Snake brains	Gift from Dr. B.Y. Soo
VIP	Gift from Dr. S. Said

were dissected out and immersed in 0.9% saline at room temperature (25 °C). After blotting on Whatman filter paper and weighing, the fat pads were sliced into small pieces of about 1 mm across and placed in Krebs-Ringer bicarbonate buffer (KRB) at pH 7.4 (3 ml/g fat pad) containing 4% bovine serum albumin (BSA) and collagenase (1 mg/ml), in Falcon polypropylene culture tubes. As suggested by Cohen (1951) the KRB used in this assay contained only half of the usual concentration of calcium. After being saturated with 95% oxygen: 5% carbon dioxide, the culture tubes were incubated at 37 °C for 45 minutes in a Dubnoff metabolic water bath with moderate shaking (60-70 cycles min.). At the end of the incubation, The tube contents were filtered through two layers of cheesecloth to remove undigested tissues. The suspension obtained was then allowed to stand still for 5 minutes at room temperature until most of the adipocytes had floated to the top. The infranatant was then removed by aspiration and replaced with the same volume of fresh KRB containing 4% BSA and 0.01% lima bean trypsin inhibitor. After mixing and standing for another 5 minutes, the infranatant was again removed. The washing procedure was repeated at least twice to remove traces of collagenase from the isolated adipocytes. The washed cells were then resuspended in fresh KRB containing 4% BSA and 0.01% lima bean trypsin inhibitor, and pre-incubated in a polypropylene beaker (Klon, England) at 37 °C under an atmosphere of 95% oxygen: 5% carbon dioxide for 30 minutes.

2.1.1b Incubation of adipocytes with hormones and fractions to

be assayed

Isolated fat cells were resuspended to achieve a cell concentration of about 2 - 4 x 10^5 cells / ml corresponding to a dry weight of 20 - 35 mg / ml suspension. One ml aliquots of the cell suspension were dispensed into 12x75 mm polypropylene culture tubes each containing hormone or the fractions to be assayed in a volume of 100 ul. During dispension the homogeneity of the cell suspension was maintained by constant swirling. To minimize damage of adipocytes during transferring, the tip of the autopipette (Gilson, P1000) was cut to make the orifice larger. Incubation was performed at 37°C under an atmosphere of 95% oxygen: 5% carbon dioxide with moderate shaking (20-30 cycles/min.) for 2 hours in a Dubnoff metabolic shaking incubator. At the end of incubation, 1 ml of 10% trichloroacetic acid (w/v) was added to each tube to stop the reaction.

2.2.1d Determination of glycerol production

Glycerol is produced as the result of lipolytic activity in the adipocytes and so can be used as an index of lipolysis. Glycerol was determined by the method of Lambert and Neish (1930) as modified by Ramachandran (1972). The tubes from the lipolysis assay were centrifuged at 1000 g in a Sorvall RC-5 centrifuge for 20 minutes to precipitate cell debris and denatured proteins. The supernatant (0.5 ml) was transferred from the assay tube to a 15x150 mm glass tube followed by addition of 0.1 ml of 50 mM sodium periodate to oxidize any

glycerol present. After 5 minutes were allowed for the oxidation to go to completion, 0.1 ml of 10% sodium metabisulfite was added to reduce the excess periodate. After standing for another 5 minutes, 3.3 ml of chromotropic acid reagent (1 g 4,5-dihydroxy-napthalene-2,7-disulfonic acid disodium salt : 100 ml H_2O : 400 ml 12.5 M H_2SO_4) were added. After vortexing the tubes were covered with marbles and placed in a boiling water bath for 30 minutes. Then one ml of water was added to each tube to stop the reaction. The tubes were vortexed and allowed to cool down to room temperature before absorbance at 570 nm was read with a Spectronic 21 spectrophotometer (Bausch & Lomb).

2.2.1e Determination of dry weight of fat cells

Six one-ml aliquots of fat cells were filtered through pre-weighed wetted glass fibre filters (Whatman GF/C). These filters together with some filters without fat cells were lyophilized and the dry weight of the fat cell aliquots was calculated from the weight difference between the two types of filters. The rate of lipolysis is computed as the number of micromoles of glycerol produced per hour per gram dry weight of fat cells. Net glycerol production rate is the increase of lipolysis due to of the test fraction over control.

2.2.2 HORMONE-INDUCED STEROIDOGENESIS ASSAY USING RAT DECAPSULAR (ZONA FASCICULATA / RETICULARIS) CELLS

2.2.2a Isolation of adrenal cells

Adrenal cells were prepared by the method of Moyle (1973) with minor modifications. Male Sprague-Dawley rats, weighing 400g - 450 g, were sacrificed by cervical dislocation. Adrenal glands were carefully removed from the rats and were trimmed free of fat. Then the capsules consisting mainly of glomerulosa cells were separated from the inner zones which were mainly made up of fasciculata and reticularis cells. The decapsulated adrenal glands were minced and suspended in Krebs-Ringer bicarbonate (KRB) buffer (1 ml/ adrenal gland) containing collagenase (3 mg/ ml), glucose (2 mg/ ml) and bovine serum albumin (4 mg/ ml), in Falcon polypropylene culture tubes. The culture tubes were then saturated with 95% oxygen : 5% carbon dioxide and incubated at 37 °C with gentle shaking (65-75 cycles/ min.) in a Dubnoff metabolic water bath for 1 hour. The tissue digest was then allowed to settle at room temperature and the supernatant removed. Incubation medium (KRB containing 0.4% BSA, 0.2% D-glucose and 0.01% lima bean trypsin inhibitor) was added (0.5 ml / adrenal gland) and the adrenal cells were dislodged from the tissue by repeatedly drawing the suspension into and out of a Pasteur pipette. The pieces of tissue were then allowed to settle and the supernatant containing the dispersed cells was collected and filtered through four layers of cheesecloth. The procedure was repeated at least twice to ensure completeness of dispersal of cells. After the cell suspension was centrifuged at 50 g for 5 min at room temperature in a MSE GF-8 centrifuge, the supernatant was removed by aspiration and the cells were resuspended in the same volume of fresh incubation medium. The

cells were washed at least twice and the final cell concentration of the cell suspension was adjusted with the incubation medium to about 1 x 10^5 cells / ml.

2.2.2b Incubation of adrenal cells with hormones and test fractions

Aliquots of the cell suspension (225 ul containing about 22,500 cells) were transferred to 12 x 75 mm polypropylene culture tubes containing hormones or fractions to be assayed in a volume of 25 ul so as to make up a final assay volume of 250 ul. The tubes were then incubated at 37 °C for 2 hours under an atmosphere of 95% oxygen : 5% carbon dioxide with moderate shaking in a Dubnoff metabolic water bath. At the end of the incubation, the tubes were frozen in a -20 °C freezer until determination of corticosterone production.

2.2.2c Determination of corticosterone production

In rats, corticotropin stimulation of zona fasciculata / zona reticularis cells leads to the production of corticosterone which can be used as an index of the hormonal activity. Corticosterone was measured by radioimmunoassay using a rabbit anti-corticosterone serum. Corticosterone standards and samples from the steroidogenesis assay were diluted with tris buffer (0.05 M tris HCl buffer with 0.01% BSA, pH 7.4). The diluted sample (100 ul) or standard (100 ul) was then incubated with 100 ul of ^{3}H-corticosterone containing about 40,000 dpm, 100 ul of bacitracin (0.5 mg / ml in 0.05 M tris HCl buffer, pH

7.4) and 200 ul of diluted anti-corticosterone serum at 4°C for 16-24 hours. At the end of incubation, 0.5 ml of an activated charcoal suspension (0.25% Norit A and 0.25% Dextran T-70 in 0.05 M tris HCl buffer, pH 7.4) was added to each assay tube. After vortexing and standing on ice for 10 minutes the tubes were centrifuged at 5000 g for 10 minutes at 4°C in a Sorvall RC-5 centrifuge. An aliquot (0.5 ml) of the supernatant was mixed with 6 ml scintillant (12 gm PPO : 1.2 gm POPOP : 2 litres toluence : 1 litre triton X-100) and counted in a Beckman liquid scintillation counter (LS-7000) after vortexing. The rate of steroidogenesis is computed as the amount of corticosterone produced per two hours per 22,500 adrenal decapsular cells.

2.2.3 OPIATE RECEPTOR BINDING ASSAY USING CRUDE RAT BRAIN MEMBRANES

2.2.3a Preparation of crude brain membranes

Membranes from rat brain homogenates were prepared as described previously by Ferrara et al (1979) with slight modifications. Male Sprague-Dawley rats, weighing 180 g- 220 g, were sacrificed by decapitation and their brains were rapidly removed and chilled in cold (0°C-4°C) 0.9% saline. After the cerebellums were removed, the brains were homogenized in 20 volumes of ice-cold 0.32 M sucrose solution by using a Polytron tissue disruptor (setting 5 for 15 seconds). The homogenate was centrifuged at 1000 g at 4°C in a Sorvall RC-5 centrifuge for 10 minutes and the supernatant was re-centrifuged at 10,000 g at 4°C

for 30 minutes. The pellet obtained was resuspended in 20 volumes of 0.05 M tris-HCl buffer (pH 7.4) at 4 °C and allowed to stand on ice for 10 minutes for the membrane to lyse. The membrane suspension was re-centrifuged at 10,000 g for 20 minutes at 4 °C and the pellet was washed once with 20 volumes of fresh tris-HCl buffer. The membrane preparation could be stored at -20 °C for a week without any significant alteration in opiate binding activity.

2.2.3b Radioreceptor binding assay using crude rat brain membranes

The crude rat brain membrane preparation was resuspended in 20 volumes of 0.05 M tris HCl buffer (pH 7.4) at 4 °C. Two hundred microlitres of this membrane preparation were added to 12 x 75 mm polystyrene tubes containing 100 ul opiate standards or samples, 100 ul bacitracin (0.5 mg / ml), and 100 ul D-ala^2 -D-leu^5 -(tyrosyl-3,5-^{3}H) enkephalin, all in 0.05 M tris buffer, pH 7.4). After incubation at 4 °C for 16 - 24 hours, the reaction was stopped by filtration under low vacuum through glass fibre filters (Whatman GF/B). The tubes were washed twice and the filters once with 3 ml portions of ice-cold tris-HCl buffer. The filters were placed in scintillation vials and 6 ml of scintillant (12 gm PPO and 1.2 gm POPOP in 2 litres toluence and 1 litre triton X-100) was added to each vial. The radioactivity was measured by liquid scintillation spectrometry in a Beckman liquid scintillation counter (LS-7000). The opiate receptor (strictly speaking, crude brain membrane) binding

activity is computed as nanomole equivalent of the binding activity of leucine-enkephalin.

2.3 EXTRACTION AND PURIFICATION OF TISSUES

2.3.1 EQUINE PANCREAS

2.3.1a Extraction

Briefly, the tissue was extracted by acetone extraction as shown in Chart 2-1. Acetone powder of the tissue was mixed with 14 volumes (w/v) of the extraction medium (Acetone : H_2O : HCl = 40 : 21 : 1 by volume) using mechanical stirring. The mixture was then homogenized with a Polytron tissue disruptor (setting 5, 20 seconds) at 4 °C in an ice-water bath and centrifuged at 15,000 g for 30 minutes in a Sorvall RC-5 centrifuge. The supernatant was saved and the pellet was re-homogenized in another 5 volumes (w/v) of 80% acetone at 4 °C. After centrifugation at 15,000 g for 15 minutes at 4 °C, the supernatants of two centrifugation steps were pooled and slowly poured into 5 volumes (v/v) of cold acetone at 4 °C with gentle stirring. The mixture was allowed to stand at 4 °C for 24 hours and the precipitate was collected on filter paper (Whatman no. 1). The precipitate was then washed with 50 ml of cold acetone and dried under vacuum in a dessicator. The dried precipitate, known as acid-acetone powder (AAP), was kept in a -20 °C freezer until further purification (Li, 1952).

2.3.1b Salt fractionation of AAP

Acid acetone powder (AAP) was dissolved in 27 volumes (w/v) of distilled water and then a 6% (v/v) saturated NaCl solution was added. After standing at 4 °C for 24 hours, the mixture was centrifuged at 15,000 g for 15 minutes at 4 °C to remove the precipitate which was saved as fraction A (Fr. A). Sodium chloride (about 36 g/ 100 ml) was added to the supernatant to saturate it with salt. The mixture was stirred with a magnetic stirrer until no solid salt was observed and was then left at 4 °C overnight. The mixture was again centrifuged at 15,000 g for 15 minutes at 4 °C and the pellet was obtained as fraction C (Fr. C) while the supernatant was designated as fraction B (Fr. B). The fractions were all stored at -20 °C (Li, 1952).

2.3.1c Desalting of fractions using a Sephadex G-10 column

Since the fractions obtained in the previous step contained a large quantity of salt, it was necessary to remove the salt from the fractions before any further purification processes could take place. Therefore, a Sephadex G-10 column was used for this purpose. Dry Sephadex G-10 was swollen in 0.1 N acetic acid for 3 hours. After degasing under vacuum for 30 minutes, the slurry was poured into a constant-bore glass column previously filled with 0.1 N acetic acid. The packed column was then equilibrated for at least 2 bed volumes of 0.1 N acetic acid.

Fractions were dissolved in 10 ml 0.1 N acetic acid and applied to the column (2.5 cm inner diameter x 70 cm). The column was developed with 0.1 N acetic acid at a flow rate of approximately 1 ml / min. and fractions were collected at 7.5 min. intervals. The column was eluted at room temperature but the eluates were stored at 4°C. Peptides were detected by measuring the u.v. absorption at 280 nm. Fractions eluted at the void volume were pooled and lyophilized.

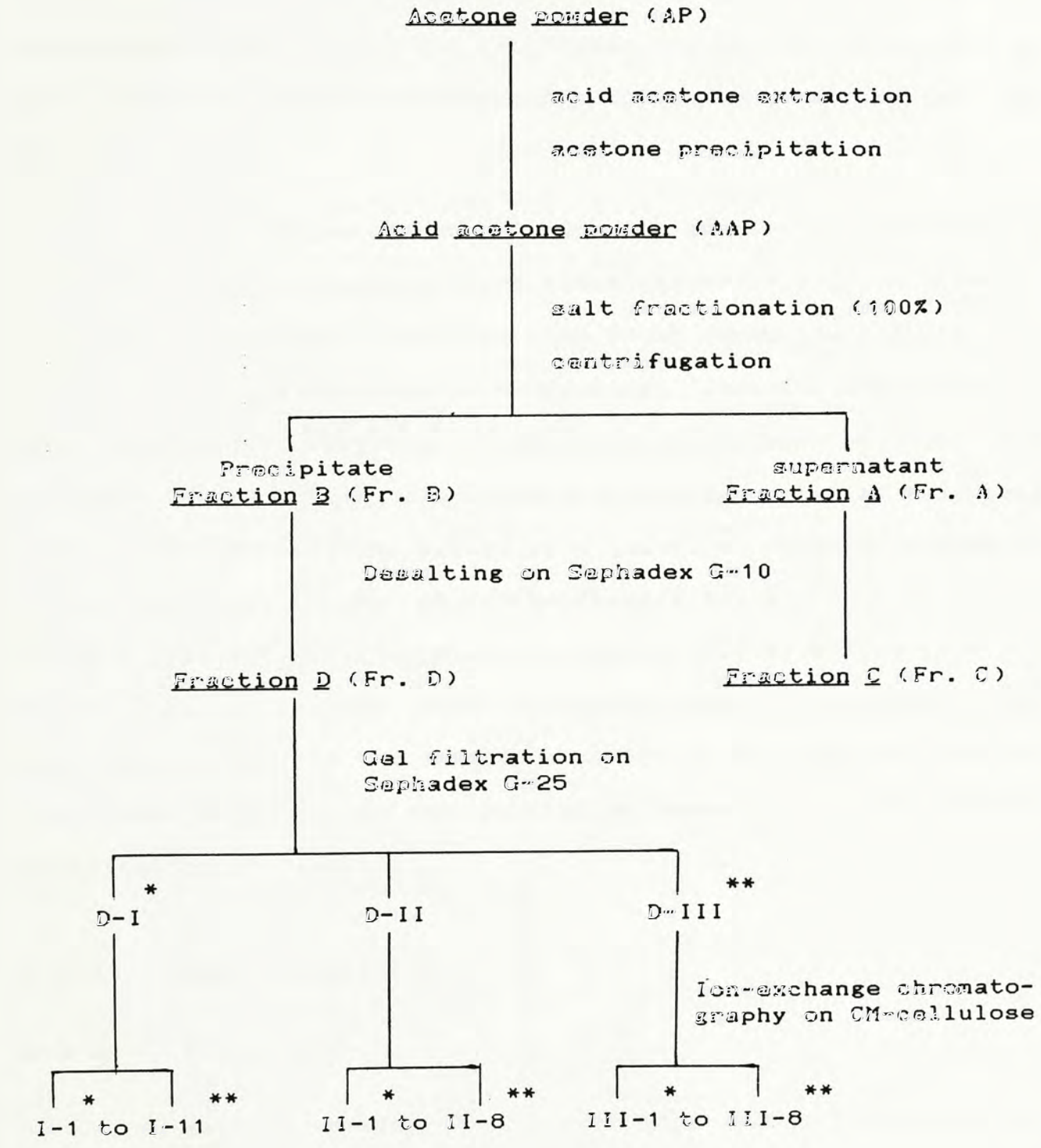
2.3.1d Gel filtration using a Sephadex G-25 column

The column (3.5 cm inner diameter x 83 cm) was prepared as previously described for the Sephadex G-10 desalting column. The desalted fractons were redissolved in 10 ml 0.1 N acetic acid and charged onto the column (3.5 cm inner diameter x 83 cm) which was then developed with 0.1 N acetic acid at a flow rate of about 1 ml / min. Fractions were collected at 7.5 min. intervals. The u.v. absorbance of the fractions was measured at 280 nm by using a Hitachi SP-20 spectrophotometer. Thereafter the fractions were pooled and lyophilized accordingly.

2.3.1e Ion exchange chromatography

CM-cellulose (0.76 meq / g) was swollen in distilled water for 2 hours and the unsettled particles were decanted. The swollen cellulose was stirred in 0.2 N NaOH (1 litre / 10 g dry cellulose) for 5 min. with a glass rod and was allowed to settle for 10 min. The supernatant was decanted together with the fine

Chart 2-1 Extraction and purification protocol of equine pancreas (Modified from Li, 1952).



* unretarded fraction
** most retarded fraction

cellulose particles. This washing procedure was repeated one more time with 0.2 N NaOH, twice with distilled water to remove traces of NaOH, twice with 20% acetic acid to activate the carboxymethyl group of the cellulose, twice with water and at last with the initial eluting buffer (10 mM NH_4 OAc buffer, pH 4.6).

After degasing under vacuum for 30 minutes, the slurry was poured into a constant-bore glass column (1.3 inner diameter x 75 cm). After equilibration with 10 mM ammonium acetate (pH 7.4), 10 ml of a solution of a fraction, from the Sephadex G-25 gel filtration step, in 10 mM ammonium acetate (pH 4.6) was applied to the column. After the unadsorbed material had come off the column was then eluted with ammonium acetate gradients (from initially 10 mM, pH 4.6 to finally 0.5 M, pH 7.0) set up using a gradient mixer as shown in figure 3-10 at a flow rate of 18 ml / h. Fractions were collected every 17 minutes. The u.v. absorption of the fractions at 280 nm was measured and the fractions were pooled and lyophilized accordingly for further purification and assays.

2.3.2 CARP PITUITARIES

2.3.2a Extraction

Acetone powder of carp (Cyprinus carpio) pituitaries was obtained from Stoller Fisheries, U.S.A. An acid acetone powder was prepared from the acetone powder with a procedure similar to that described above for equine pancreas.

2.3.2b Ion-exchange chromatography

The carp pituitary acid acetone powder was dissolved in 10 mM ammonium acetate buffer (pH 4.6) and applied to a CM-cellulose (1.3 cm inner diameter x 42 cm) column previously equilibrated and eluted with the same buffer. Subsequently, conditions of chromatography were the same as those described above for equine pancreas. The eluates were pooled and lyophilized according to their u.v. absorption at 280 nm.

In this procedure the salt fractionation and gel filtration steps were skipped owing to the small amount of acid acetone powder available.

2.3.3 SALMON PITUITARIES

Acid acetone powder of salmon (Oncorhynchus tshawytscha) pituitaries was prepared by extracting the frozen pituitaries with 2.3 volumes (w/v) of acetone-H_2O-HCl (40 : 5 : 1 by volume), re-extracting with 1 volume of 80% acetone, and precipitating peptides in the combined extract with 5 volumes of pre-chilled acetone to form the acid acetone powder (AAP). An extraction medium with a lower percentage of water than that used for the extraction of pancreas acetone powder was used because acetone removes water from the tissue during the preparation of tissue acetone powder. The acid acetone powder was fractionated by ion-exchange chromatography using CM-cellulose. CM-cellulose

was prepared by the procedures previously described in the section of equine pancreas and packed into a glass tube (0.7 cm inner diameter x 16 cm). The AAP was dissolved in 10 mM ammonium acetate buffer (pH 4.6) and applied onto the CM-cellulose column previously equilibrated with the same buffer. After the unadsorbed material had come off, the column was developed with stepwise gradients of ammonium acetate buffer (10 mM, pH 4.6; 0.1 M, pH 7.0; 0.2 M, pH 7.0 and 0.5 M pH 7.0) and fractions were pooled and freeze dried. The stepwise gradient method of elution was employed instead of the continuous gradient method described for equine pancreas and salmon pituitaries in order to avoid elution of the adsorbed material over a large number of tubes and consequently low ultraviolet absortption of the fractions which would render the task of pooling the fractions according to the u.v. absorption peaks a difficult one.

2.3.4 RAT PLACENTAS

2.3.4a Extraction

Adult female Sprague-Dawley rats, weighing 400 g - 450 g and pregnant for 18 - 20 days, were sacrificed by cervical dislocation. The placentas were carefully removed and frozen at -20 °C until extraction. The placentas were then thawed, rinsed in 0.9% saline to remove blood, cut into small pieces of 2 mm across and heated in 0.3 volume (w/v) of acid mixture (water : 35% hydrochloric acid = 2 : 1, v : v) for 20 minutes in a water bath at 95 °C. The mixture was then cooled, two volumes of acetone were added, and the mixture was homogenized with a

Polytron tissue disruptor (setting 5 for 30 seconds) and centrifuged at 10,000 g for 10 minutes at 4 °C. The residue was re-extracted with 1 volume (w/v) of 80% acetone and re-centrifuged at 10,000 g for another 10 minutes at 4 °C. The two supernatants from the centrifugation steps were pooled and added to 5 volumes (v/v) of cold acetone which had been prechilled at 4 °C for at least 24 hours, and allowed to stand at 4 °C for overnight. The precipitate was collected on a circle of Whatman no. 1 filter paper and washed twice with prechilled acetone. The precipitate, known as acid acetone powder, was removed from the filter with a spatula, dried under vacuum and kept at -20 °C.

2.3.4b Gel filtration

Sephadex G-25 was swollen, packed into a constant-bore glass column and equilibrated with 0.1 N acetic acid. The acid acetone powder of rat placentas was dissolved in 0.1 N acetic acid and applied onto the column which was eluted with 0.1 N acetic acid. Fractions were monitored by u.v. absorbance at 280 nm, pooled and freeze-dried.

2.3.5 BOVINE PLACENTAS

2.3.5a extraction

An acid acetone powder was prepared from bovine placental acetone powder with a procedure similar to that described previously in the section of equine pancreas. The placental acetone powder was purchased from Sigma.

2.3.5b Gel filtration

The acid acetone powder of bovine placentas was next fractionated by gel filtration. Sephadex G-25 and G-100 were swollen in 0.1 N acetic acid, degassed under vacuum and packed into glass columns. The acid acetone powder of bovine placentas was dissolved in 0.1 N acetic acid and charged onto the G-25 column previously equilibrated and eluted with 0.1 N acetic acid. The collected fractions were pooled and lyophilized according to peaks of absorbance at 280 nm. The void volume peak was then subjected to chromatography on Sephadex G-100 in 0.1 N acetic acid. The eluted peaks were saved and lyophilized.

3.3.6 OTHER TISSUES

The other tissue studied including snake brains, mouse testes, livers, spleens, kidneys, brains, and lungs from rats, were extracted in a similar way as described for salmon pituitaries, using the acid acetone extraction and acetone precipitation procedure. The acid acetone powders of these tissues were then dried under vacuum and kept at -20 °C for further purifications and assays.

RESULTS AND DATA ANALSIS

Chapter 3 RESULTS AND DATA ANALYSIS

3.1 ESTABLISHMENT OF ASSAY SYSTEM

3.1.1 LIPOLYSIS ASSAY

Although both products of lipolysis, glycerol and free fatty acid, can be used as an index of lipolytic activity, glycerol production was chosen throughout this project because unlike free fatty acid it does not reesterify after lipolysis (Patten, 1970). By a colorimetric method involving chromotropic acid reagent (Korn, 1955), the glycerol released can be measured to a very high degree of accuracy. It was shown, in figure 3-1, that the relationship between absorbance at 570 nm and the quantity of glycerol was a linear one at least up to 0.2 micromole glycerol per tube assayed.

Figure 3-2 shows a typical lipolytic response curve of hamster epididymal adipocytes under the stimulation of porcine corticotropin. It was noticed that hamster adipocytes responded in a dose-dependent manner only within the range of ACTH concentrations from 10^{-10} M to 10^{-8} M with an ED_{50} of about 6 x 10^{-10}M. Above the concentration of 10^{-8} M, the lipolytic response of hamster adipocytes remained at the maximal level which, though varying among experiments, was in the range of 7 umole glycerol hr^{-1} g^{-1} fat cell to 8 umole glycerol hr^{-1} g^{-1} fat cell.

Besides corticotropin, the lipolytic activities of related peptide hormones were also studied with this assay. Figure 3-3 presents the dose response curves of, α-MSH and β-MSH,

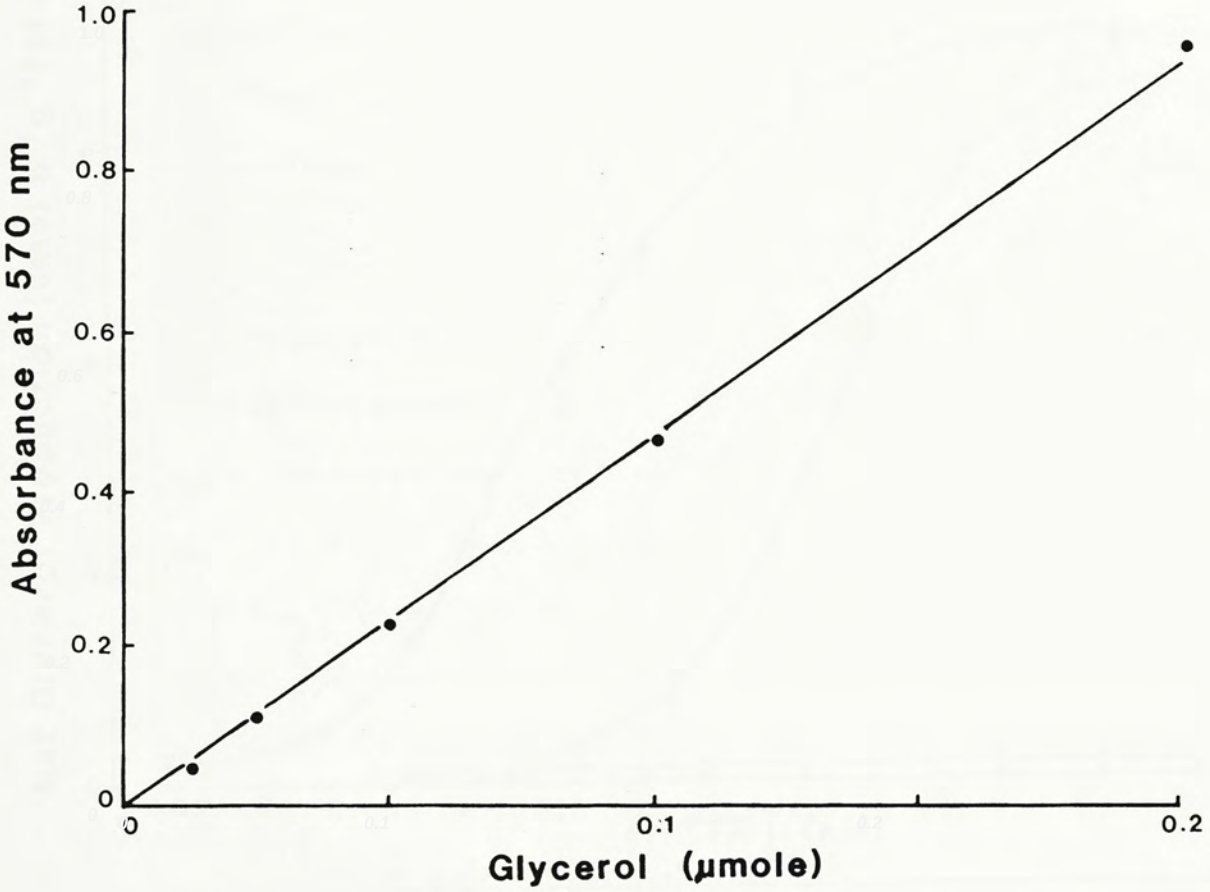


Figure 3-1. Standard curve of glycerol determination

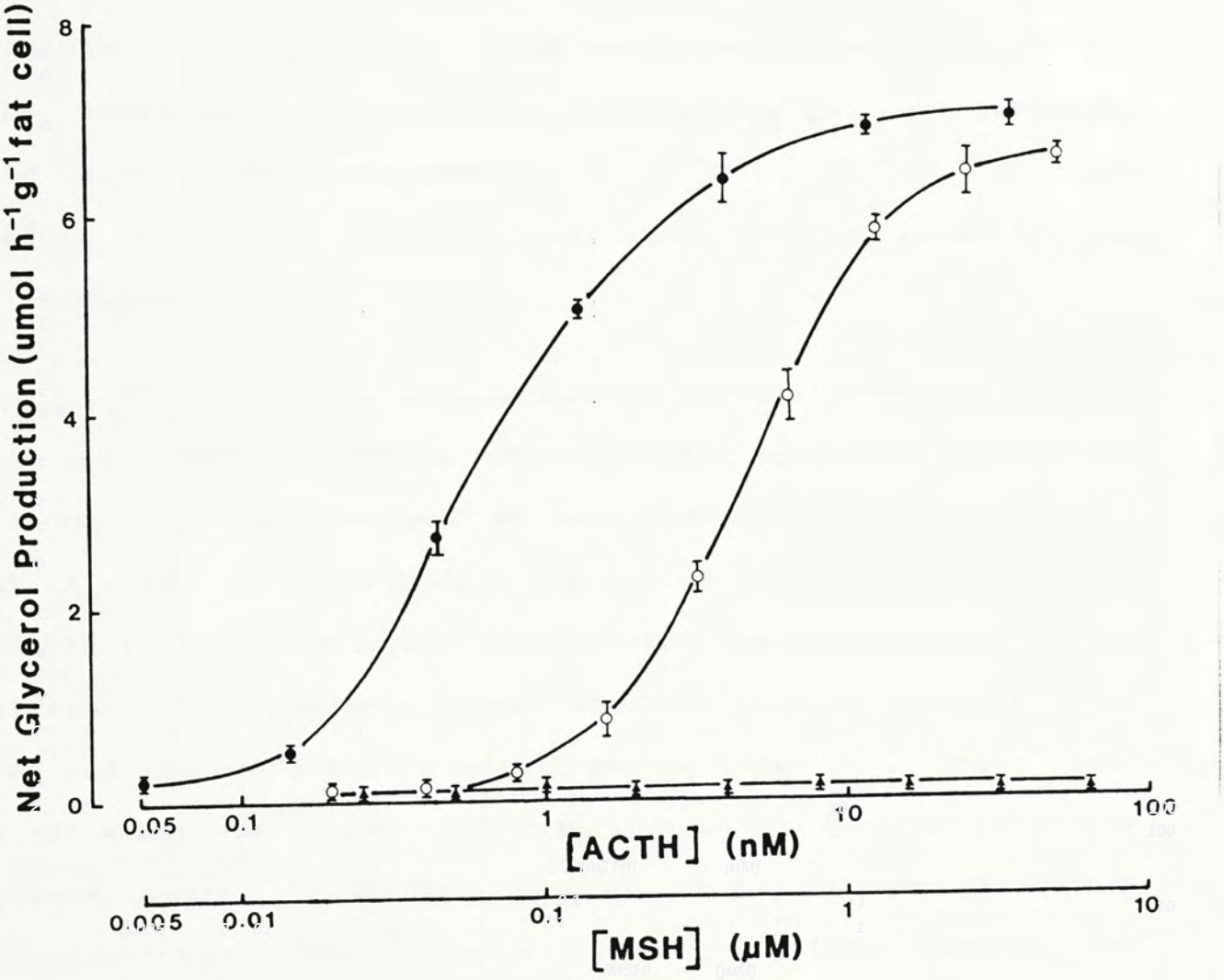


Figure 3-2. Effects of porcine ACTH (●), α-MSH (▲) and β-MSH (△) on lipolysis in hamster adipocytes.

two peptide hormones structurally related to corticotropin. α-MSH was regarded as inactive in stimulating lipolysis in hamster adipocytes because, as shown in figure 3-2, β-MSH showed no stimulatory effects in the adipocytes up to a concentration of 10^{-5} M which was unlikely to be encountered under physiological conditions. However, when β-MSH was investigated, it was found to be effective in stimulating lipolysis in a dose-dependent manner within the concentration of 10^{-8} M to 10^{-6} M. It was therefore much less potent, only about 1% as effective as corticotropin.

Glucagon and β-LPH, two known potent lipolysis inducers in rat and rabbit adipocytes respectively, were also tested and was found to be inactive even at very high concentrations: 1.1 ug / ml (3×10^{-7} M) for glucagon and 0.5 ug / ml (0.5×10^{-7} M) of -LPH (Table 3-1). In order to determine the specificity of this lipolysis assay, a large number of other peptide hormones were tested and the results were summarized in Table 3-1. Among them, only VIP was found to be slightly active in the hamster adipocyte lipolysis assay. From these results, it may be concluded that this lipolysis assay is a fairly specific assay for conticotropin. However, even when a compound is found to be active in stimulating lipolysis in hamster adipocytes, we still cannot arrive at the conclusion that it is corticotropin or β-MSH because lipolysis is the result of a cascade of reactions and regulation can occur in many steps of the cascade other than at the level of the hormonal receptors.

Table 3-1. Lipolytic activity of various peptides in hamster epididymal adipocytes.

Peptide	Dose (uM)	% of control
β-endorphin	1.0	98.05 ± 0.78
β-lipotropin	0.05	88.55 ± 1.91
dynorphin (1-13)	6.0	82.10 ± 4.6
Met-enkephalin	6.0	98.73 ± 1.90
Leu-enkephalin	6.0	95.05 ± 0.49
Glucagon	0.3	93.90 ± 18.97
α-melanotropin	6.0	135.27 ± 10.09 *
β-melanotropin	6.0	1253.88 ± 52.40
γ-melanotropin	6.0	97.79 ± 3.43
neurotensin	6.0	92.60 ± 2.31
somatostatin	6.0	76.65 ± 7.28 *
VIP	1.5	282.22 ± 3.83 *
corticotropin	10^{-3}	532.92 ± 36.20 **
corticotropin	10^{-4}	151.20 ± 6.90

The values represent mean ± S.E.M. of triplicate determination.

VIP vasoactive intestinal peptide

* $p < 0.001$

** $p < 0.01$ compared with control.

3.1.2 STEROIDOGENESIS ASSAY

When rat decapsular adrenal cells were incubated with corticotropin, corticosterone production was stimulated. To determine this stimulation of steroid production, the most commonly used and most convenient method was radioimmunoassay e.g. Li et al (1982). Figure 3-3 presents a typical displacement curve of ^{3}H-corticosterone using an anti-corticosterone serum purchased from Miles. As shown in the figure, this assay could detect corticosterone within the range of 100 pg to 10 ng per tube and hence the amount of corticosterone produced in the corticosteroidogenesis assay could be accurately measured.

A response curve of rat adrenal decapsular cells to corticotropin was shown in figure 3-4. A dose dependent response was observed in the dose range of 4×10^{-11} M to 4×10^{-9} M beyond which the response leveled off. Besides corticotropin and its analogs, other hormones known to have corticosteroidogenic activity were α-MSH and β-MSH but at dosages much higher than that of corticotropin (Li et al, 1982). The effect of diverse peptides on steroidogenesis were investigated and the results were summarized in Table 3-2. It was shown that these peptides were inactive in rat adrenal decapsular cells even at very high concentrations. It has been demonstrated previously that many other hormones lacked steroidogenic activity (Rafferty et al, 1983). Thus the steroidogenesis assay appeared to be a very sensitive and specific assay for corticotropin and its related hormones.

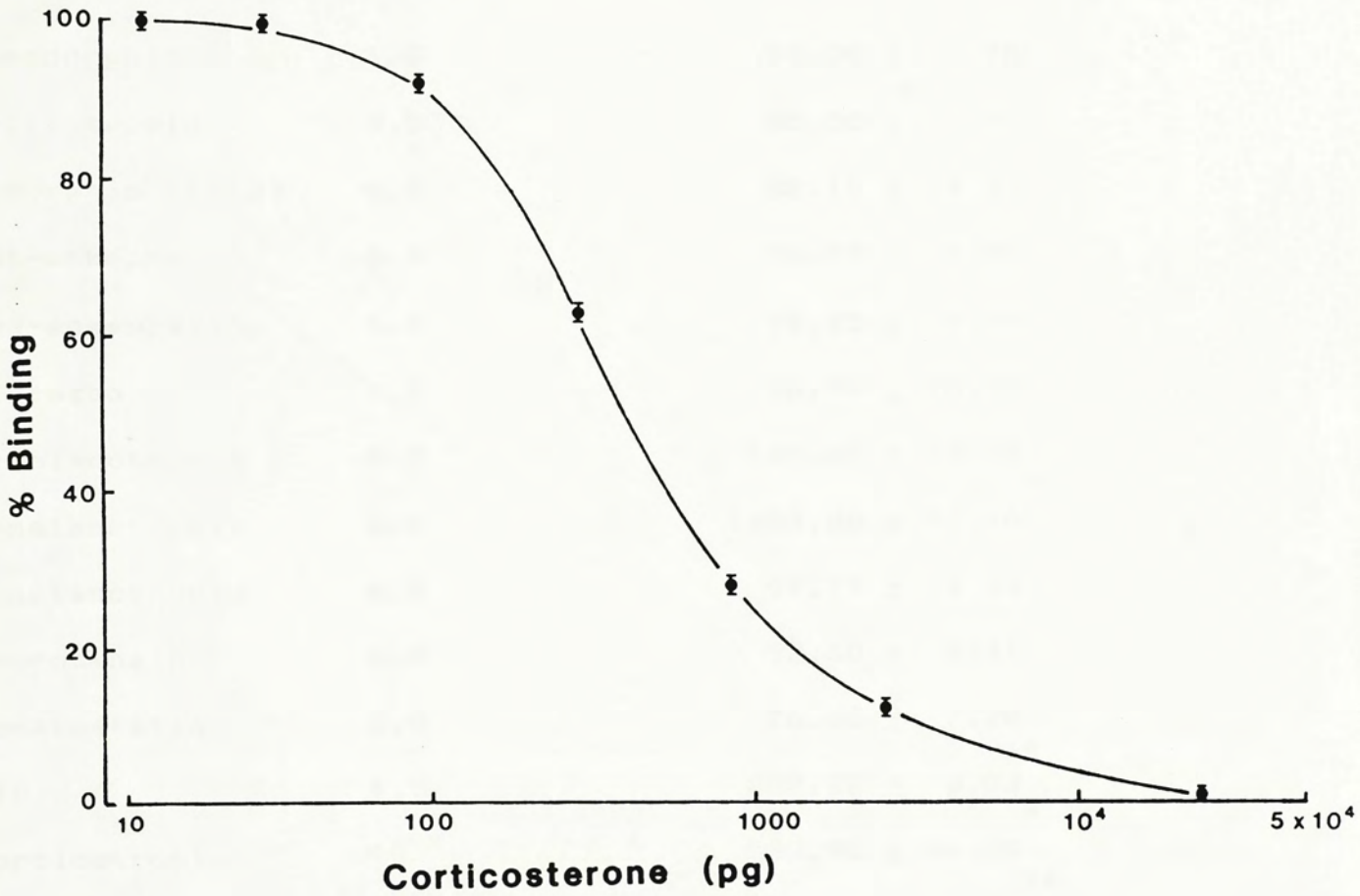


Figure 3-3. Standard curve of corticosterone RIA

Table 3-1. Lipolytic activity of various peptides in hamster epididymal adipocytes.

Peptide	Dose (uM)	% of control
β-endorphin	1.0	98.05 ± 0.78
β-lipotropin	0.05	88.55 ± 1.91
dynorphin (1-13)	6.0	82.10 ± 4.6
Met-enkephalin	6.0	98.73 ± 1.90
Leu-enkephalin	6.0	95.05 ± 0.49
Glucagon	0.3	93.90 ± 18.97
α-melanotropin	6.0	135.27 ± 10.09 *
β-melanotropin	6.0	1253.88 ± 52.40
γ-melanotropin	6.0	97.79 ± 3.43
neurotensin	6.0	92.60 ± 2.31
somatostatin	6.0	76.65 ± 7.28 *
VIP	1.5	282.22 ± 3.83 *
corticotropin	10^{-3}	532.92 ± 36.20 **
corticotropin	10^{-4}	151.20 ± 6.90

The values represent mean ± S.E.M. of triplicate determination.

VIP vasoactive intestinal peptide

* $p < 0.001$

** $p < 0.01$ compared with control.

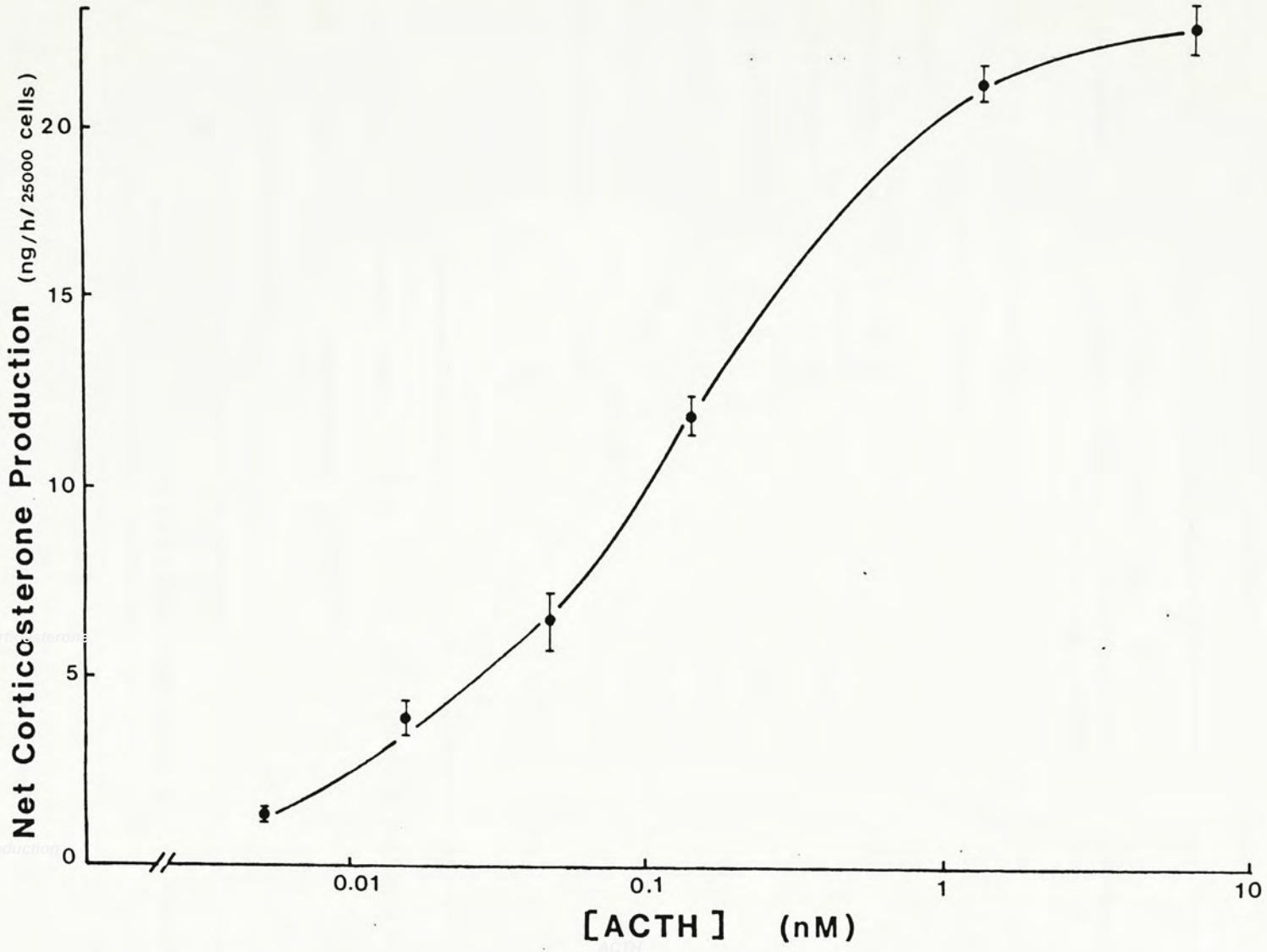


Figure 3-4. Effect of porcine ACTH on steroidogenesis in rat adrenal decapsular cells

Table 3-2. Steroidogenic activities of various peptides in rat adrenal decapsular cells

Hormone	Dose (uM)	Corticosterone production (ng /h/25,000 cells)
Control	-	ND
corticotropin	4.0×10^{-3}	4.38 ± 0.25^{a}
β-endorphin	1.0	ND
β-lipotropin	0.2	ND
dynorphin (1-13)	1.0	ND
Leu-enkephalin	10.0	1.74 ± 1.64^{b}
Met-enkephalin	10.0	ND
VIP	1.0	0.47 ± 0.09^{b}

The values represent mean ± S.E.M. of triplicate determinations.

VIP : vasoactive intestinal peptide.

ND : undetectable.

a : $p < 0.001$ compared with control.

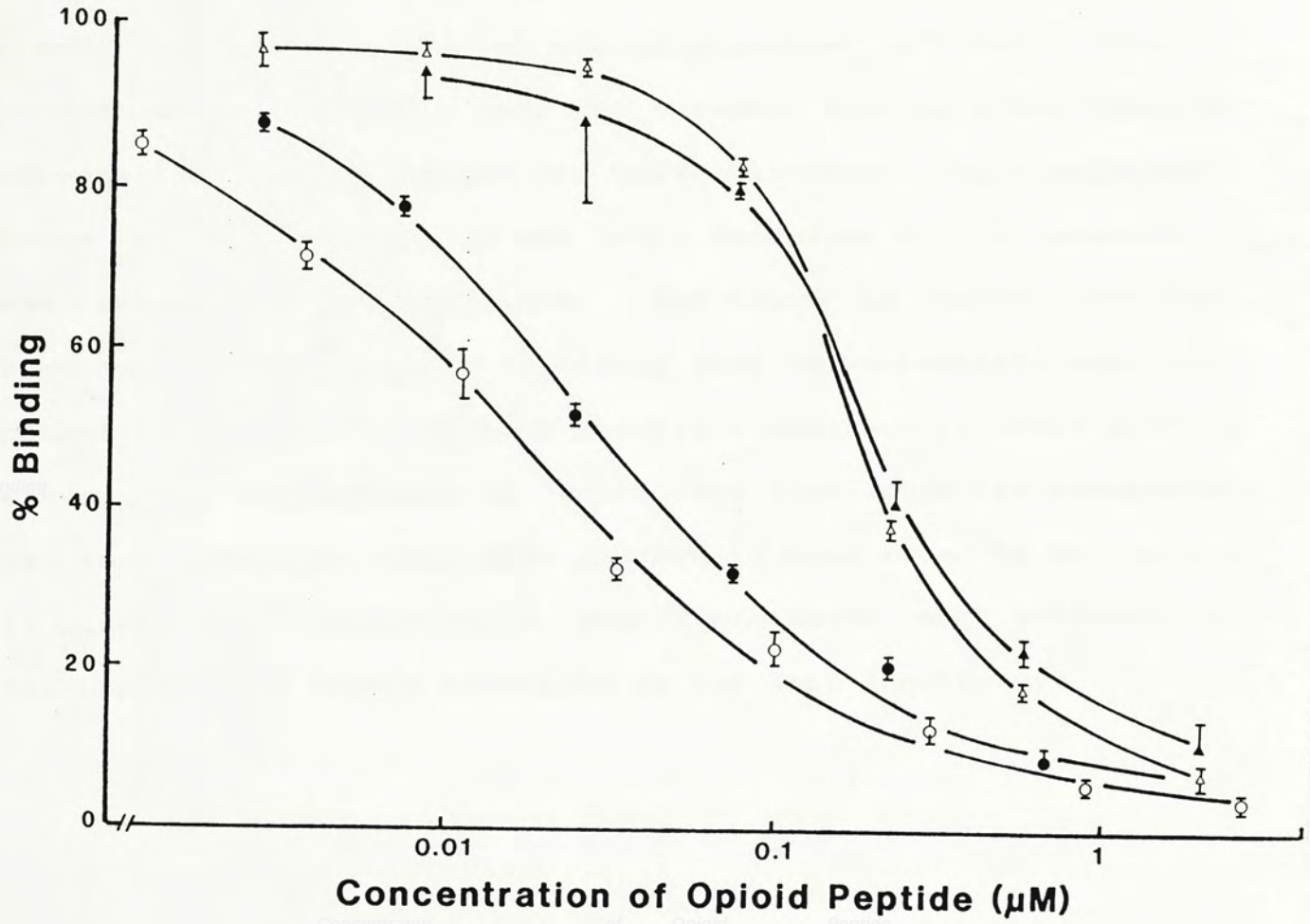
b : not statistically significant compared with control

3.1.3 OPIATE RECEPTOR BINDING ASSAY

It was well known that the cerebral cortex contains different types of opiate receptors (Martin. 1967). The σ-receptors and χ-receptors are only distinguishable by synthetic opiate analogs like SKF 10,047 and benzomorphans respectively (Andrea, 1982); μ-receptors are specific for morphine-like alkaloids and δ-receptors are highly specific for short-chain endogenous opioid peptides such as enkephalins but display lower affinity for the other classes of opiates. To detect the presence of opioid peptides in tissue extracts and chromatographic fractions, a leucine-enkephalin analog, DADLE (D-ala^2-D-leu^5-(tyrosyl-3,5-^{3}H) enkephalin), was used as the competitive ligand so that opioid peptides present in the test fractions and extracts would be able to displace this ligand from the δ-receptors.

Figure 3-5 shows a displacement curve of ^{3}H-DADLE by its natural counterpart leucine-enkephalin. It was a mono-component saturable binding curve with an apparent Kd of 29.0 nM indicating that ^{3}H-DADLE was displaced apparently from a single type of binding sites. Since peptide bonds involving amino acid(s) of D-configuration were very unlikely to be broken by common peptidases, the displacement of ^{3}H-DADLE by tissue extracts or fractions are unlikely to be due to the degradation of this labelled peptide by enzymes present in the tissue extracts or fractions. When other opioid peptides including β-endorphin, dynorphin (1-13) and methionine-enkephalin, were tested, the resulting competition curves could all be well

Figure 3-5. Inhibition of [^{3}H] DADLE binding to rat brain membranes by leu-enkephalin (●), met-enkephalin (○), β-endorphin (▲) and dynorphin (△).


100
80
60
40
20
0
% Binding
0.01
0.1
1
Concentration of Opioid Peptide (μM)

fitted into the single site model as shown in figure 3-5 and the Kd values of the different opioid peptides were quite different from one another as listed in table 3-3.

From the results listed above, the enkephalins, leu-enkephalin and met-enkephalin, were found to be more potent than β-endorphin and dynorphin in the displacement of ^{3}H-DADLE from its binding sites indicating that the receptor binding properties of enkephalins and β-endorphin are quite different. The displacement curve of ^{3}H-naloxone from rat brain membranes by leu-enkephalin was presented in figure 3-6. The higher Kd value for leu-enkephalin in this assay indicated that leu-enkephalin was less potent in binding to naloxone binding sites than to DADLE binding sites. The displacement of ^{3}H-naloxone from crude rat membrances by test fractions which have previously been found to be active in displacing ^{3}H-DADLE would constitute unequivocal evidence for the presence of opioid materials in the test fractions.

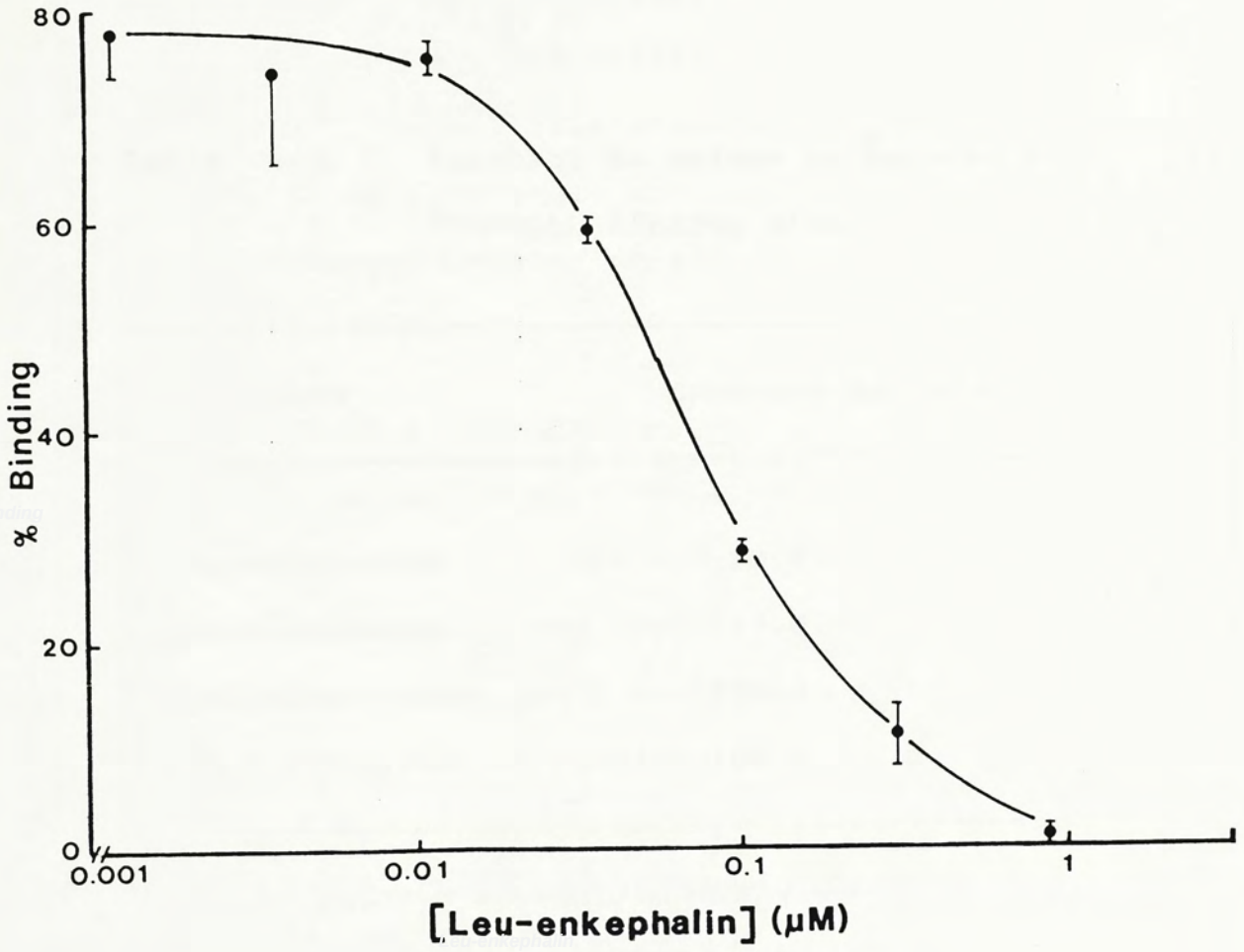


Figure 3-6. Inhibition of [^{3}H] naloxone to rat brain membranes by leucine enkephalin.

Table 3-3. Apparant Kd values of opiates for ^{3}H-DADLE binding site

Ligand	Apparant Kd (nM)
Leu-enkephalin	29.0
Met-enkephalin	14.5
Dynorphin (1-13)	195.0
β-endorphin	185.0

3.2 EXTRACTIONS AND PURIFICATIONS

3.2.1 EQUINE PANCREAS

From 50 g of equine pancreatic acetone powder, 14 g of acid acetone powder (AAP) was obtained by the acid acetone extraction method. The AAP thus prepared was tested in various assays and found to be moderately active in the lipolysis assay using hamster epididymal adipocytes and in the opiate receptor binding assay (Table 3-4).

After salt fractionation and desalting on a Sephadex G-10 column (Figure 3-7), 14 g of fraction D was collected. The other fraction (fraction C), was discarded after finding that it was virtually inactive in the lipolysis and opiate receptor binding (Table 3-4). Fraction D was further fractionated on a Sephadex G-25 column into 3 fractions, D-I, D-II and D-III as shown in figure 3-8. Two of these fractions, D-I and D-II, exhibited weak activities in the lipolysis assay but D-III was almost completely inactive. However, all three fractions were able to displace ^{3}H-DADLE from rat brain membrane (Table 3-4).

The opioid activity located in D-I was unlikely β-endorphin but more likely a larger polypeptide or an aggregate of smaller opioid-like polypeptides. For the other 2 peaks, D-II and D-III, the presence of smaller opioid materials was strongly suggested by their high potencies in displacing ^{3}H-DADLE from rat brain membrane (Table 3-4). Because they were retarded on G-

Figure 3-7. Elution profile of equine pancreas fraction B from a Sephadex G-10 column (2.5 x 70 cm). Eluent : 0.1 M acetic acid. Flow rate: 72 ml/h. Fraction size: 7.5 ml. Sample: Fr. B obtained from 1.1 g of AAP. Yield : 0.9 g of fraction D.

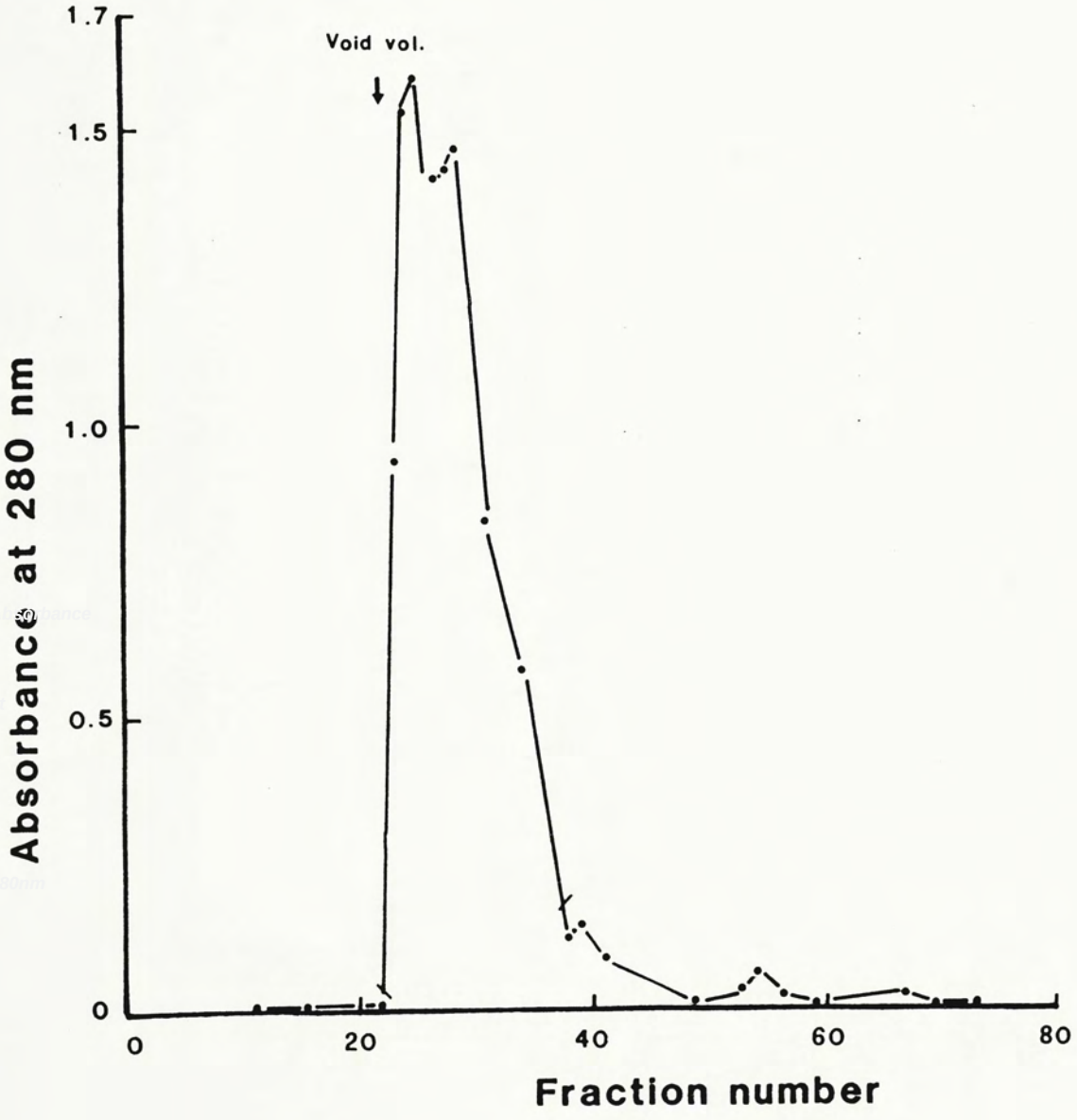

Void vol.
1.7
1.5
1.0
0.5
0
Absorbance at 280 nm
0
20
40
60
80
Fraction number

Figure 3-8. Elution profile of 300 mg equine pancreas fraction D from a Sephadex G-25 column (3.5 x 83 cm). Eluent : 0.1 M acetic acid. Flow rate : 60 ml/hr. Fraction size : 7.5 ml. Yield : D-I, 70 mg; D-II, 6 mg; D-III, 13 mg.

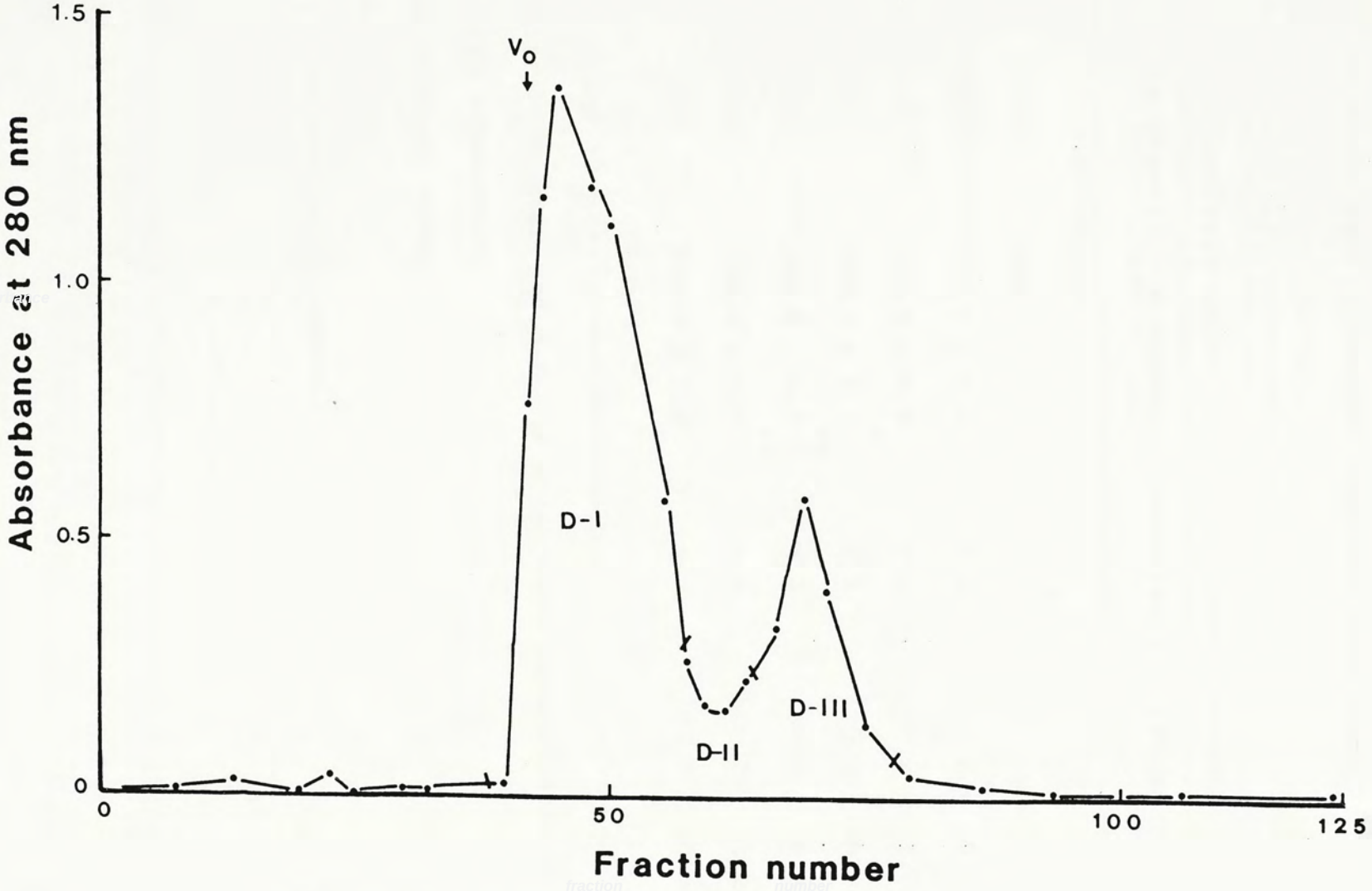

1.5
1.0
0.5
0
Absorbance at 280 nm
V_O
D-I
D-II
D-III
0
50
100
125
Fraction number

Table 3-4. Lipolytic and opiate receptor binding (RRA) activities of equine pancreas fractions.

	Lipolysis assay		RRA (^{3}H-DADLE)	
fraction	Dose (mg/ml)	% control	dose (mg/ml)	LEK eq (nM)
Control	-	100	-	ND
AAP	2.0	178.0 ± 5.1	4.0	110.0 ± 10.0
Fr. C	4.0	111.2 ± 8.5	4.0	20.0 ± 1.0
Fr. D	2.0	134.3 ± 0.1 *	4.0	115.0 ± 15.0
D-I	2.0	144.8 ± 8.3 **	4.0	69.0 ± 18.4
D-II	2.0	136.6 ± 4.1 **	4.0	126.7 ± 11.5
D-III	2.0	103.8 ± 2.0	4.0	123.3 ± 5.8

All values represent mean ± S.E.M. of triplicate determinations.

LEK : leucine enkephalin.

AAP : acid acetone powder

* $p < 0.001$

** $p < 0.01$ compared with control.

25, their molecular weights were estimated to be smaller than 5,000, the exclusion volume of Sephadex G-25.

All three fractions were submitted to ion-exchange chromatography on CM-cellulose and the chromatograms were shown in figures 3-9, 3-10, and 3-11. The CMC fractions were assayed for lipolytic, steroidogenic and opiate receptor binding activitites. Thus fractions II-7 and III-7 were found to be highly potent in the opiate receptor binding assay (Table 3-5). To further investigate the binding properties of these fractions to rat brain membranes, the effects of different doses of II-7 were tested in the opiate receptor binding assay and the results were presented in figure 3-12. The Kd value found in the ^{3}H-DADLE binding site for II-7 was 32 μg and the shape of the displacement curve very much resembled that of leu-enkephalin indicating that the binding properties of this fraction were quite similar to those of leu-enkephalin. In another radioreceptor assay, displacement of ^{3}H-naloxone by this fraction was also observed confirming the presence of opiate-like materials (Table 3-5). For the other fraction with opiate receptor binding activity, III-7, the amount obtained was too small to permit further assays.

The fractions with opiate receptor binding activity including II-7 and III-7 were found to be strongly adsorbed on the column and were eluted only by high concentrations of ammonium acetate. this finding revealed that these opioid polypeptides were quite basic and that they might contain basic amino acid residues like glutamine and histidine as in the case

Figure 3-9. Elution profile of 500 mg of equine pancreas fraction D-I from CM-cellulose column (1.3x75 cm). Eluent: (a) 10 mM NH_4OAc, pH 4.6; (b) 10 mM NH_4OAc pH 4.6 to 0.1 M, pH 6.7; (c) 0.1 M NH_4OAc, pH 6.7 to 0.2 M, pH 7.0; (d) 0.2 M NH_4OAc to 0.5 M, pH 7.0. Yield : I-1, 37 mg; I-2, 6 mg; I-3, 95 mg; I-4, 33 mg; I-5, 40 mg; I-6, 32 mg; I-7, 13 mg; I-8, 11 mg; I-9, 14 mg; I-10, 14 mg; I-11, 11 mg.

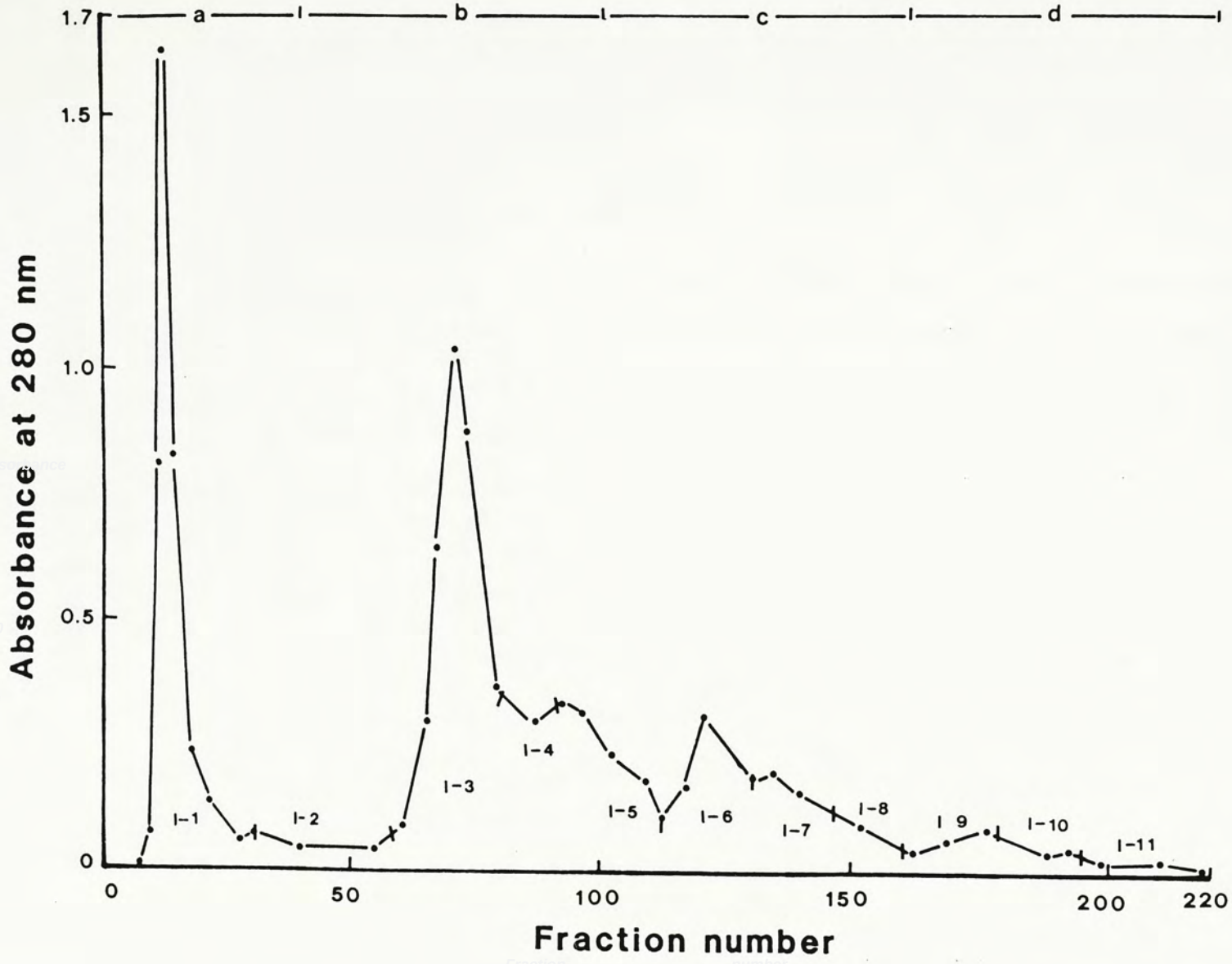

a
b
c
d
1.7
1.5
1.0
0.5
0
Absorbance at 280 nm
I-1
I-2
I-3
I-4
I-5
I-6
I-7
I-8
I 9
I-10
I-11
0
50
100
150
200
220
Fraction number

Figure 3-10. Elution profile of 250 mg of equine pancreas D-II from CM-cellulose column (1.3 x 75 cm). Eluent : (a) 0.01 M NH_4OAc, pH 4.6; (b) 10 mM NH_4OAc, pH 4.6 to 0.1 M, pH 6.7; (c) 0.1 M NH_4OAc, pH 6.7 to 0.2 M, pH 7.0; (d) 0.2 M NH_4OAc to 0.5 M, pH 7.0. Yield : II-1, 90 mg; II-2, 36 mg; II-3, 63 mg; II-4, 30 mg; II-5, 12 mg; II-6, 2 mg; II-7, 6 mg; II-8, 7 mg.

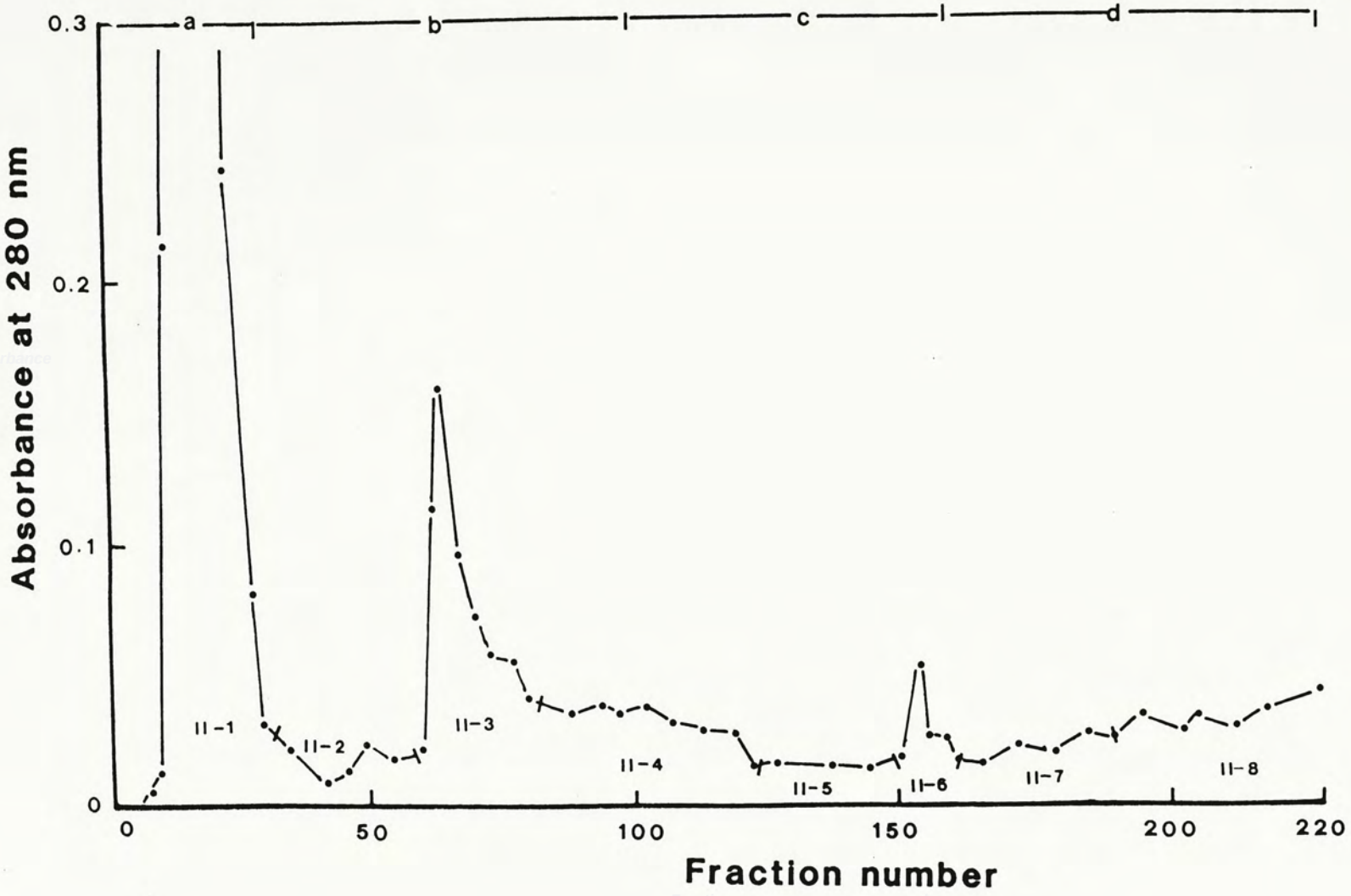

a
b
c
d
0.3
0.2
0.1
0
Absorbance at 280 nm
II-1
II-2
II-3
II-4
II-5
II-6
II-7
II-8
0
50
100
150
200
220
Fraction number

Figure 3-11. Elution profile of 580 mg of equine pancreas D-III from CM-cellulose column (1.3 x 75 cm). Eluant : (a) 0.01 M NH_4OAc, pH 4.6; (b) 10 mM NH_4OAc, pH 4.6 to 0.1 M, pH 6.7; (c) 0.1 M NH_4OAc, pH 6.7 to 0.2 M, pH 7.0; (d) 0.2 M NH_4OAc to 0.5 M, pH 7.0. Yield : III-1, 5 mg; III-2, 39 mg; III-3, 49 mg; III-4, 6 mg; III-5, 2 mg; III-6, 1 mg; III-7, 1 mg; III-8, 2 mg.

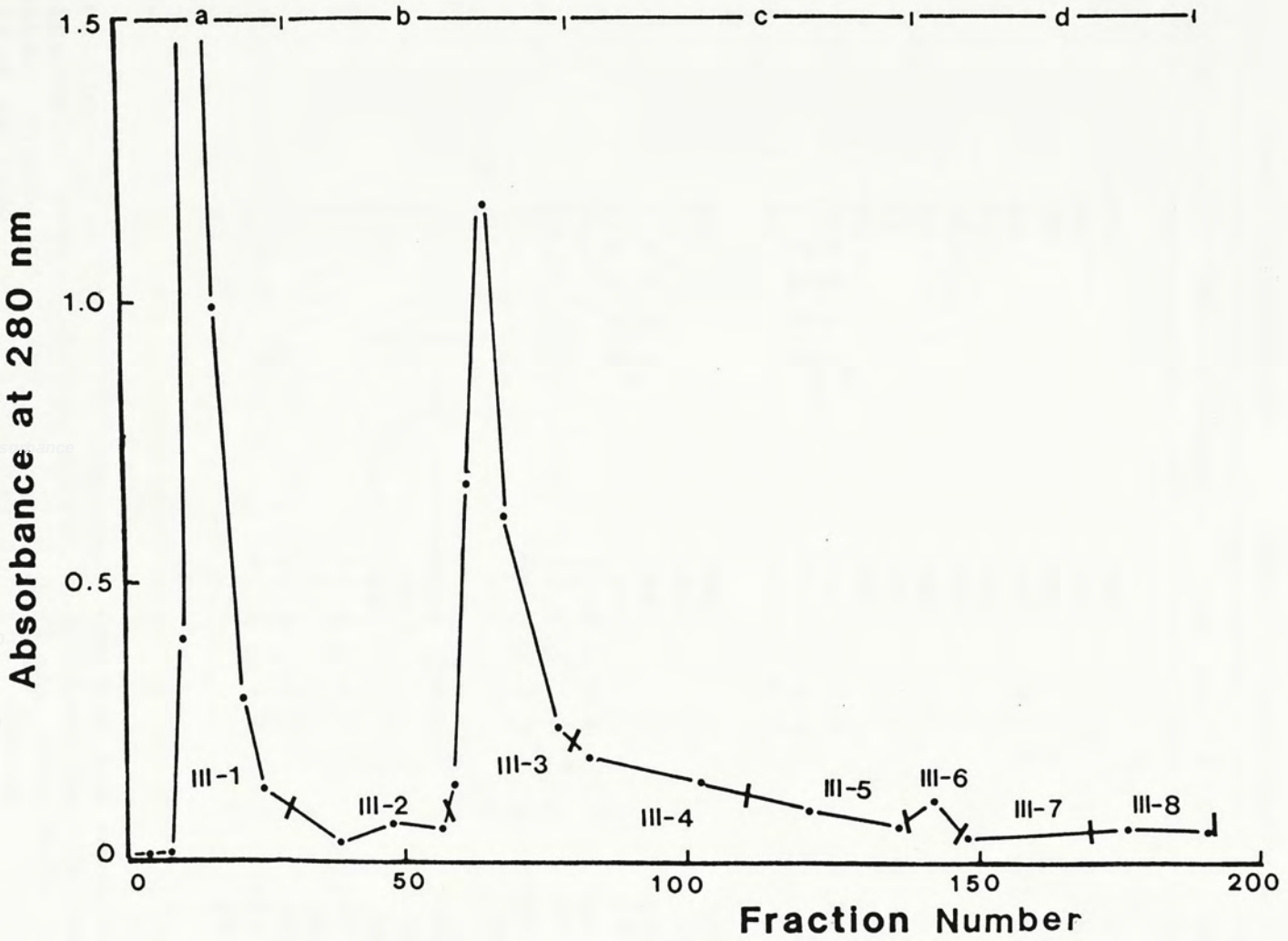

a
b
c
d
1.5
1.0
0.5
0
Absorbance at 280 nm
III-1
III-2
III-3
III-4
III-5
III-6
III-7
III-8
0
50
100
150
200
Fraction Number

Table 3-5. Steroidogenic and opiate receptor binding (RRA) activities of pancreas CM-cellulose fractions of equine pancreas

Fraction	Steroidogenesis (ng cort./hr/25,000 cells)	RRA (^{3}H-naloxone) LEK eq. (nM)	RRA (^{3}H-DADLE) LEK eq. (nM)
Control	ND	ND	ND
I-1	ND	ND	ND
I-2	ND	ND	ND
I-3	ND	ND	10.0 ± 0.0
I-4	ND	ND	5.3 ± 0.7
I-5	ND	ND	7.7 ± 2.7
I-6	ND	ND	8.4 ± 5.1
I-7	ND	ND	20.3 ± 2.5
I-8	ND	ND	25.0 ± 0.0
I-9	4.4 ± 0.1*	6.0 ± 5.3	30.5 ± 9.2
I-10	2.3 ± 0.3**	25.0 ± 0.0	ND
I-11	ND	34.3 ± 6.7	ND
II-1	ND	ND	ND
II-2	ND	ND	ND
II-3	1.7 ± 0.4	ND	11.3 ± 1.1
II-4	1.6 ± 0.0*	ND	17.3 ± 1.8
II-5	ND	31.5 ± 8.5	26.6 ± 5.4
II-6	ND	15.6 ± 3.5**	27.0 ± 7.1
II-7	ND	46.5 ± 0.0*	150.0 ±50.0
II-8	ND	UD	ND
III-1	ND	48.6 ±10.7	40.0 ± 0.0
III-2	ND	UD	ND
III-3	ND	UD	ND
III-4	ND	17.8 ±10.7	22.8 ± 3.2
III-5	ND	ND	38.5 ±16.6
III-6	UD	17.0 ±17.5	18.0 ± 2.8
III-7	UD	51.2 ±19.6	109.0 ±58.0
III-8	ND	UD	29.3 ± 5.3

a : fractions I-1 to I-11 derived from D-I had been tested in the lipolysis assay at 0.1 ug/ml and found to be inactive. All other fractions (II-1 to II-8 derived from D-II and III-1 to III-8 derived from D-III) had not been tested.

b : All fractions were tested at 160 ug/ml in steroidogenesis assay, 160 ug/ml in RRA with ^{3}H-naloxone and 340 ug/ml in RRA with ^{3}H-DADLE as labelled ligand.

LEK : leucine enkephalin

ND : undetectable.

UD : undetermined because of insufficient materials.

* $p < 0.001$ and ** $p < 0.01$ compared with the controls.

all values shown are mean ± S.E.M of triplicate determination.

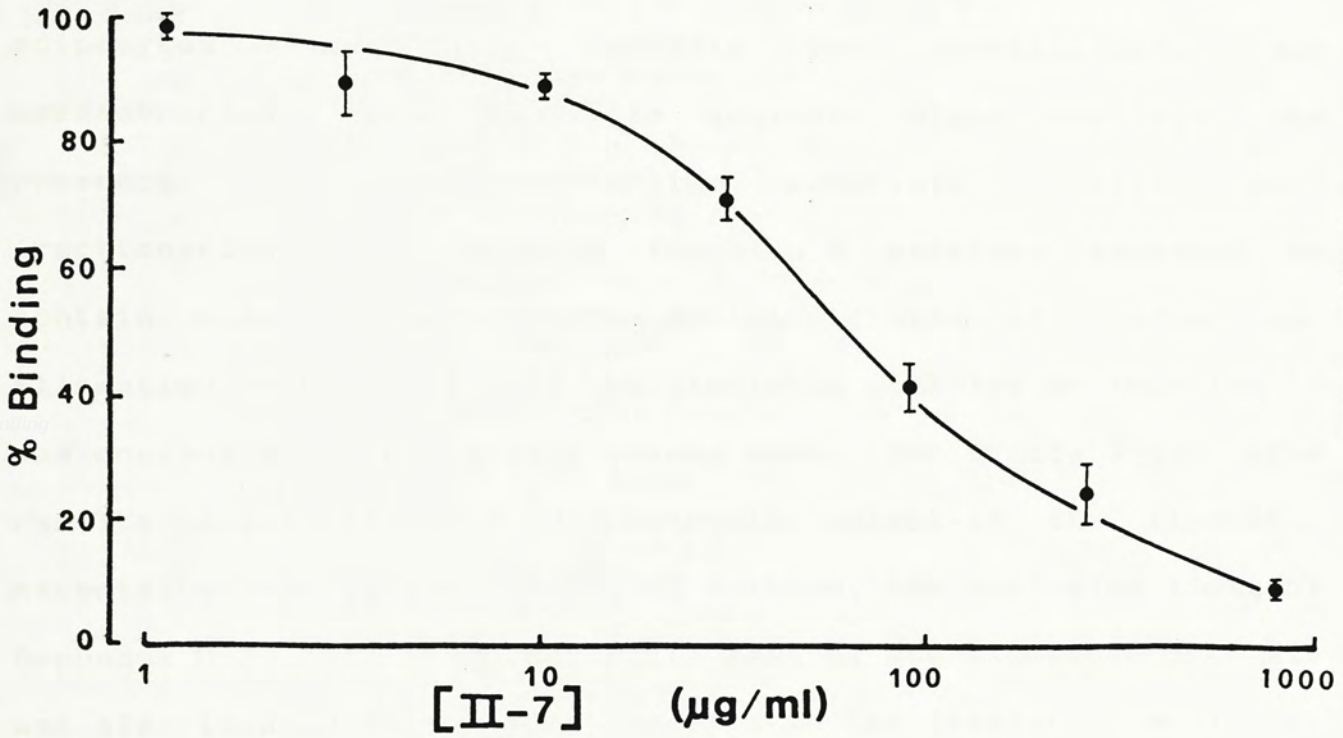


Figure 3-12. Inhibition of [^{3}H] DADLE to rat brain membrane by equine pancreas fraction II-7.

of β-endorphin.

Besides endorphin-like materials, corticotropin-like activity was also detected in the equine pancreatic extract. The acid acetone powder of equine pancreas was shown to be slightly lipolytic indicating the presence of lipolytic hormone(s). Since it was demonstrated that the lipolysis assay utilizing hamster adipocytes was quite specific for corticotropin and melanotropins, this lipolytic activity might indicate the presence of corticotropin-like materials. After salt fractionation, the desalted fraction D obtained appeared to contain most of the lipolytic activity (Table 3-4). After gel filtration on Sephadex G-25 the lipolytic activity of fraction D was concentrated in the void volume peak, D-I (Table 3-4). The results suggested that the molecular weight of the lipolytic material(s) was larger than 5,000 daltons, the exclusion limit of Sephadex G-25. In steroidogenesis assays, steroidogenic activity was also located in the same fraction as the lipolytic activity. This coincidence strongly suggested that a corticotropin-like material was present in this fraction since the steroidogenesis assay has been proven to be a very specific test for corticotropin-like materials.

In the CM-cellulose chromatography, the corticotropin-like activity was tightly adsorbed on the column and was eluted only by buffer of very high ionic strength (Table 3-5 and figure 3-9). Based on this result, we speculated that this corticotropin-like material was quite basic in nature as human

corticotropin which contains a large proportion of basic amino acid residues such as lysine and arginine (Cheng et al, 1980).

When 50 ug of some CM-cellulose fractions, including I-9, I-10, II-3 and II-4 were incubated with isolated rat adrenal decapsular cells, stimulation of corticosterone production was demonstrated (Table 3-5). The response of rat adrenal decapsular cells to different doses of I-9 was shown in figure 3-13. It was observed that a dose-dependent response was demonstrated and the ED_{50} of this fraction was estimated to be 65 ug. The dose response curve for porcine corticotropin and I-9 were very similar in shape showing that I-9 stimulated corticosterone production in rat decapsular cells in a manner similar to corticotropin. Although the corticotropin-like activity in fraction I-9 has not yet been completely purified and its structure has not been elucidated, this corticotropin-like activity in equine pancreas seemed to be structurally and functionally similar to corticotropin.

In two CM-cellulose fractions of D-II, II-3 and II-4, steroidogenic activities were also observed but at a much lower potency (Table 3-5). This result revealed the possibility of the presence of steroidogenic materials of smaller molecular size in the equine pancreas.

3.2.2 RAT PLACENTA

Placenta, a multifunctional organ, was known to contain a high proportion of proteolytic enzymes which might degrade

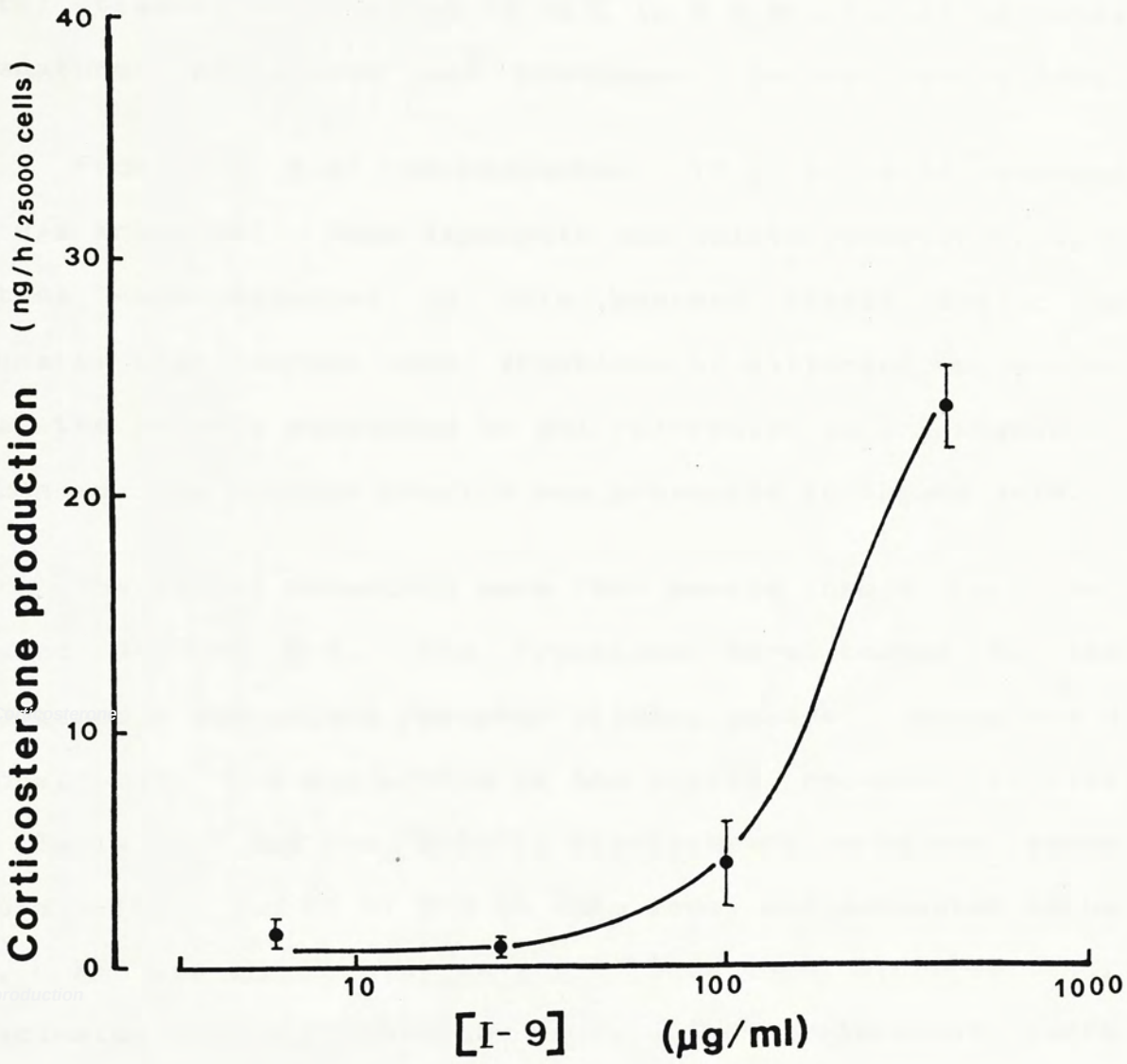


Figure 3-13 Effect of equine pancreas fraction I-9 on steroidogenesis in rat adrenal decapsular cells.

peptide hormones such as corticotropin and endorphins during the processes of extraction. To prevent this from happening, the placental tissues were heated to 95 °C in 2 N HCl for 20 minutes to denature peptidases and proteases before extraction.

From 290 g of rat placentas, 17 g of acid acetone powder was prepared. Weak lipolytic and opiate receptor binding activities were detected in this extract (Table 3-6). To fractionate this extract into fractions of different molecular weights, the AAP was submitted to gel filtration on a Sephadex G-25 column and the elution profile was presented in figure 3-14.

The eluted materials were then pooled into 4 fractions, designated R-1 to R-4. The fractions were tested in the steroidogenesis and opiate receptor binding assays. Among the 4 fractions, only R-3 was active in the opiate receptor binding assay (Table 3-6) and its ^{3}H-DADLE displacement curve was shown in figure 3-15. The Kd of R-3 in this assay was estimated to be 250 µg. R-3 has a molecular weight of less than 5,000 daltons, the exclusion volume of Sephadex G-25. The displacement curve shown in figure 3-16 strongly supports the proposal of the presence of opioid material in this fraction, since all the proteolytic activities in the placental tissues should have been destroyed and a false displacement curve generated by enzymatic degradation of the labelled ligand, ^{3}H-DADLE, would be very unlikely.

Very weak steroidogenic activity was detected in R-4 (Table 3-6). Because of the small size of this activity, much

Figure 3-14. Elution profile of 200 mg rat placenta AAP from a Sephadex G-25 column (3 x 80 cm). Eluent : 0.1 M acetic acid. Flow rat : 26.7 ml/h. Fraction size: 4 ml.

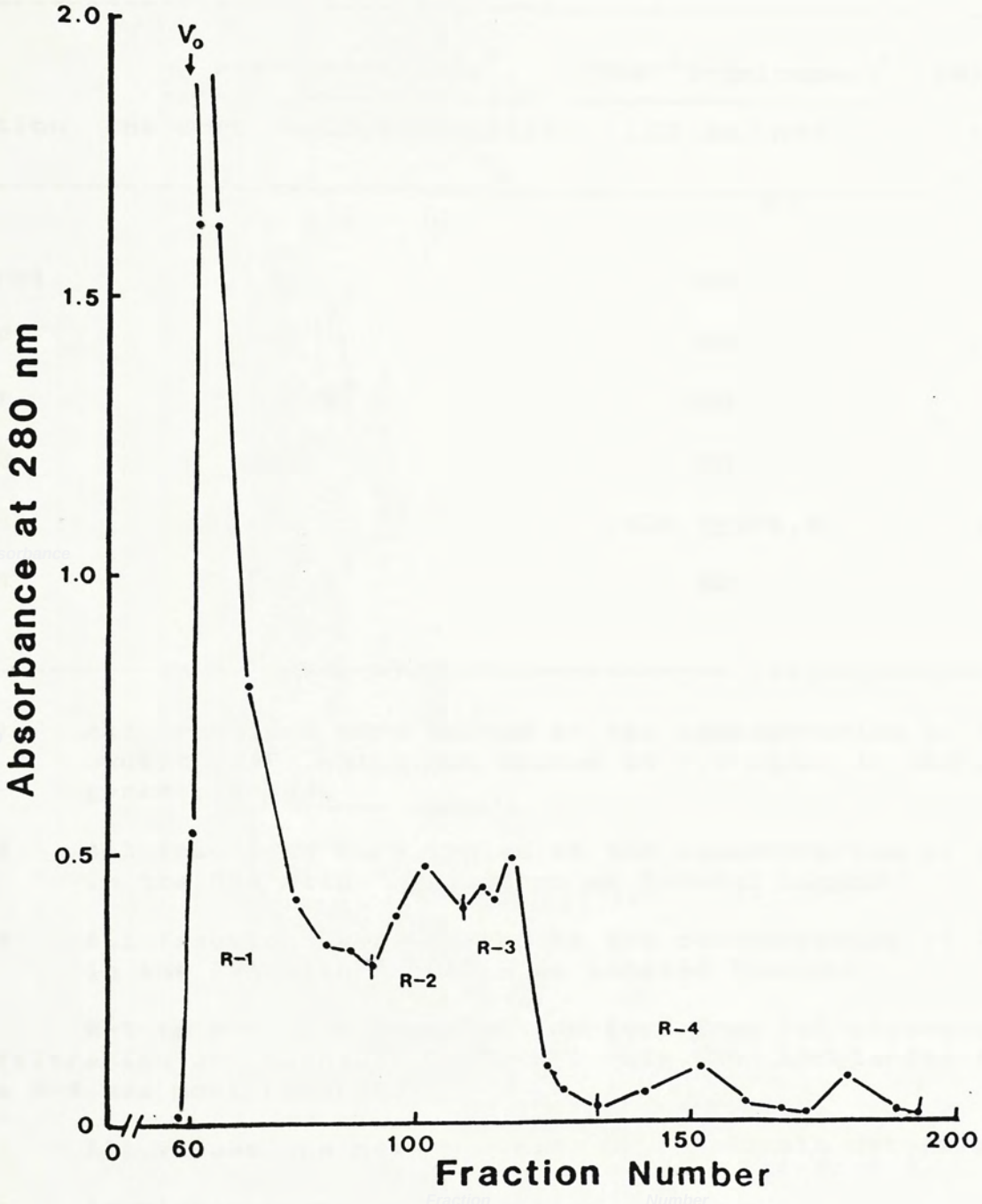

2.0
1.5
1.0
0.5
0
V_o
Absorbance at 280 nm
R-1
R-2
R-3
R-4
0
60
100
150
200
Fraction Number

Table 3-6. Steroidogenic and opiate receptor binding (RRA) activities of rat placental fractions.

Fraction	steroidogenesis[a] (ng cort./h/25,000 cells)	RRA(^{3}H-naloxone)[b] LEK eq.(nM)	RRA(^{3}H-DADLE)[c] LEK eq.(nM)
Control	ND	ND	ND
AAP	ND	ND	259.7±10.2*
R-1	5.8±0.3*	ND	ND
R-2	ND	ND	ND
R-3	UD	536.7±376.3	67.7± 6.6**
R-4	37.5±0.0*	ND	ND

a : All fractions were tested at the concentration of 160 ug/ml except AAP which was tested at 1.6 mg/ml in the steroidogenesis assay.

b : All fractions were tested at the concentration of 160 ug/ml in the RRA with ^{3}H-naloxone as labelel ligand.

c : All fractions were tested at the concentration of 0.2 ml/ml in the RRA with ^{3}H-DADLE as labeled ligand.

R-1 to R-4 are fractions derived from rat placental AAP by gel filtration on Sephadex G-25, R-1 was the unretarded fractions while R-4 was most retarded.

All values are mean ± S.E.M. of triplicate determinations.

LEK : leucine-enkephalin.

ND : undetectable.

* $p < 0.001$ and ** $p < 0.01$

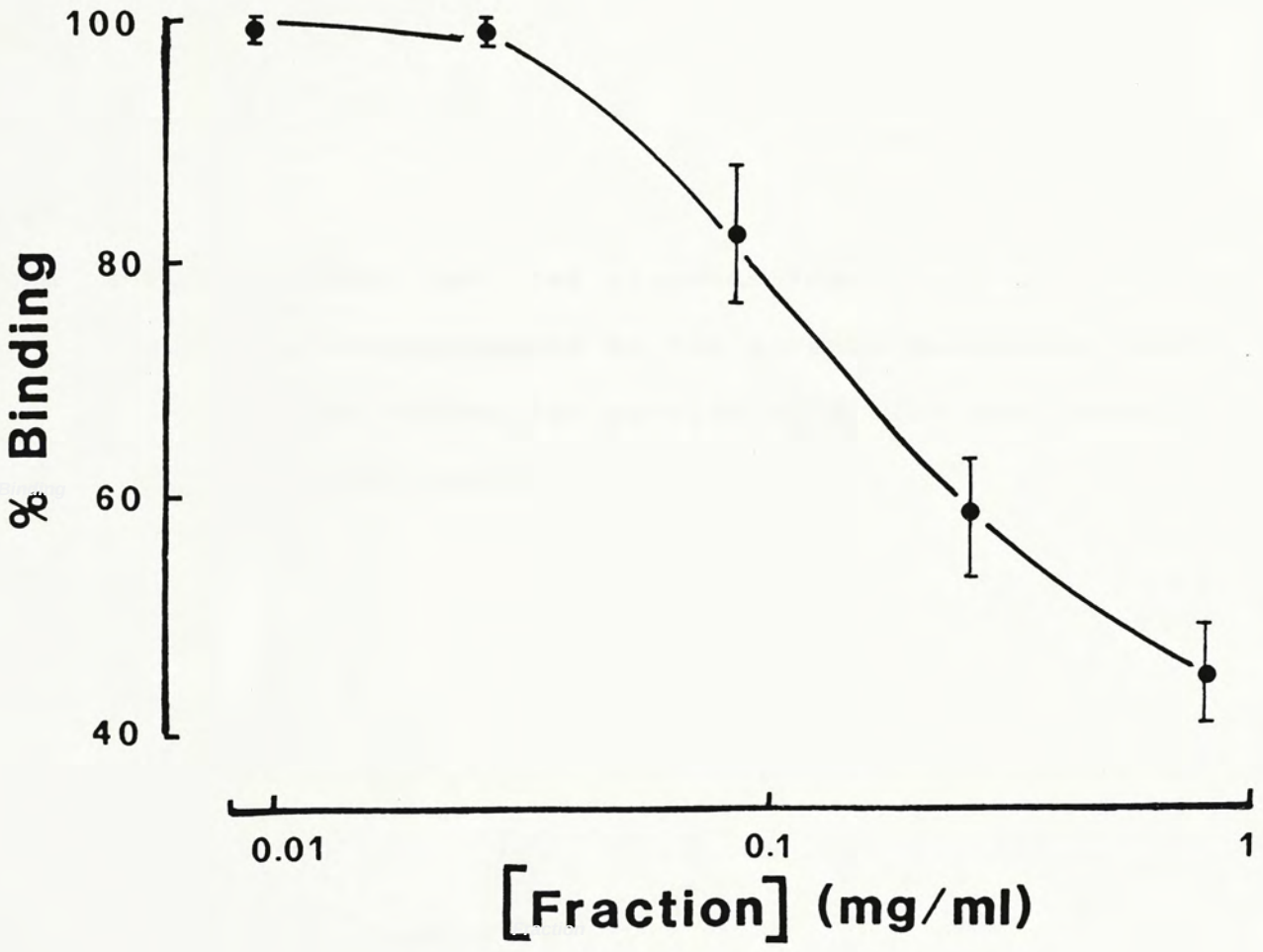
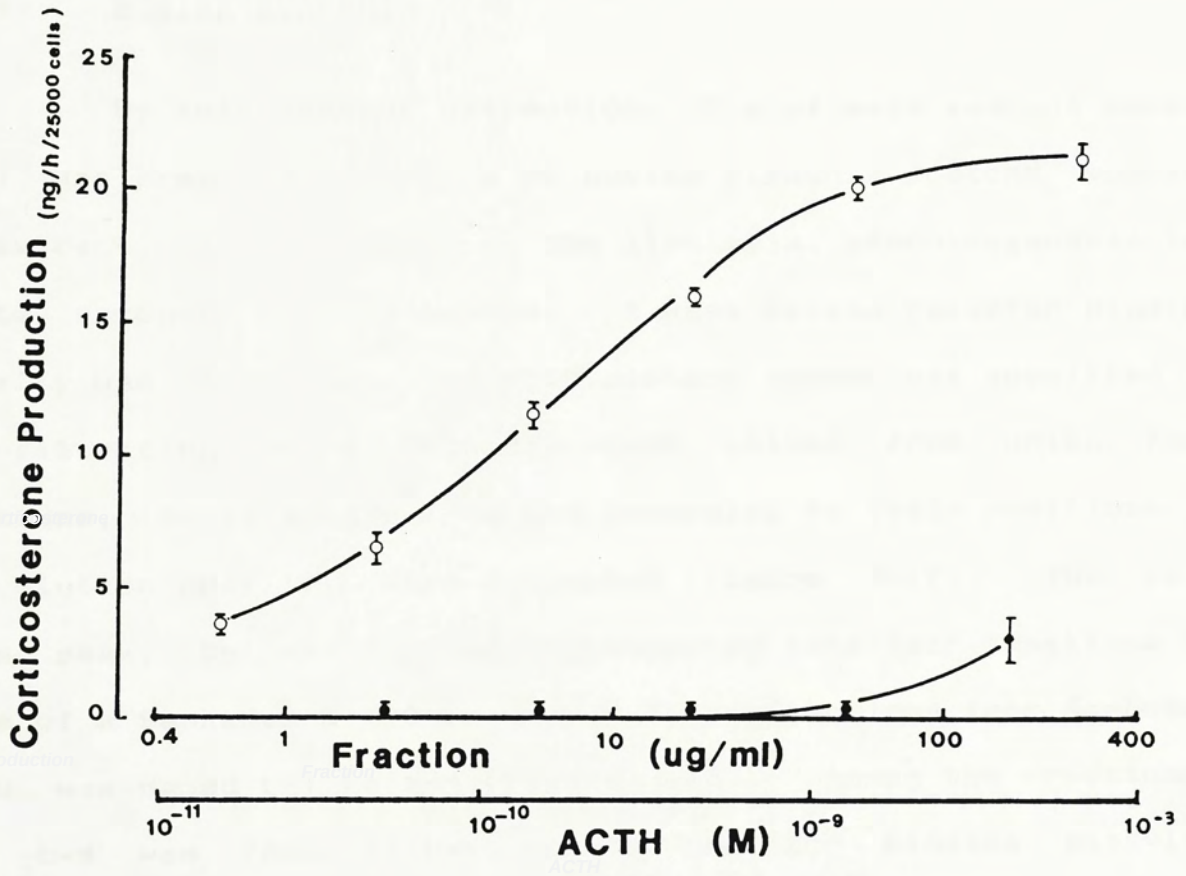


Figure 3-15 Inhibition of [^{3}H] DADLE binding to rat brain membranes by rat placenta fraction R-3.

Figure 3-16. Effect of rat placenta fraction R-4 (●) on steroidogenesis in rat adrenal decapsular cells. (The curve for porcine ACTH (○) was shown for comparison).


Corticosterone Production (ng/h/25000 cells)
25
20
15
10
5
0
0.4
1
10
100
400
Fraction (ug/ml)
10^{-11}
10^{-10}
10^{-9}
10^{-8}
ACTH (M)

less than 5,000 daltons, it was unlikely the 4,500 dalton corticotropin. We suggested that it was a shorter peptide than corticotropin (1-39) but contained the active core of corticotropin. However, because the yield of this fraction, was too small and the potency of this fraction was too low a complete dose-response curve of this fraction in rat decapsular adrenal cells could not be demonstrated (Figure 3-16).

3.2.3 BOVINE PLACENTA

By acid acetone extraction, 15 g of acid acetone powder (AAP) was prepared from 20 g of bovine placenta acetone powder. The extract was then tested in the lipolysis, steroidogenesis and opiate receptor binding assays. A weak opiate receptor binding activity was detected. The acid acetone powder was submitted to gel filtration on a Sephadex G-25 column from which four fractions, designated B-1 to B-4 according to their positions in the elution profile, were collected (Figure 3-17). The void volume peak, B-1 was further fractionated into four fractions by means of a Sephadex G-100 column. These fractions from Sephadex G-100 was named b-1 to b-4 (Figure 3-18). Among the fractions, only b-4 was found to have opiate receptor binding activity (Table 3-7). From the chromatographic behaviour of the opiate receptor binding activity on the gels it appears that the opioid substances in bovine placenta possess a molecular weight larger than 5,000 daltons, the exclusion volume of Sephadex G-25 but much less than 100,000 daltons, the exclusion volume of Sephadex G-100. This finding indicates that the endorphin-like activity

Figure 3-17. Elution profile of 300 mg bovine placenta AAP from a Sephadex G-25 column (3 x 80 cm). Eluent: 0.1 M acetic acid. Flow rate : 30 ml/h. Fraction size : 4 ml.

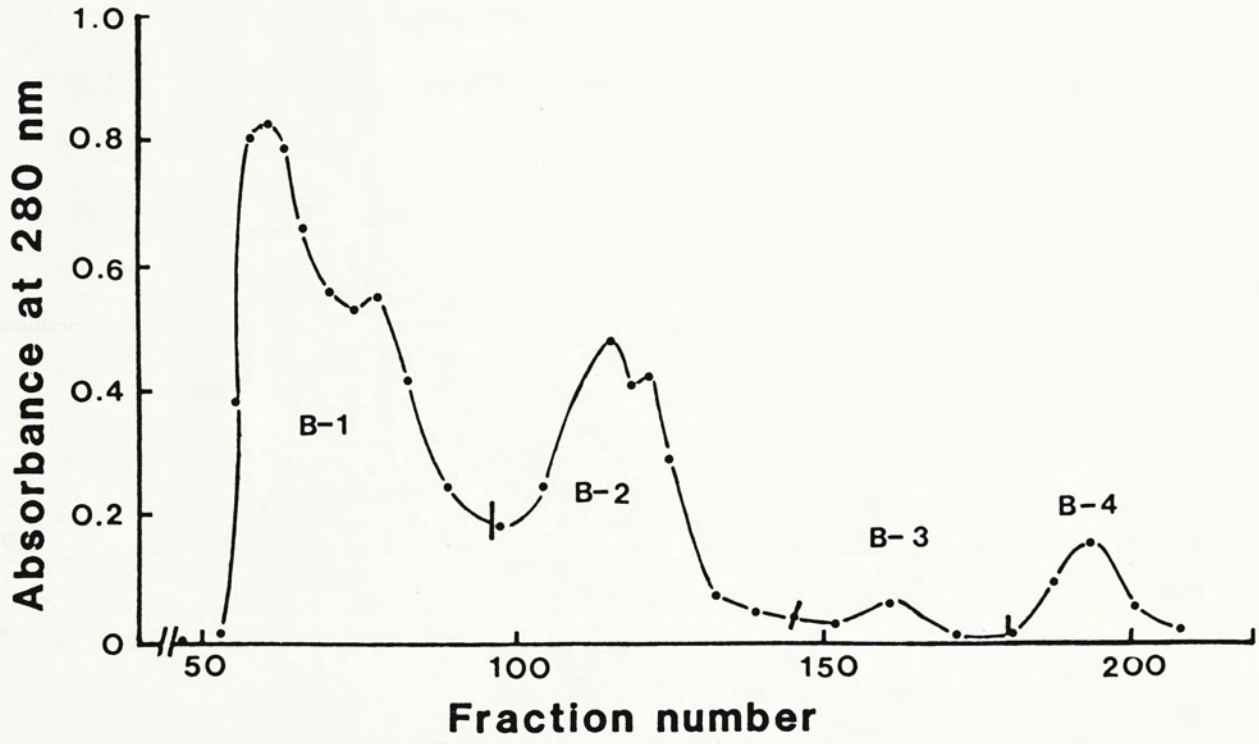

Absorbance at 280 nm
1.0
0.8
0.6
0.4
0.2
0
50
100
150
200
Fraction number
B-1
B-2
B-3
B-4

Figure 3-18. Elution profile of 49 mg bovine placenta AAP from a Sephadex G-100 column (2.5 x 77 cm). Eluent : 0.1 M acetic acid. Flow rate : 24.7 ml / hr. fraction size : 3.5 ml.

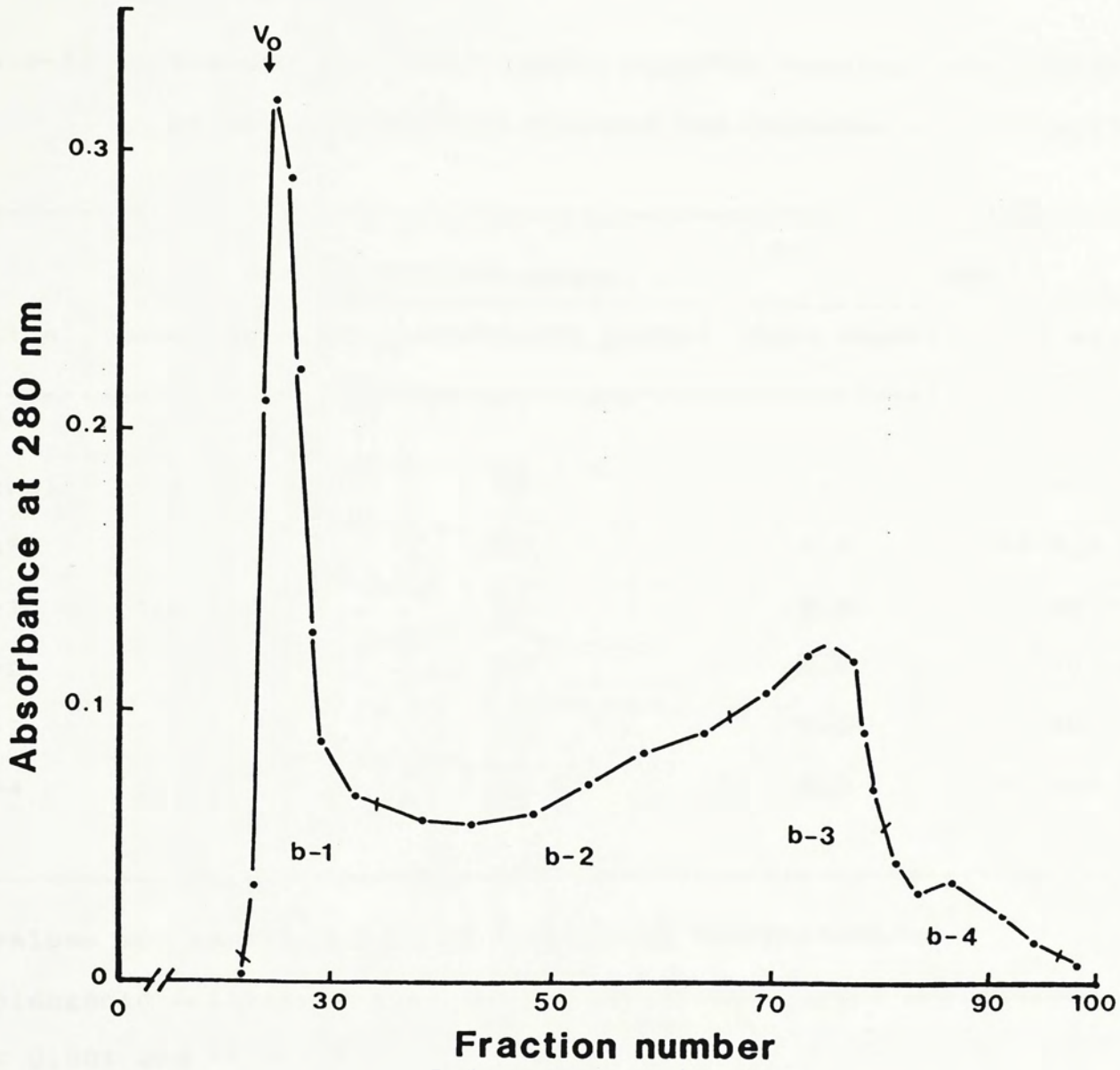

V_O
0.3
0.2
0.1
0
Absorbance at 280 nm
b-1
b-2
b-3
b-4
0
30
50
70
90
100
Fraction number

Table 3-7. Steroidogenic and opiate receptor binding (RRA) activities of bovine placental AAP and its Sephadex G-100 fractions.

	Steroidogenesis assay		RRA	
Fraction	dose (ug/ml)	(ng /h/25,000 cells)	dose (mg/ml)	LEK eq (nM)
Control	-	ND	-	ND
AAP	-	UD	4.0	33.0±3.8**
b-1	160.0	ND	0.2	ND
b-2	160.0	ND	0.2	ND
b-3	160.0	ND	0.2	ND
b-4	160.0	ND	0.2	71.0±0.0*

All values are mean ± S.E.M. of triplicate determinations.

Steroidogenic activity was expressed in corticosterone production.

* $p < 0.001$ and ** $p < 0.01$

ND : undetectable.

UD : undetermined.

Bovine placental AAP was not lipolytic even when a concentration 8.0 mg/ml was used. The sephadex G-25 fractions, B-2 to B-4, were unable to displace ^{3}H-DADLE from its binding sites when the concentration of 0.2 mg/ml was used. Fraction b-1 to b-4 were derived by gel filtration of B-1 on Sephadex G-100.

in bovine placenta might be structurally quite different from β-endorphin which has a molecular weight of only 3,500 daltons.

3.2.4 MOUSE TESTIS

Mouse testis acid acetone powder was also investigated for the presence of opioid substances and corticotropin-like substances. However, after testing with the lipolysis assay, steroidogenesis assay and radioreceptor assay for opiates, only opiate receptor binding and weak steroidogenic activities were detected. Figure 3-19 demonstrates the displacement curve of ^{3}H-DADLE by mouse testis acid acetone powder. On this graph, a dose-dependent displacement of ^{3}H-DADLE was observed within the dose range of 2 - 50 µg and the Kd was estimated to be 10 µg. From these results, the presence of opioid substances in mouse testis was suggested.

3.2.5 RAT TISSUES

The acid acetone powder of some rat tissues, including the brains, kidneys, livers and lungs were prepared with the acid acetone extraction method. The AAPs obtained were then tested in the steroidogenesis assay and radioreceptor assay for opiates. As shown in table 3-8, rat brain was the only tissue found to contain corticotropin-like and opiate receptor binding activities. The displacement curve of ^{3}H-DADLE by rat brain AAP from rat brain membranes was shown in figure 3-20. Based on the

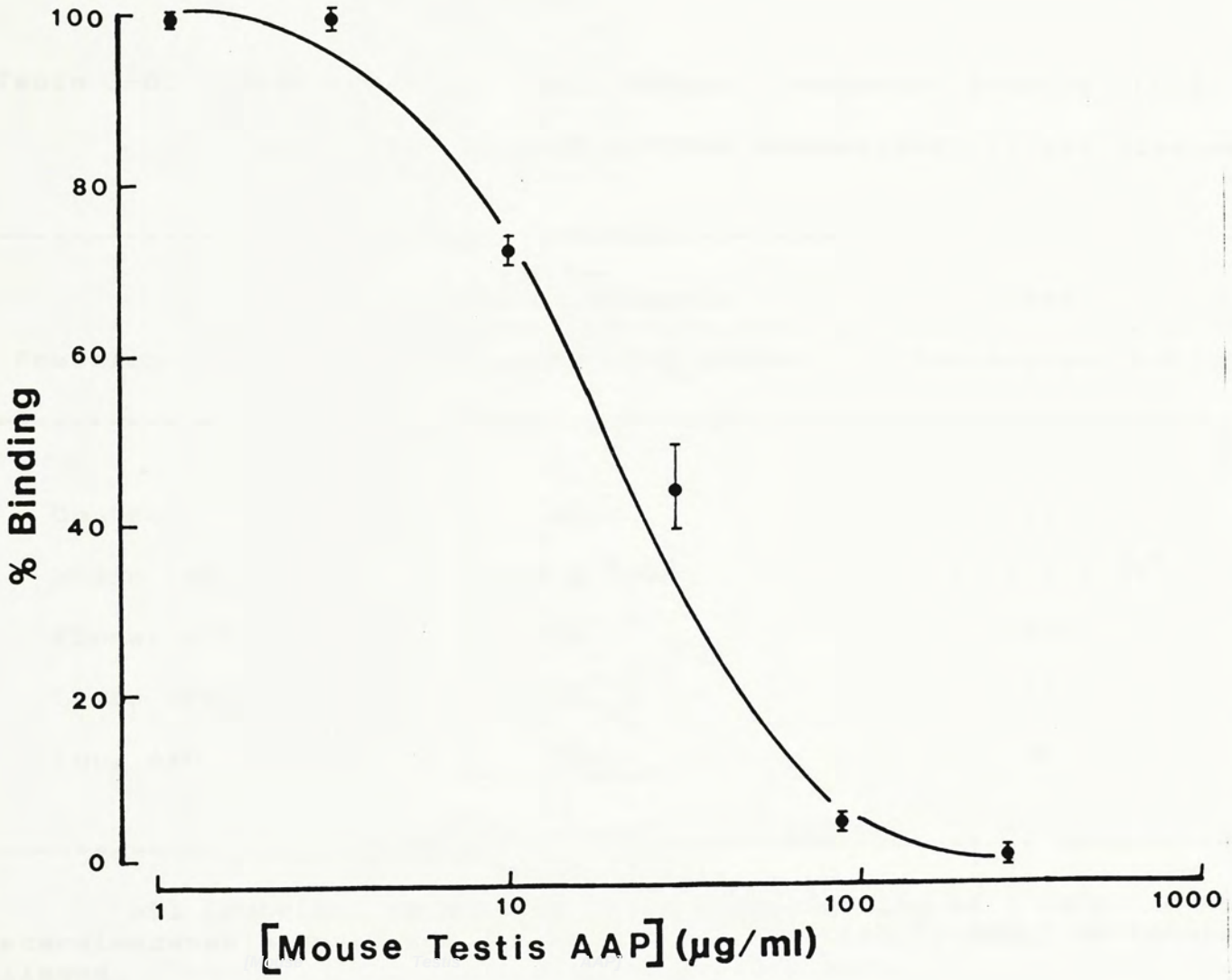


Figure 3-19. Inhibition of [^{3}H] DADLE binding to rat brain membranes by mouse testis AAP.

Table 3-8. Steroidogenic and opiate receptor binding (RRA) activities in acid acetone powder (AAP) of rat tissues.

Fraction	steroidogenesis (ng cort./h/25,000 cells)	RRA Leu-enk eq. (nM)
Control	ND	ND
Brain AAP	2.44 ± 0.80	24.1 ± 0.04*
Kidney APP	ND	ND
Liver APP	ND	ND
Lung AAP	ND	ND

All fractions were tested at a concentration of 1 mg/ml in the steroidogenesis assay and 2 mg/ml in the RRA with ^{3}H-DADLE as labeled ligand.

The values are mean ± S.E.M. of triplicate determination.

* $p < 0.001$ compared to control.

ND : undetectable

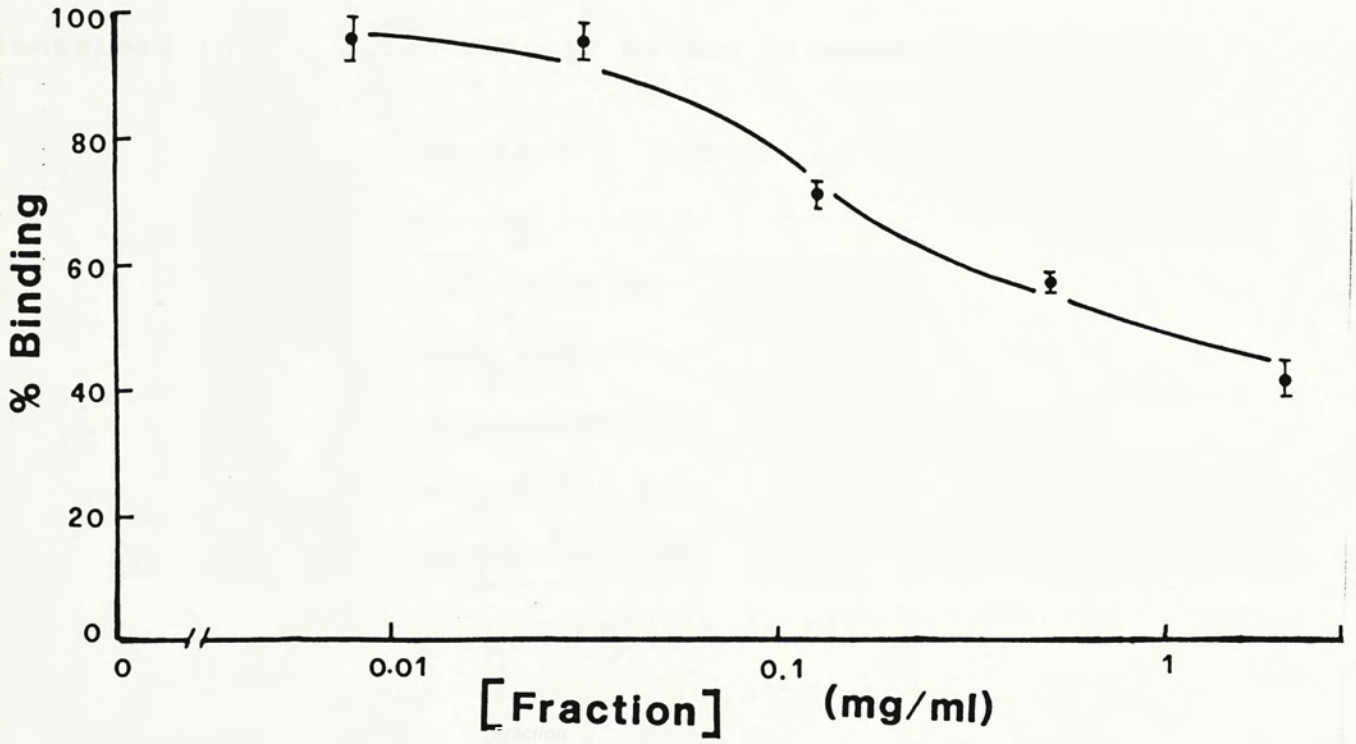


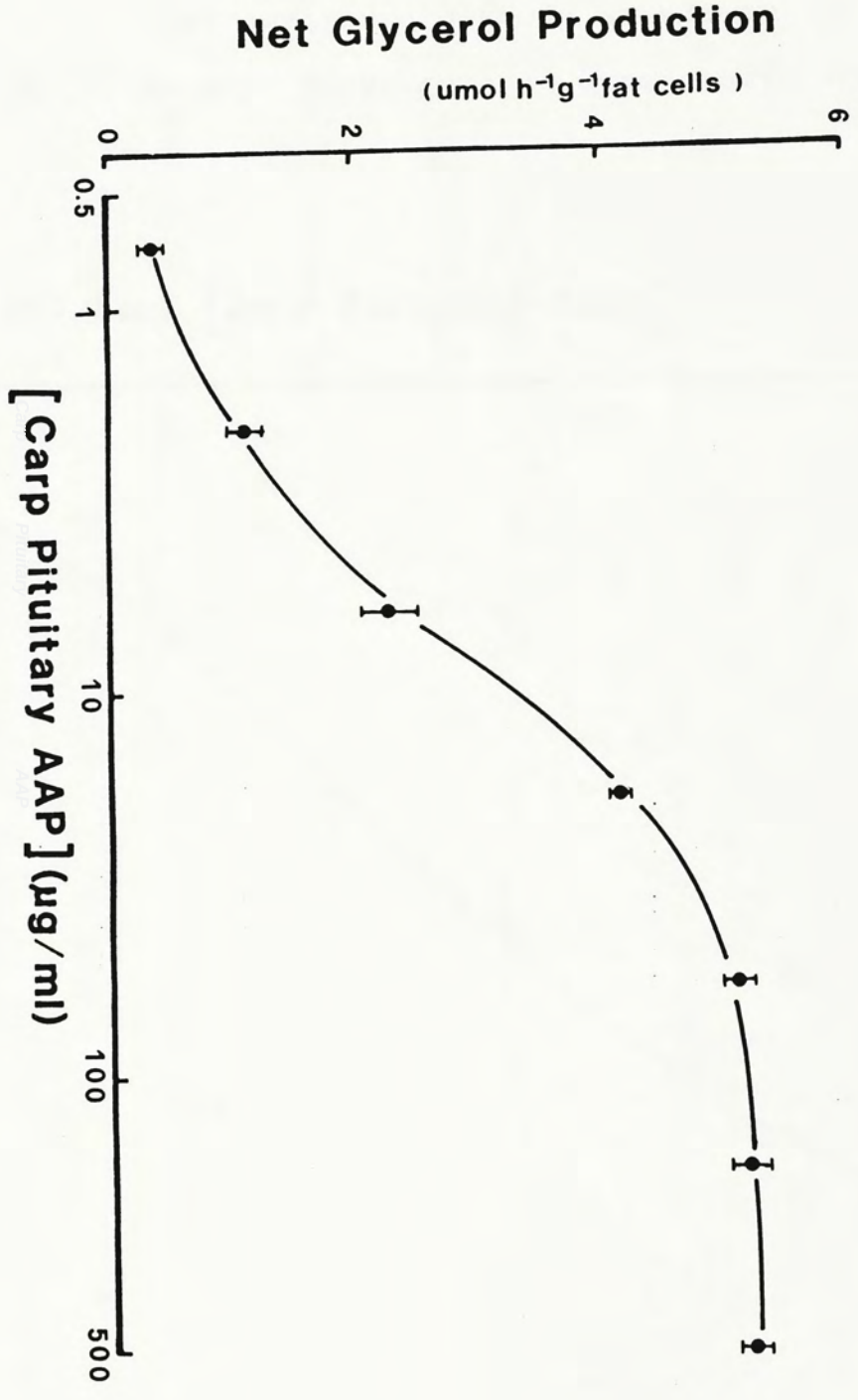
figure 3-20. Inhibition of [^{3}H] DADLE binding to rat brain membranes by rat brain AAP.

results, rat brains was found to contain corticotropin-like activity and opioid activity while the other tissues tested contained little if any of such activities. These results suggest that the corticotropin-like and opioid activities demonstrated in equine pancreas, carp and salmon pituitaries, rat and bovine placentas, snake brains and mouse testes are real, either synthesized or internalized by the tissues, and not due to artifacts caused by enzymes present in the tissues or activitiess contained in the blood trapped by the tissues.

3.2.6 CARP PITUITARY

From 7.0 g of carp (Cyprinus carpio) pituitary acetone powder, 1.5 g of acid acetone powder was obtained by acid acetone extraction. In the lipolysis, steroidogenesis and opiate receptor binding assays, this carp pituitary AAP was found to be highly active. In the lipolysis assay, carp pituitary AAP displayed a dose dependent response in the hamster adipocytes with an ED_{50} at 6.6 µg (Figure 3-21) suggesting the presence of corticotropin-like materials. Figure 3-22 was a displacement curve of ^{3}H-DADLE from rat brain membranes using carp pituitary AAP as displacer. Its high potency in this assay with an IC_{50} at 70 µg also suggested the presence of opioid materials. The dose-response curve of hamster adipocytes to the acid acetone powder of carp pituitaries was almost identical in shape to the dose-response curve of corticotropin. These results suggested that the corticotropin-like activity in carp pituitary AAP might interact with the adipocytes in a similar way to corticotropin. Hence the corticotropin-like material in this carp pituitary AAP might be structurally and functionally related to corticotropin. On CM-cellulose column, carp pituitary AAP was fractionated into 16 fractions designated C-1 to C-16 according to the u.v. absorption profile (Figure 3-23). When 200 µg of each of these fractions was incubated with hamster adipocytes, stimulation of lipolysis occured in many fractions including C-6 to C-16 (Table 3-9). The observation suggested that there were not only one lipolytic material in carp piutuitary but a large number of them.

Figure 3-21. Dose-response curve of carp pituitary AAP in Stimulating lipolysis in hamster epididymal adipocytes.


Net Glycerol Production
(umol h⁻¹g⁻¹fat cells)
0
2
4
6
0.5
1
10
100
500
[Carp Pituitary AAP] (µg/ml)

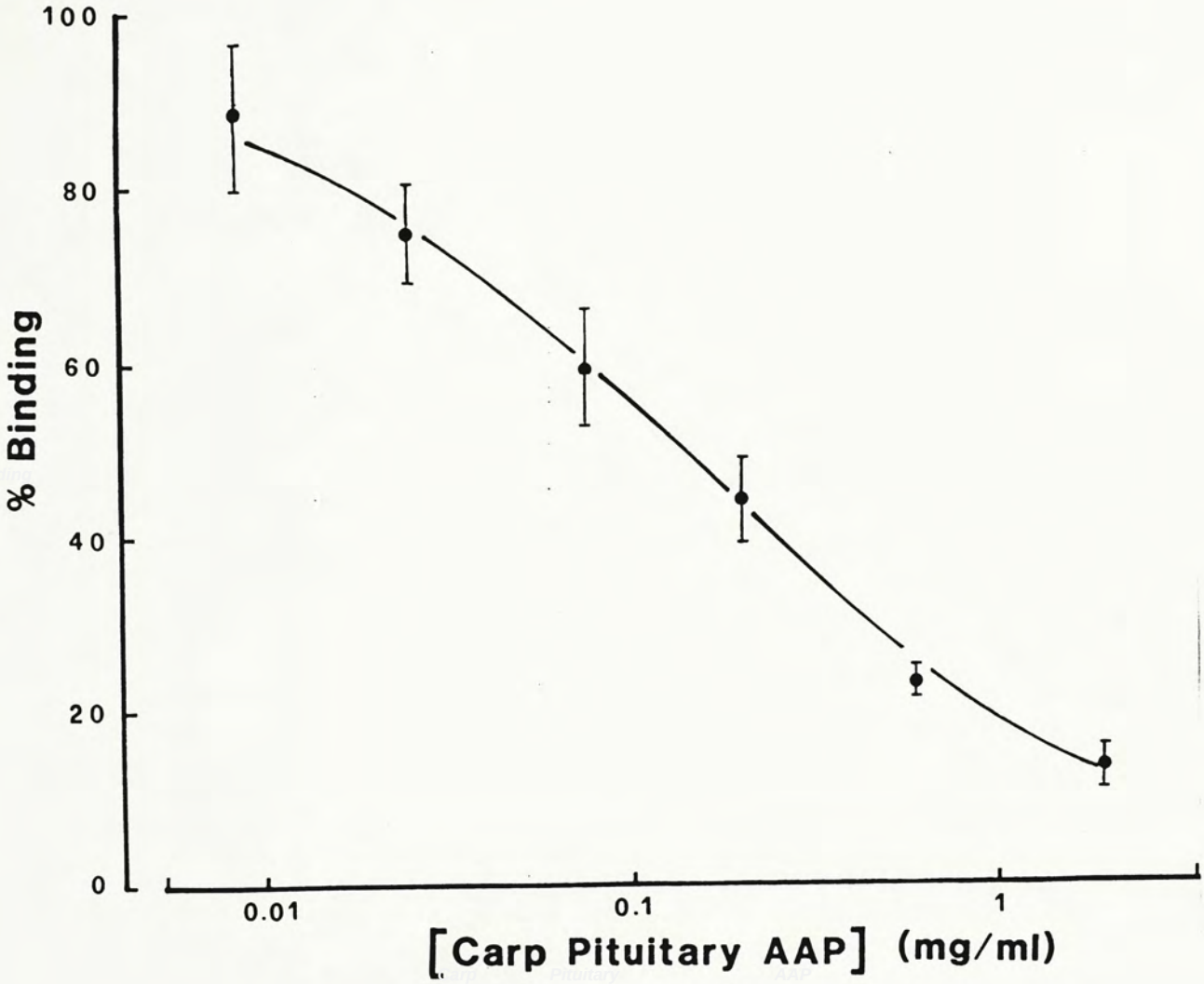


Figure 3-22. Inhibition of [^{3}H] DADLE binding to rat brain membranes by carp pituitary AAP.

Figure 3-23. Elution profile of carp pituitary AAP from a CM-cellulose column (1.3 x 42 cm). Eluent: (a) 0.01 M NH_4OAc, pH 4.6; (b) 0.01 M NH_4OAc, pH 4.6 to 0.1 M, pH 6.7; (c) 0.1 M NH_4OAc, pH 6.7 to 0.2 M pH 7.0; (d) 0.2 M NH_4OAc to 0.5 M, pH 7.0; (e) 1.0 M NH_4OAc, pH 7.0; (f) 2.0 M NH_4OAc, pH 7.0; (g) 0.1 M $(NH_4)HCO_3$, pH 10.0. Flow rate : 17.5 ml/h. Fraction size : 5 ml. Sample : 750 mg carp pituitary AAP. Yield : C-1, 172 mg; C-2, 15 mg; C-3, 5 mg; C-4, 38 mg; C-5, 21 mg; C-6, 49 mg; C-7, 47 mg; C-8, 30 mg; C-9, 11 mg; C-10, 8 mg; C-11, 18 mg; C-12, 5 mg; C-13, 9 mg; C-14, 2 mg; C-15, 21 mg; C-16, 39 mg.

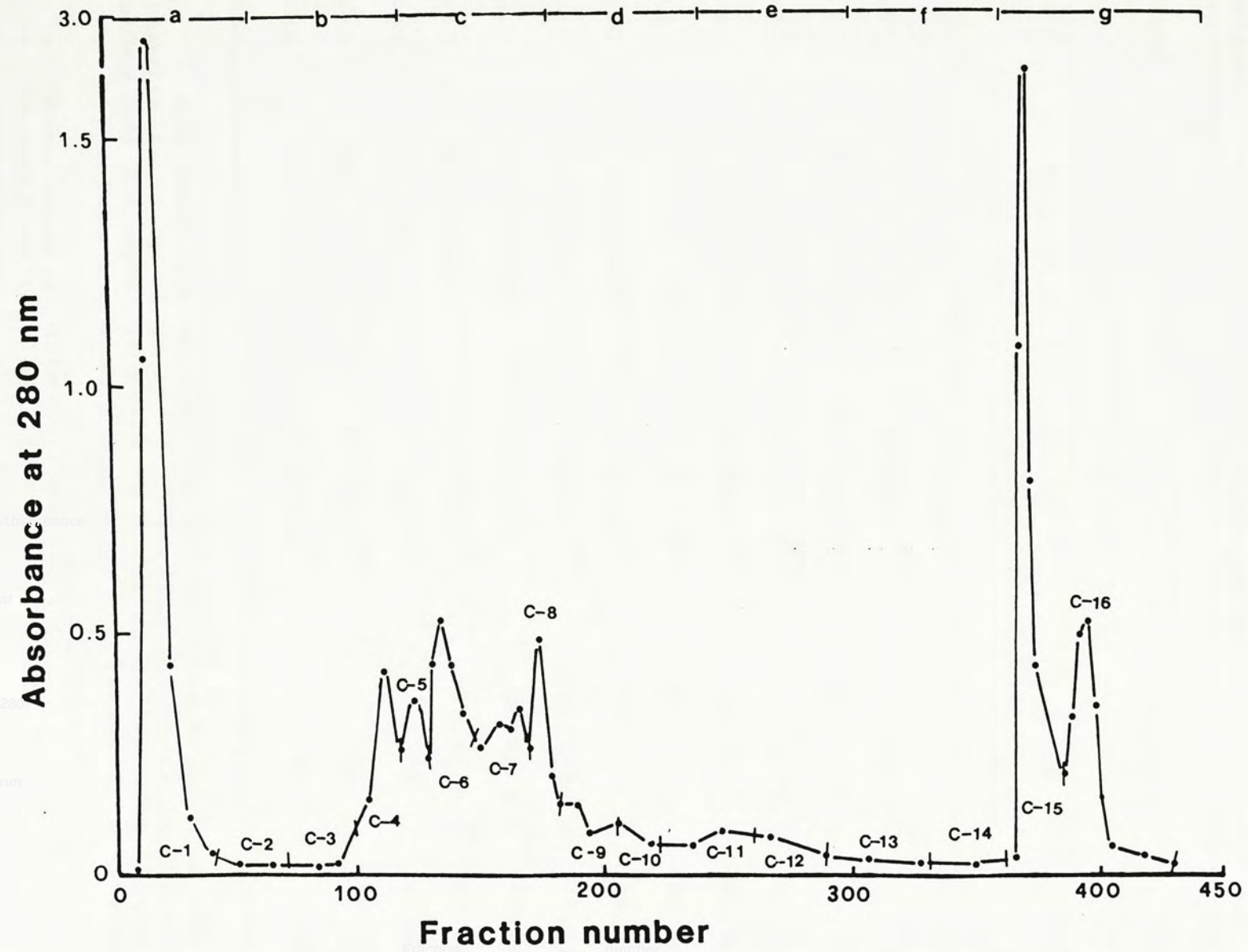

a
b
c
d
e
f
g
3.0
1.5
1.0
0.5
0
Absorbance at 280 nm
C-1
C-2
C-3
C-4
C-5
C-6
C-7
C-8
C-9
C-10
C-11
C-12
C-13
C-14
C-15
C-16
0
100
200
300
400
450
Fraction number

Table 3-9. Steroidogenic, lipolytic and opiate receptor binding (RRA) activities of CM-cellulose fractions of carp pituitary

Fraction	Lipolysis	steroidogenesis	RRA[a]	RRA[b]
	% control	(ng /h/25,000 cells)	LEK eq.(nM)	LEK eq.(nM)
Control	100	ND	ND	ND
C-1	150.2±10.8	17.9±4.4	ND	ND
C-2	99.1± 4.3	3.5±0.4	ND	ND
C-3	106.6± 3.0	6.6±0.2	ND	ND
C-4	101.7± 0.0	2.3±0.1	ND	ND
C-5	147.4± 1.3	13.7±0.9*	ND	ND
C-6	542.9±22.8**	23.1±0.6*	31.0±17.0	40.3± 6.8
C-7	772.7± 7.0*	18.8±1.3*	430.0±70.7	83.3± 9.0
C-8	741.1±17.2*	15.3±0.4*	28.0± 7.1	63.3± 7.2
C-9	815.8± 4.9*	16.3±0.0*	>1000	185.0±20.0
C-10	805.3±39.8*	16.9±0.6*	>1000	79.3± 3.5*
C-11	939.4±83.1**	11.0±0.1*	106.0±31.6	77.5± 9.7**
C-12	854.8±54.3	17.3±4.4	36.5± 3.5**	48.3± 5.7**
C-13	830.2±131.8	16.7±1.4*	102.5± 3.5*	67.0± 5.2**
C-14	UD	UD	UD	41.0± 1.4*
C-15	353.3± 0.0*	18.3±2.9**	ND	ND
C-16	792.8±12.2*	20.8±2.0**	ND	ND

All fractions were tested at the concentration of 200 ug/ml in lipolysis, 40 ug/ml in steroidogenesis, 160 ug/ml in RRA with ^{3}H-naloxone and 200 ug/ml in RRA with ^{3}H-DADLE as labelled ligand.
All values are mean ± S.E.M. of triplicate determination.

a : ^{3}H-naloxone as labeled ligand.
b : ^{3}H-DADLE as labeled ligand.
ND : undetectable.
UD : undetermined due to insufficient materials.
* $p < 0.001$ and ** $p < 0.01$

They were different in ionic properties and they were eluted in different positions of the elution profile. It was pointed out that the lipolysis assay utilizing hamster adipocytes was only fairly specific for corticotropin, and so we could not conclude that the lipolytic activity in these fractions were all corticotropin-like polypeptides before their structures were further studied. Since steroidogenesis assay was a more specific test for corticotropin, the fractions were tested in this assay. When 10 µg of these fractions was used, two fractions, C-6 and C-16 , were found to be active in stimulating corticosterone production in rat adrenal decapsular cells while the other fractions were less active (Table 3-9). This finding supported the suggestion of more than one corticotropin-like material in the carp pituitary. Figure 3-24 shows the response curve of rat adrenal decapsular cells to C-6 and C-16. The 2 fractions were found to be approximately equipotent in stimulating adrenal steroidogenesis, and their dose-response curves were quite parallel to that of porcine corticotropin. Furthermore, when submaximal doses of these steroidogenic fractions were incubated with adrenal cells in the presence of corticotropin inhibiting peptide (CIP), inhibitions of 90% and 75% of the activity of C-6 and C-16 respectively was demonstrated (Table 3-10). These results confirmed the suggestion that these fractions acted on the corticotropin receptors on the adrenal decapsular cells.

On the other hand, opioid peptides, probably including endorphin which is produced from the same precursor as corticotropin in the mammalian system, were also detected in the

Figure 3-24. Effects of carp pituitary fraction C-6 (O) and C-16 (●) on steroidogenesis in rat adrenal decapsular cells.

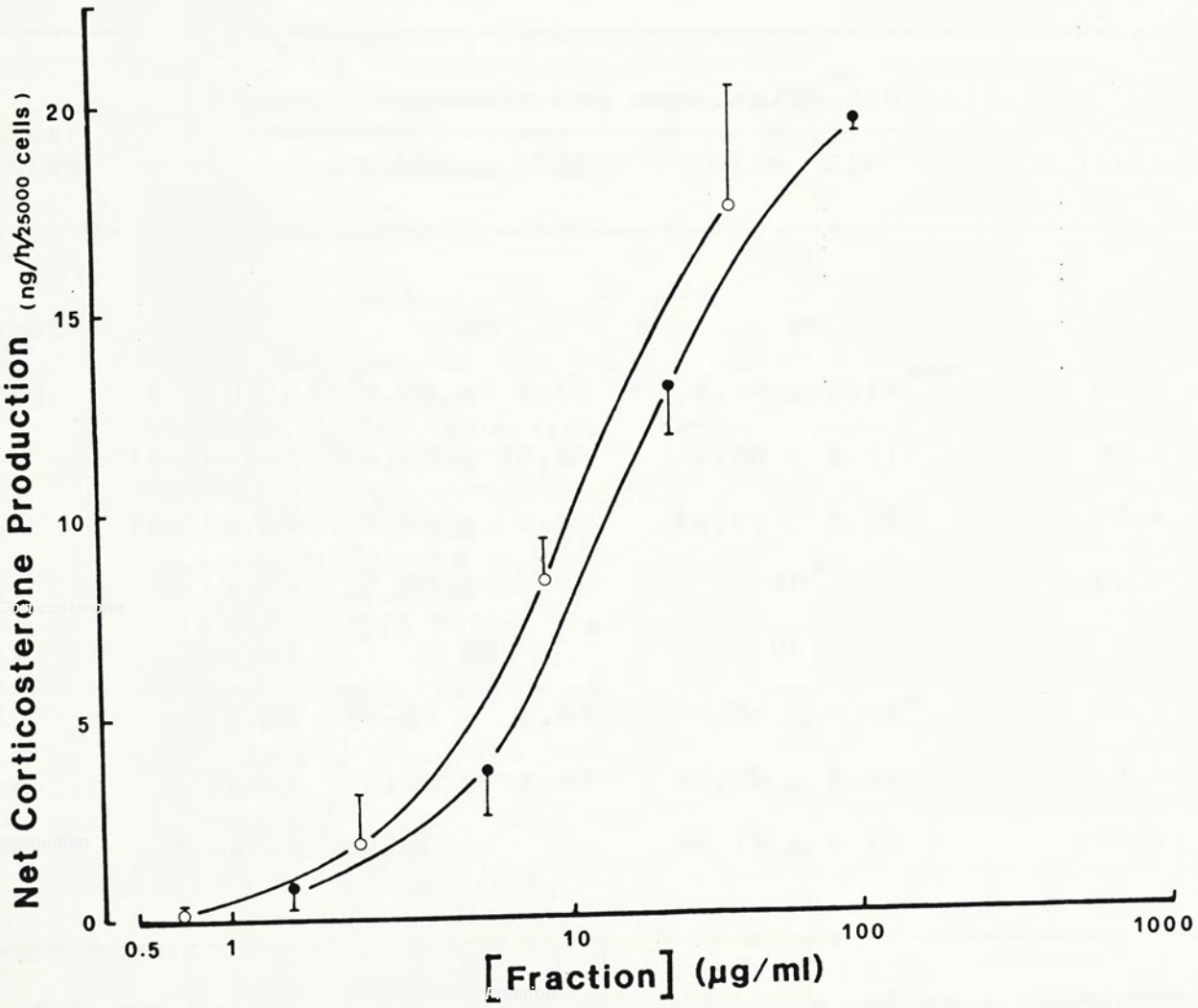

Net Corticosterone Production (ng/h/25000 cells)
20
15
10
5
0
0.5
1
10
100
1000
[Fraction] (µg/ml)

Table 3-10. Effects of corticotropin-inhibiting peptide (CIP) on steroidogenic activities of carp and salmon pituitary chromatographic fractions.

Hormone/ fraction	dose	Steroidogenesis (ng cort./h/25,000 cells) without CIP	with CIP	% inhibition
Control	-	ND	ND	-
ACTH	10 M	9.75 ± 3.18	1.44 ± 0.19***	85.3
C-6	100 ug/ml	16.88 ± 17.68	1.08 ± 0.41**	93.6
C-16	100 ug/ml	39.38 ± 0.63	10.00 ± 0.35*	74.6
S-1	20 ug/ml	7.25 ± 1.06	ND**	100.0
S-2	2 ug/ml	ND	UD	-
S-3	2 ug/ml	19.69 ± 0.94	2.94 ± 0.62*	83.1
S-4	2 ug/ml	24.58 ± 1.44	23.75 ± 5.30	3.4
S-5	2 ug/ml	>37.50	28.13 ± 4.42	>25.0

C-6 and C-16 are CM-cellulose fractions of carp pituitary AAP and S-1 to S-5 are CM-cellulose fractions of sockeye salmon pituitary fraction D.

* $P < 0.001$, ** $P < 0.01$ and *** $P < 0.1$ compared to steroidogenesis in the absence of CIP by Students' t-test.

carp pituitary AAP. Since it has been reported that endorphins were present in pituitaries of the chum salmon, Oncorhynchus keta (Kawauchi et al, 1980; Rodrigues et al, 1982), the acid acetone powder of carp pituitaries was tested in the opiate radioreceptor assay and was found to be active. To preliminarily estimate the binding properties of this opioid substance in carp pituitaries, different doses of the carp pituitary AAP were tested in the opiate receptor binding assay and the results were summarized in figure 3-25. The displacement curve obtained was quite parallel to that of leucine-enkephalin showing that the binding property of the opioid materials was quite similar to that of leucine-enkephalin. After the carp pituitary AAP was fractionated on the CM-cellulose column, the fractions were scanned for opiate receptor binding activity. The opiate receptor binding activity was found to be distributed among a large number of fractions from C-6 to C-14 with C-9 being the most potent one (Table 3-9). A displacement curve of this fraction fraction was demonstrated in figure 3-25 showing that the Kd of this fraction was 3.5 mg which is 20 times more potent than AAP. C-9 was adsorbed on CM-cellulose and eluted at a position in the elution profile similar to that of salmon endorphin I reported by Kawauchi et al (1980), showing that the ionic property of this carp pituitary opioid material might be similar to chum salmon endorphin I. In the brain radioreceptor assay, a dose-dependent displacement was elicited by this fraction showing that this opioid activity in carp pituitary had a high affinity to the opiate receptors in rat brain. However, its function and structure remain to be determined.

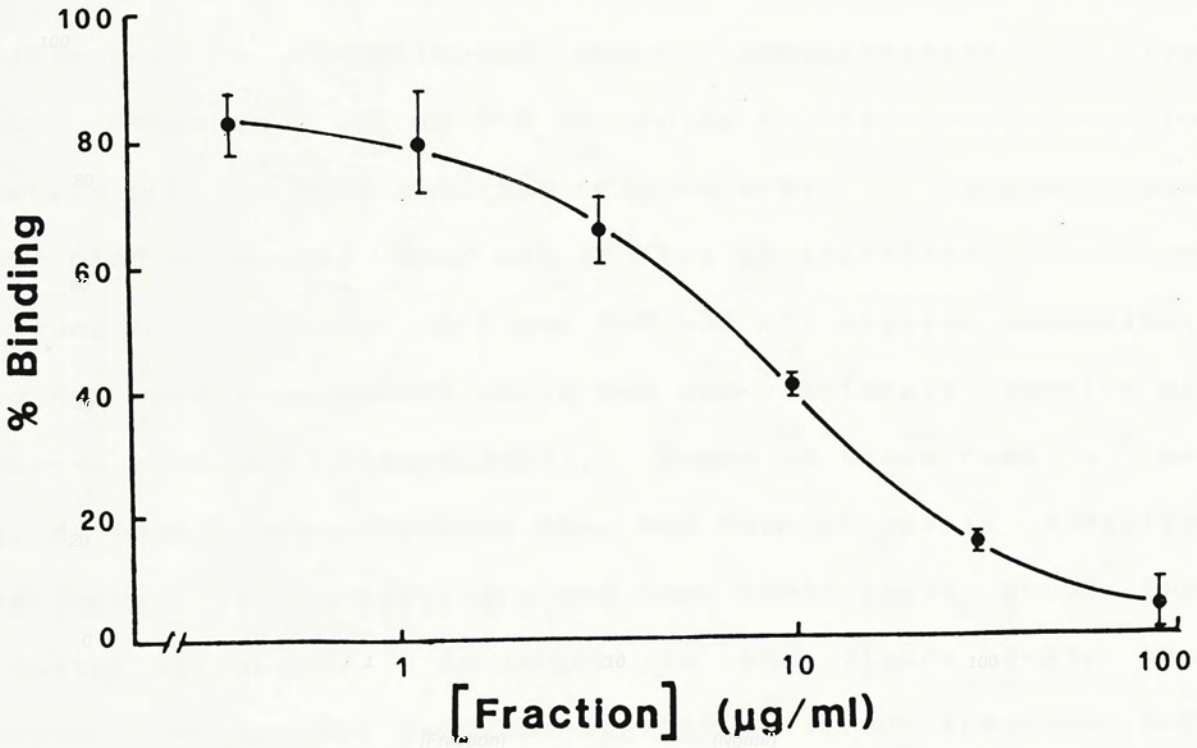


Figure 3-25. Inhibition of [^{3}H] DADLE binding to rat brain membranes by carp pituitary fraction C-9.

3.2.7 SALMON PITUITARY

Only a small amount (30 mg) of Fraction D of the sockeye salmon *Oncorhynchus nerka* pituitary was available for investigation on opioid and corticotropin-like materials. This Fr. D was found to be lipolytic (411.1% ± 11.7% of control at the concentration of 0.5 mg/ml used) in hamster adipocytes and so was subjected to CM-cellulose column chromatography. Five fractions designated S-1 to S-5 according to their positions in the elution profile were obtained (Figure 3-26). In the opiate receptor binding assay, four out of five CM-cellulose fractions were found to be active: S-1 and S-2 had the highest potencies, S-4 and S-5 were less potent while S-3 was completely inactive at the dosages employed (figure 3-27). Based on these results, we suggested that there were more than one form of opioid activity in the sockeye salmon pituitary and that their ionic properties were quite different. As shown in the figure 3-27, the unadsorbed fraction S-1 and the slightly adsorbed fraction S-2 were the fractions with the highest potencies in the opiate receptor binding assay indicating that most of the opioid materials in the chinook salmon pituitary were neutral or acidic peptides. However, there were still some basic opioid activities as demonstrated in the fractions S-4 and S-5. When ^{3}H-naloxone was used as ligand, the opioid activities of the fractions were presented in Table 3-11.

In the search for corticotropin-like activity in the CM-cellulose fractions of salmon pituitary Fr. D by using a

Figure 3-26. Elution profile of 24 mg salmon pituitary fraction D from a CM-cellulose column (0.7 x 16 cm). Eluent : (a) 0.01 M NH_4OAc, pH 4.6; (b) 0.1 M NH_4OAc, pH 6.7; (c) 0.2 M NH_4OAc, pH 7.0 (d) 0.5 M NH_4OAc, pH 7.0. Flow rate : 6 ml/h. Fraction size : 1 ml. Yield : S-1, 5 mg; S-2, 8 mg; S-3, 1 mg; S-4, 1 mg and S-5, 0.5 mg.

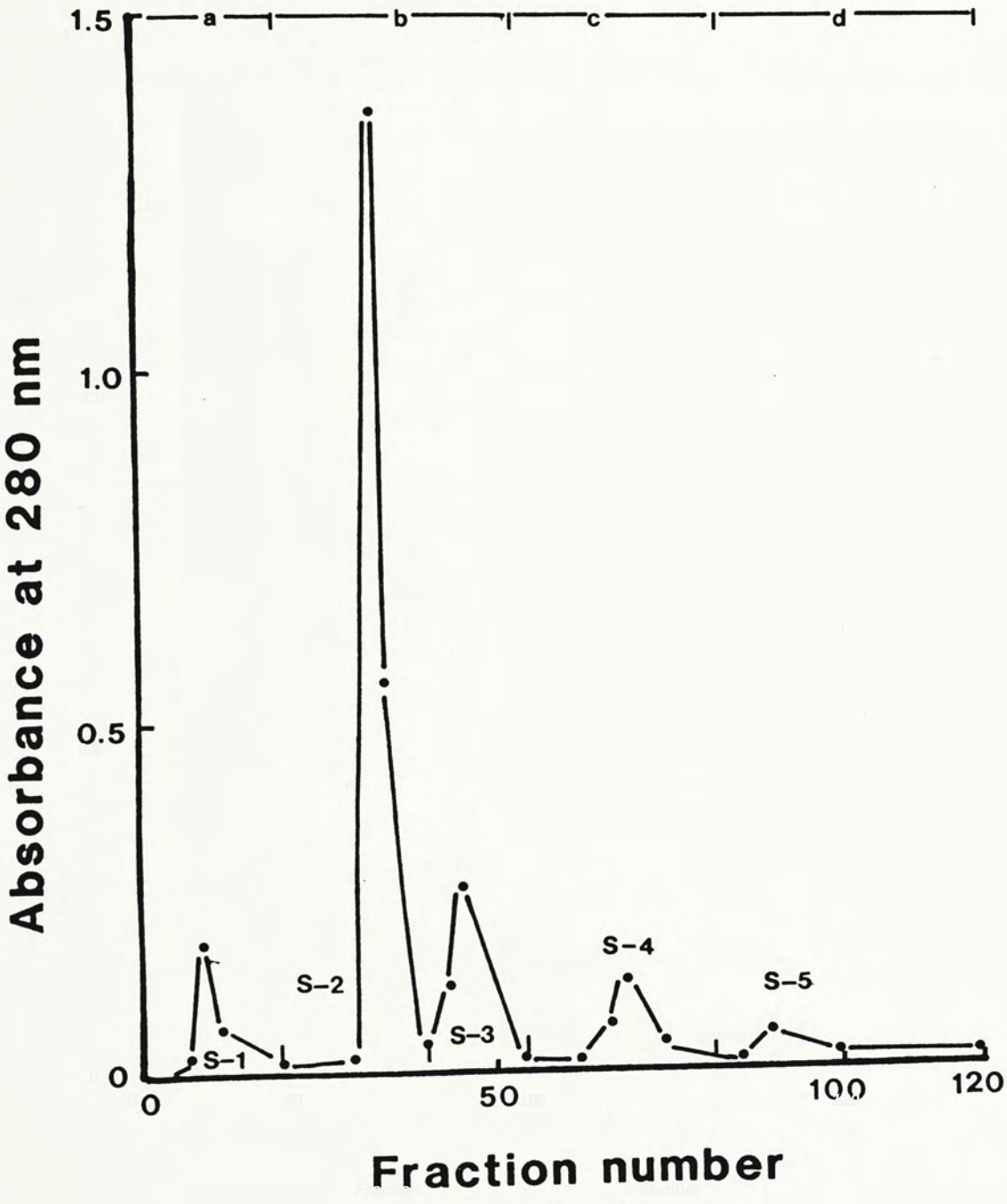

a
b
c
d
1.5
1.0
0.5
0
0
50
100
120
S-1
S-2
S-3
S-4
S-5
Absorbance at 280 nm
Fraction number

Figure 3-27. Inhibition of [^{3}H] DADLE binding to rat brain membranes by salmon pituitary fractions : S-1 (●), S-2 (○), S-3 (▲), S-4 (△) and S-5 (■).

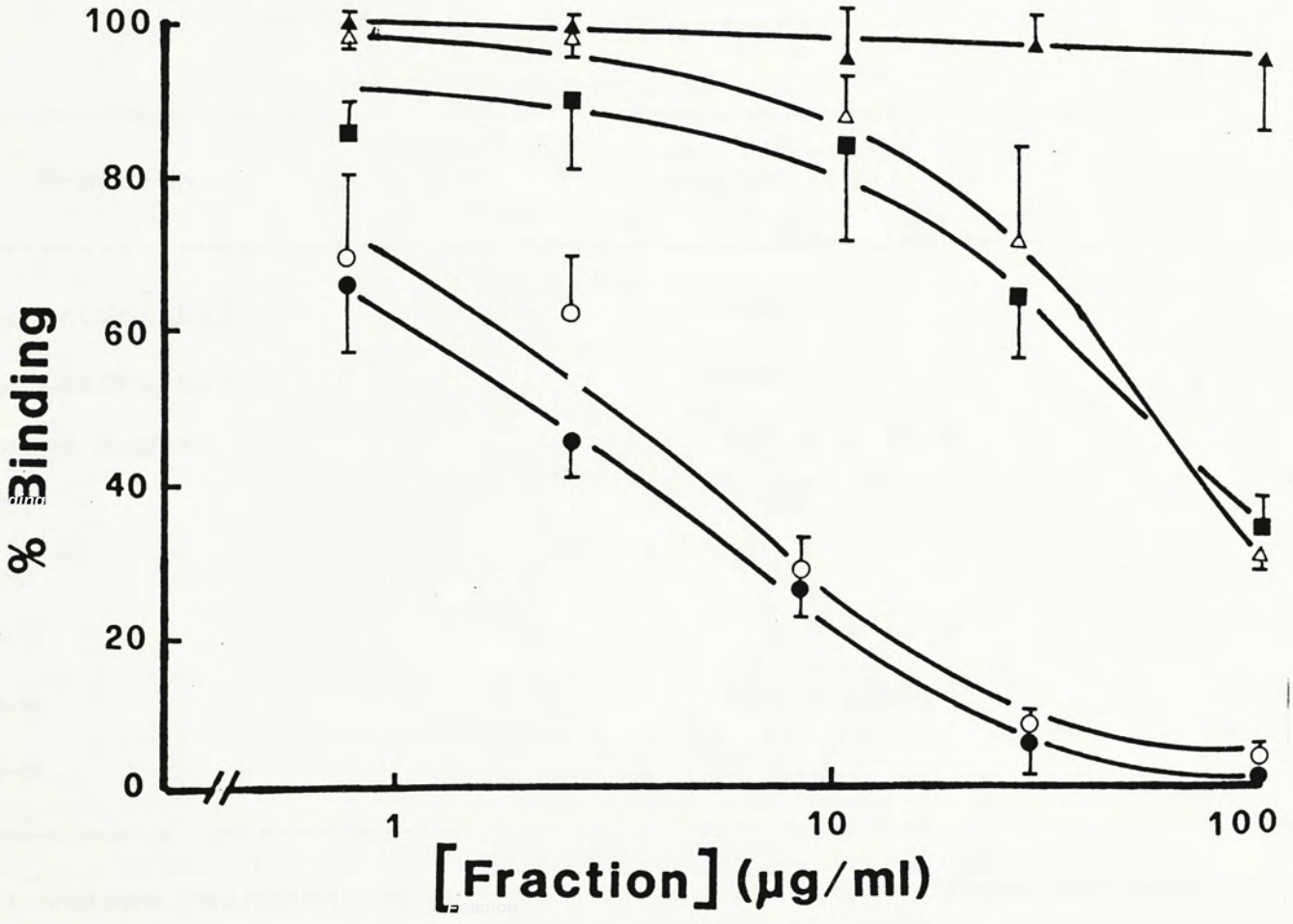

100
80
60
40
20
0
% Binding
1
10
100
[Fraction] (µg/ml)

Table 3-11. Activities of various tissue fractions in displacing ^{3}H-naloxone from binding to rat brain membrane.

Fraction	LEK eq. (nM)
L. hardwickii AAP	>1000
H. cyanocinctus AAP	>1000
Mouse testes AAP	200.0 ± 35.5
S-1	ND
S-2	ND
S-3	9.9 ± 10.5
S-4	473.5 ±150.0
S-5	>1000

All values represent mean ± S.E.M. of triplicate determinations.

All fractions were tested at 160 ug/ml.

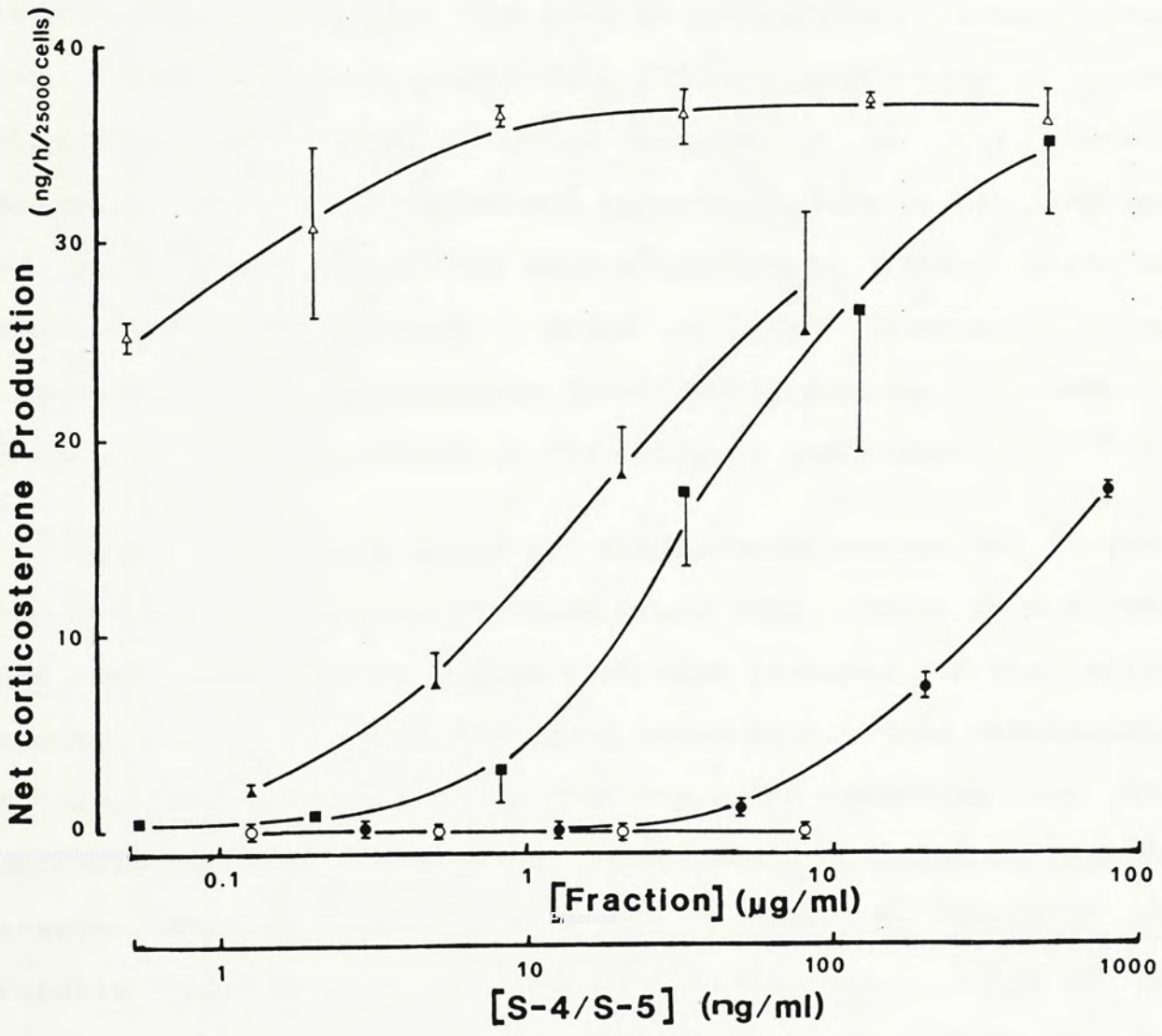
LEK : leucine enkephalin

ND : undetectable.

S-1 to S-5 denoted the CM-cellulose fractions of sockeye salmon fraction D.

steroidogenesis assay, again not one but four out of the five fractions were found to be active (Figure 3-28). Only S-2 was inactive in stimulating rat adrenal decapsular cells. The active fractions can be divided into two groups: a highly active group and a group of lower potency. For the less potent group including S-1 and S-3, the ED_{50}s were estimated to be 22.5 ug and 510 ng respectively from the curves shown on figure 3-28. When these fractions were incubated with rat adrenal decapsular cells in the presence of corticotropin inhibiting peptide (CIP), the steroidogenic activities of these fractions were strongly inhibited (Table 3-10). The inhibition of the steroidogenic activities of of these fractions by CIP, a competitive inhibitor of corticotropin, suggested that they might act through a similar mechanism as corticotropin. S-4 and S-5, the two strongly absorbed fractions from the CM-cellulose column, were found to be extraordinarily active in the stimulation of corticosterone production in rat adrenal cells and their ED_5s in the steroidogenesis assay were estimated to be about 500 pg for S-4 and 10 ng for S-5 from figure 3-28 by extrapolation. When compared on the weight basis, S-4 was found to be even more potent than pure porcine corticotropin in this steroido genesis assay. This extraordinary high activity might indicate the presence of a highly potent steroidogenic material in salmon pituitaries. However only about 20% of the steroidogenic activities of fractions S-4 and S-5 was inhibited by CIP (Table 3-10) suggesting that the action of these fractions might be different from that of mammalian corticotropin. Further

Figure 3-28. Effects of salmon pituitary fractions S-1 (●), S-2 (○), S-3 (▲), S-4 (△) and S-5 (■) on steroidogenesis in rat adrenal decapsular cells.


Net corticosterone Production (ng/h/25000 cells)
40
30
20
10
0
0.1
1
10
100
[Fraction] (µg/ml)
1
10
100
1000
[S-4/S-5] (ng/ml)

investigation will be necessary to clarify the issue.

3.2.8 SNAKE BRAIN

Brains of two species of sea snakes, Hydrophis cyanocinctus and Lapemis hardwickii were obtained and extracted to yield acid acetone powders. In the lipolysis and steroidogenesis assays, the AAPs of both kinds of snake brains were highly potent in stimulating glycerol production in hamster adipocytes and corticosterone production in rat adrenal decapsular cells. The response curves obtained in the lipolysis and steroidogenesis assays were presented in figure 3-29 and figure 3-30 respectively. Based on these findings, it was suggested that some materials functionally related to mammalian corticotropin were present in the brain of the snakes.

Figure 3-31 shows the displacement curves of ^{3}H-DADLE from rat brain membranes by snake brain AAPs. These curves shows that there were some materials with high affinity for the opiate receptor binding site in the snake brain AAPs. This displacement of labelled opiate ligands from rat brain membranes was also confirmed by using ^{3}H-naloxone as the labelled ligand in another receptor binding assay (Table 3-11) so as to eliminate the probable interference from enzymatic activities. Based on the above data, opioid substances were shown to be present in the brains of the snakes.

Figure 3-29. Stimulation of lipolysis in hamster adipocytes by brain AAP of Hydrophis cyanocintus (○) and Laticauda hardwickii (●).

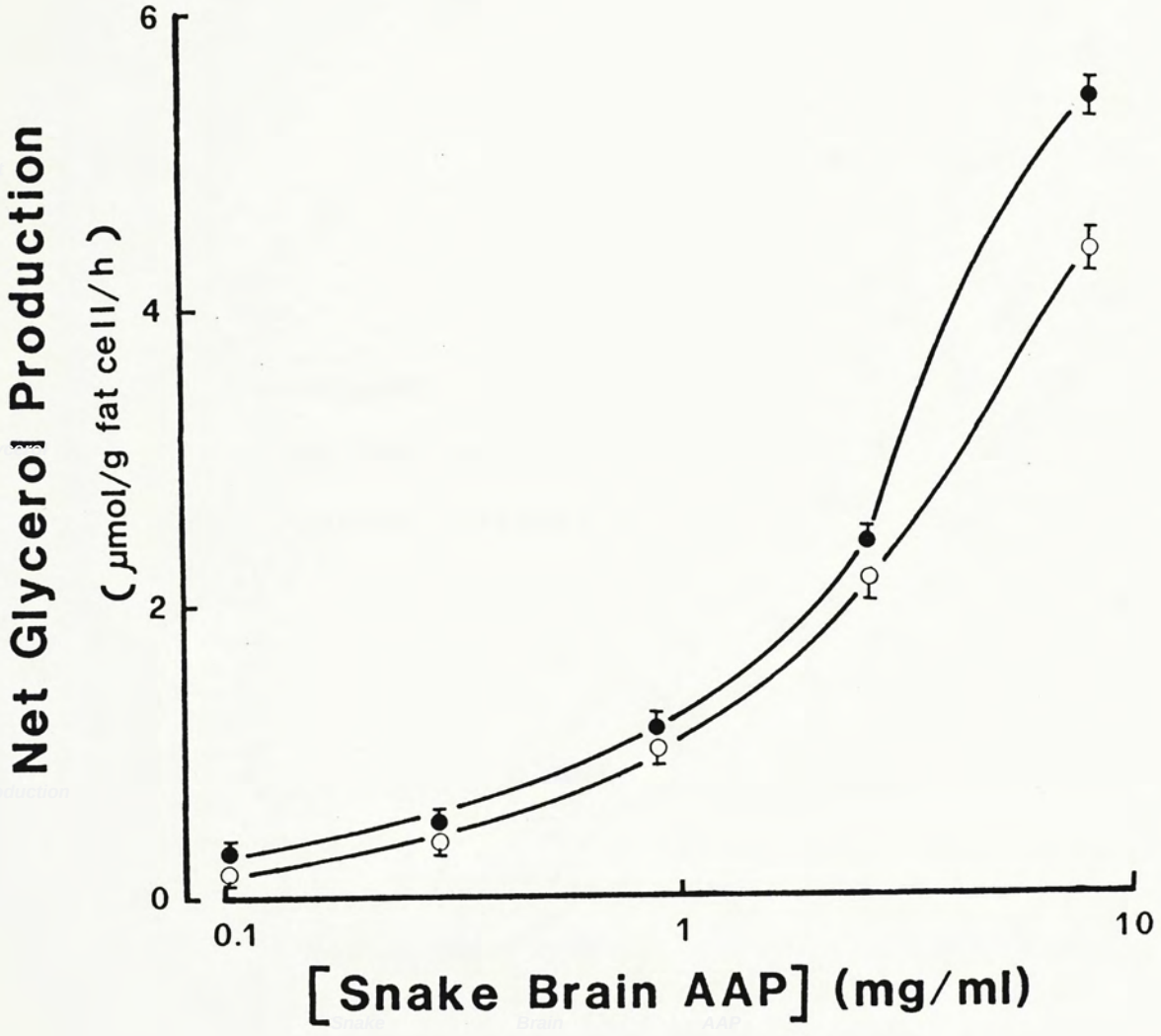

Net Glycerol Production
(μmol/g fat cell/h)
6
4
2
0
0.1
1
10
[Snake Brain AAP] (mg/ml)

Figure 3-30. Effects of *Hydrophis cyanocintus* (○) and *Lapemis hardwickii* (●) brain AAP on steroidogenesis in rat adrenal decapsular cells.

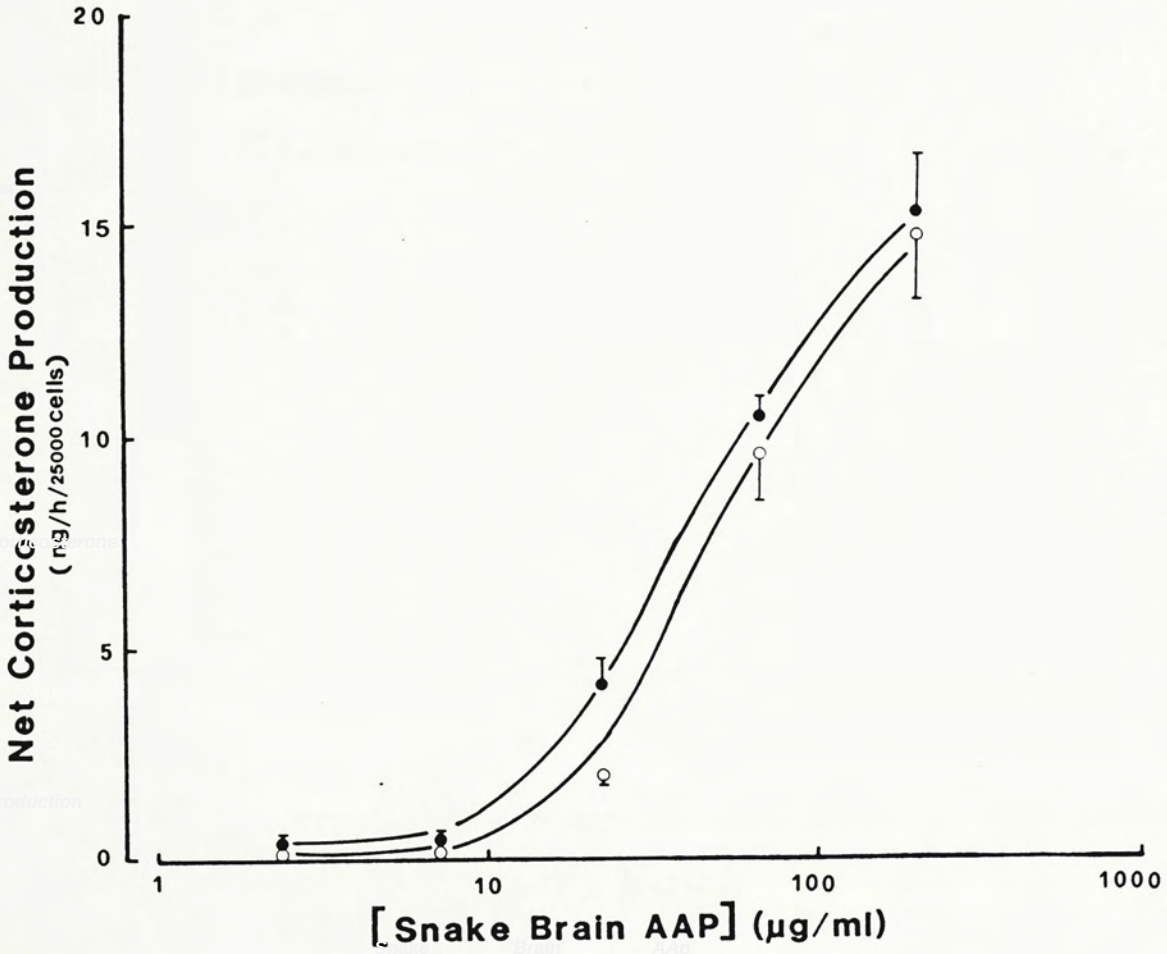
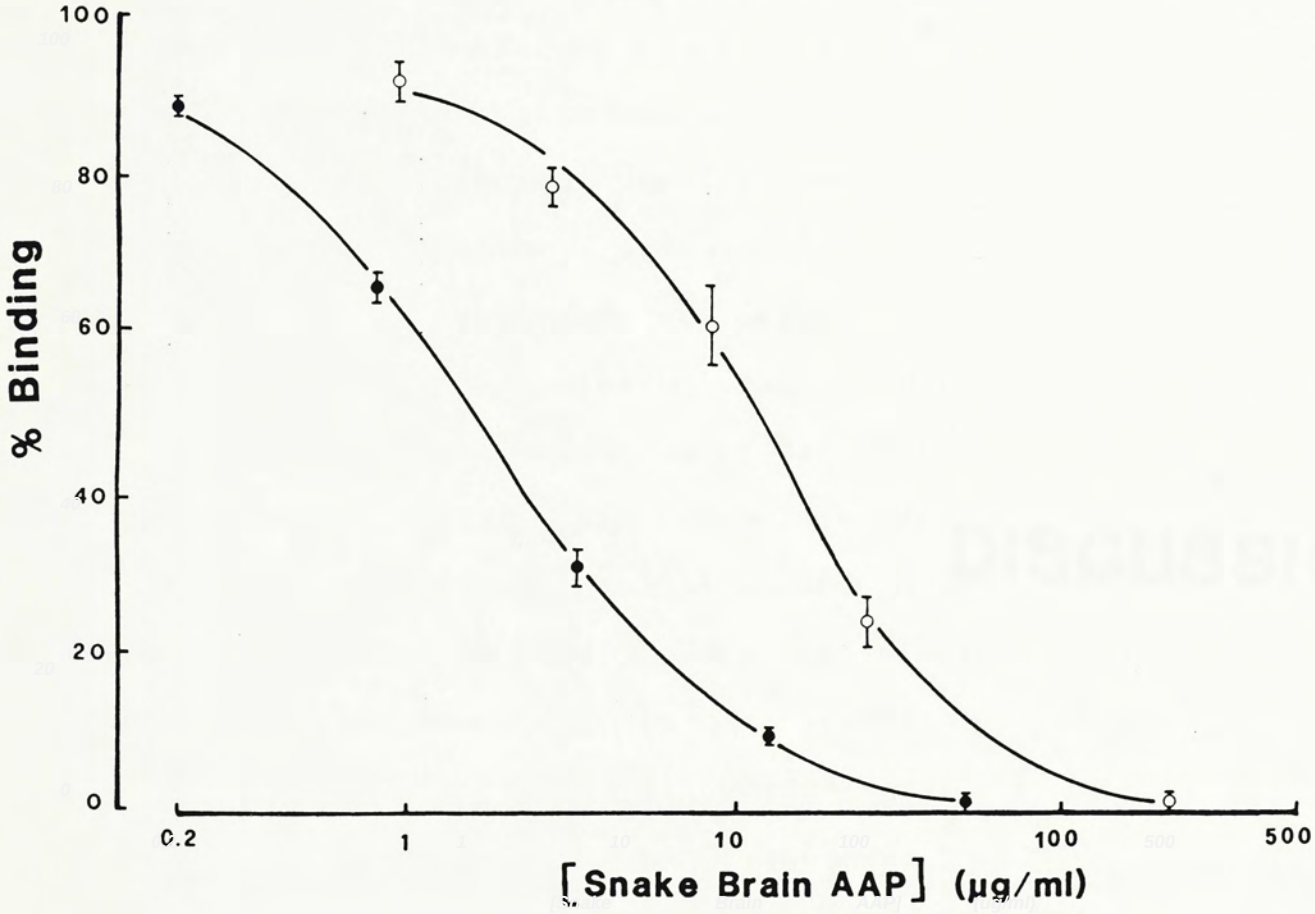

Net Corticosterone Production
(ng/h/25000 cells)
20
15
10
5
0
1
10
100
1000
[Snake Brain AAP] (µg/ml)

Figure 3-31. Inhibition of [^{3}H] DADLE binding to rat brain membranes by brain AAP of *Hydrophis cyanocinctus* (●) and *Lapemis hardwickii* (○).


100
80
60
40
20
0
% Binding
0.2
1
10
100
500
[Snake Brain AAP] (µg/ml)

DISCUSSION

CHAPTER 4. DISCUSSION

4.1 DISCUSSION ON THE ASSAY SYSTEM UTILIZED IN THIS STUDY

Radioimmunoassays have been used extensively for the detection and characterization of peptides in tissues (Jegou et al, 1983; Odagiri et al, 1979) and for the localization of certain peptides within tissues (Sjolund et al, 1983; Alumets et al, 1983) The assay results could be used as evidence for the presence or absence of certain peptide hormones (Follenius et al, 1985; Rodrigues and Sumpter, 1984). However, a lot of latent dangers and drawbacks accompany the usage of radioimmunoassay. The specificity of the antiserum used, the stability of the labelled ligand and the structures of target materials have all to be considered. Since the primary principle of antibody binding is the 3-dimensional spatial conformation of the antigen which may be achieved in many different ways, the possession of such a spatial conformation by an unrelated molecule may significantly alter the results of immunoassays. Radioimmunoassay results are based on the relative proportions of antibody-bound labelled antigen and the free labeled antigen. However, the degradation of such labelled compound in the assay will significantly reduce the quantity of labelled antigen bound to the antibody and thus produce an apparent displacement of the labelled antigen. The aforementioned problems can be circumvented by using a specific antiserum, enzyme-resistant labeled ligand and enzyme inhibitors. In addition, when radio-immunoassay is used for the detection of related material whose

structures have not been elucidated, caution must be taken in the interpretation of results because materials expected to be immunoreactive may be immunologically completely different from the antigen. For example, when radioimmunoassay for mammalian β-endorphin was used for the measurement of endorphins in salmon pituitary, the fish endorphins were found to be immunologically completely different from their mammalian counterpart (Takahashi et al, 1984).

Having considered these problems, we decided to use bioassays for the detection and characterization of peptide hormones in our study. Since bioassays are specific for the biologically functional portion of the molecules, positive results obtained in these assays would at least indicate the likely presence of such an active core. Moreover, by this approach, only the biologically active forms of expected materials would be measured and these forms may represent the physiologically active forms rather than the inactive precursors or degraded fragments. Hence concrete conclusions can be drawn about the presence and identitiy of the biologically active materials in a tissue only when positive results in radioimmunoassays using antisera of high specificity are backed up by bioactivity.

4.2 PROOPIOMELANOCORTIN-RELATED PEPTIDES IN EXTRA-PITUITARY TISSUES

4.2.1 Pancreas

In an immunocytochemical study, Alumets et al (1983) observed the presence and development of corticotropin containing cells in the exocrine parenchyma of porcine pancreas. This finding strongly suggested that pancreas may be a corticotropin producing organ. However, in study on human pancreas, not a trace of corticotropin immunoreactivity was detected (Bruni et al, 1979). Based on this finding, Bruni et al (1979) suggested that corticotropin is not synthesized in human pancreas. Although contradicting results were obtained on the presence of corticotropin in mammalian pancreas, no isolation or further investigation has been reported. In this study the presence of corticotropin-like material in equine pancreas was demonstrated by assays for steroidogenic and lipolytic activities. Because of the limitation of material available and time available, the structure of this corticotropin-like material has not yet been determined and so we can only deduce its properties from its chromatographic behaviour on Sephadex G-10, G-25 and CM-cellulose. This equine pancreatic corticotropin-like material may be a basic peptide of molecular weight larger than 5,000 as it appeared in the void volume of Sephadex G-25 and was strongly adsorbed on CM-cellulose. Being a large molecule, this pancreatic corticotropin-like activity is obviously quite different from its pituitary counterpart which has a molecular

weight of 4,500 daltons. However, it has been shown that the corticotropin-like material released *in vitro* was of higher molecular weight than corticotropin (1-39) (Kraicer *et al*, 1978) suggesting that large corticotropin might also be biologically functional. On the other hand, the basic character of this pancreatic corticotropin-like material agrees with the ionic properties of mammalian corticotropin (1-39) which was shown to contain large quantities of basic amino acid residues including lysine and arginine (Chang *et al*, 1980). However, before the primary structure of this corticotropin-like material can be determined, there is little we can say about its chemical nature.

The presence of this corticotropin-like activity in equine pancreas suggested it might play some physiological role in the pancreas. Although its role has yet to be determined, the observations of Knudtzon (1983) that exogenous administration of α-MSH, β-MSH and corticotropin increased the plasma levels of insulin and glucagon suggested a regulatory role, either direct or indirect, of these peptides in the pancreas.

Besides corticotropin, another class of peptides, the opioid peptides, have also been suggested to be physiologically significant in the pancreas (Hermansen, 1983; Reid *et al*, 1984). Enkephalins, the oligopeptides with opioid activity, were detected in porcine pancreas by immunocytochemistry (Alumets *et al*, 1983) and in guinea pig pancreas by radioimmunoassay and HPLC (Stern *et al*, 1982) while β-endorphin-like immunoreactivities were observed in human and porcine pancreas (Houck *et al*, 1981;

Bruni et al, 1979; Watkins et al,1980). In our study, an opioid substance(s) was also detected in the equine pancreatic extract by radioreceptor assay. Based on its chromatographic behaviour on Sephadex G-10 and Sephadex G-25, the molecular weight of this opioid material was estimated to be within the range of 700 to 5,000 daltons. However, we still cannot eliminate the possibility of this opioid material being enkephalin-like because it has been shown that pancreatic enkephalins could be hexa-, hepta- and octapeptides (Stern et al, 1982) containing the amino acid sequence of enkephalns. The adsorption of this opioid material on CM-cellulose revealed the fact that this peptide may consist of a large number of basic amino acid residues but its exact structure is still unknown. From the finding of Watkins et al (1980) that the immunoreactivities of β-endorphin and somatostatin are co-localized in the D-cells of pancreas, it appears that the insulin-secreting cells and glucagon secreting cells might be the most direct target for any regulatory role of β-endorphin. Furthermore, recent studies of the effects of opioid peptides and opiate antagonists on pancreatic functions (Ried et al, 1984; Rudman et al, 1983; Hermansen, 1983) strongly suggest that opiate peptides may be important in the regulation of the endocrine function of pancreas.

4.1.2 Placentas

Beta-endorphin-like material has been detected in human placentas by radioimmunoassay (Odagiri et al, 1979) and radioreceptor assay (Houck et al, 1980) and they were proposed to

have physiological functions in the placenta e.g. they may act as a natural antidote for the pain and stress of parturition. However, in other mammalian species in which parturition is apparently not as painful as that of human beings and consequently an natural analgesic may not be necessary, opioid peptides in the placental tissues have not been studied. Therefore we studied the placental tissues of two species, the rat and the bovine.

In the rat placental extract, we observed the presence of opioid peptide(s) by radioreceptor assay. This opioid activity, apparently due to peptide(s) of molecular weight less than 5,000, was capable of displacing ^{3}H-DADLE and ^{3}H-naloxone from their binding sites on rat brain membranes. Since it was found to be more potent in displacement of ^{3}H-naloxone than H-DADLE, we suggested that it may be a μ-receptor directing ligand. Simultaneously, opioid activity was also observed in bovine placental extracts. However, from the apparent molecular weight estimated from its chromatographic behaviour on Sephadex G-25 and Sephadex G-100, this bovine placental opioid material was unlikely to be structurally similar to that of rat placenta. Based on the above observations, it is suggested that placental opioid peptides were also presented in mammalian species in which natural analgesia during parturition seems to be of lesser importance and so opioid peptides might also have some other physiological functions in these placentas. Moreover, based on the results that the opioid peptides in the two species studied may be structurally different, it is proposed that different

species might contain different opioid peptides in their placentas.

In our study, we also detected the presence of corticotropin-like activity in rat placenta by means of the lipolysis and steroidogenesis assays. As it has previously been reported that corticotropin, β-lipotropin and β-endorphin are synthesized in the form of a common precursor (Mains et al, 1977), the co-localization of corticotropin-like activity and opioid peptides in the placenta suggests that the placenta may synthesize these peptides from a common precursor molecule.

4.2.3 Testes

By radioimmunoassasy and immunocytochemistry, opioid peptides have been reported to be present in the testes of a large variety mammalian species (Margioris et al, 1983; Tsong et al, 1982) and partially characterized by HPLC (Margioris et al, 1983). However, the opioid activity or opiate receptor binding activity of the testicular opioid peptides was not tested. Herein we report an opiate receptor binding activity in the acid acetone powder of mouse testes.

This opioid activity, found to displace ^{3}H-DADLE from rat brain membranes, might be able to interact with the opiate receptors in the male reproductive tract because a paracrine function has been suggested by Tsong et al (1982). The detection of a weak steroidogenic activity in mouse testicular AAP is in line with the presence of opioid peptides in mouse testes since

corticotropin and endorphins are synthesized in the same precursor in pituitaries (Roberts and Herbert, 1977). Although the physiological function of opioid peptides in testes has yet to be determined, the finding of Gerendai et al (1984) that intra-testicular administration of opiate antagonists significantly reduced the testosterone output in vitro suggested that Leydig cell function may be under the regulation of testicular opioid peptides. Since it has been reported that corticotropin administration can also regulate testicular testosterone secretion in pigs (Liptrap and Raeside, 1975; Juniewica and Johnson, 1984) and rabbits (Pitzel et al, 1981), a paracrine function is also suggested for the steroidogenic activity detected in mouse testicular AAP.

4.3 PROOPIOMELANOCORTIN RELATED PEPTIDES IN NON-MAMMALIAN VERTEBRATES

4.3.1 Carp and salmon pituitaries

Although pro-opiomelanocortin-related peptides have been detected by radioimmunoassay in the pituitaries of fish including elasmobranch (Pezalla et al, 1977; Hugh et al, 1974) and teleosts (Rodrigues and Sumpter, 1982, 1984), the pro-opiomelanocortin-related peptide system in teleost remained largely obscure until Kawauchi et al (1979, 1980, 1982) isolated and characterized a large number of pro-opiomelanocortin-related peptides from chum salmon (Oncorhynchus keta) pituitries. From their results, chum salmon was the first species found to have two sets of pro-opiomelanocortin-derived peptides which suggested that this system in chum salmon is a very complicated one. However, to study fish hormones with a radioimmunoassay using an antiserum raised against the mammalian hormone, erroneous conclusions might be obtained since it has been shown that the immunoreactivity of salmon endorphins is completely different from that of mammalian β-endorphin (Takahashi et al, 1984). Hence, we used bioassays and radioreceptor assays for the detection and functional characterization of peptides from two species of teleost, a fresh water species, the carp Cyprinus carpio and a marine species, the sockeye salmon, Oncorhynchus nerka.

Although pituitaries of several teleost species have

been studied by a number of groups (Hugh et al, 1974; Kawauchi et al, 1979), only the endorphin molecules of chum salmon have been isolated and fully characterized. The salmon endorphins resemble mammalian endorphins in the possession of the amino acid sequence of methionine enkephalin (Tyr-Gly-Gly-Phe-Met) at the amino-terminal and in the possession of a large proportion of basic amino acid residues. However, the N-acetylation at their amino termini is not found in mammalian endorphins. In the present study, results different from those of Kawauchi et al (1982) was obtained. The observations that the C. carpio pituitary extract and chromatographic fraction could displace ^{3}H-DADLE and ^{3}H-naloxone from binding to rat brain membranes suggests that the carp opioid peptides, unlike chum salmon endorphins, were not acetylated at their amino termini because it has been shown that N-acetylation of the amino terminus in opioid peptides would abolish receptor-binding and analgesic activities (Li et al, 1980). A basic nature for the carp opioid peptides can be inferred from the observation that the majority of the carp opioid activities was strongly adsorbed on CM-cellulose. With the time available, we have only demonstrated the receptor binding properties of the major peak of opioid material using a radioreceptor assay. The co-presence of other activities in the CM-cellulose fractions indicates that the C. carpio pituitary may contain more than one form of opioid peptide. In the sockeye salmon pituitary extract that we studied, we also observed the presence of a number of chromatographic fractions with radioreceptor binding activity. Surprisingly, opioid activity was also detected in the fraction of sockeye salmon fraction D

unadsorbed on CM-cellulose suggesting the presence of neutral or acidic opioid peptide in sockeye salmon pituitaries. The detection of opiate receptor binding activity in the CM-cellulose fractions of the sockeye salmon pituitary extract again shows that the opioid peptide(s) in sockeye salmon were unlikely N-acetylated.

Besides opioid activity, we also detected the presence of steroidogenic and lipolytic activities in the CM-cellulose fractions of carp (C. carpio) pituitaries. Although melanotropins and corticotropin have been detected in many species of fish by radioimmunoassay (Follenius et al, 1985; Hugh et al, 1974; Rodrigue and Sumpter, 1982, 1984), no teleost corticotropin has been isolated and characterized. Identification of corticotropin in teleost based on activity in radioimmunoassays employing antiserum raised against mammalian corticotropin and of unknown cross-reactivity to fish hormones is not always conclusive. So, using two specific bioassays for corticotropin-like activity, the hamster adipocyte lipolysis assay and the rat adrenal cell corticosteroidogenesis assay, we obviously demonstrated the presence of corticotropin-like materials in pituitary of C. carpio and O. nerka. Although it has been shown that teleost β-MSH and its derivatives can also stimulate corticosterone production in rat decapsular adrenal cells (Kawauchi et al, 1984) their potencies were only approximately 0.01% of that of corticotropin. So the steroidogenic and lipolytic activities of the CM-cellulose fractions of carp pituitary extract and salmon pituitary extract

were unlikely all mediated by MSHs though we cannot exclude the possibility that some of the minor steroidogenic activities may be due to melanotropins and this may be the cause of the widespread distribution of corticotropin-like activities among the CM-cellulose fractions.

4.2.2 Snake brain

Although pro-opiomelanotropin-related peptides have been extensively studied in mammalian tissues (Nouch et al, 1981; Saito et al, 1983) and pituitaries of many non-mammalian species including turkey (Li et al, 1977), salmon (Kawauchi et al, 1979, 1980, 1982) and dogfish (Lowry et al, 1974), little if any attention has been directed to a common reptile, the snakes. In this studies, we report the presence of these peptides in the snake brain for the first time.

It is well known that pro-opiomelanocortin-related peptides are mainly produced in the pituitaries of mammalian and non-mammalian vertebrates. Only recently were these peptides isolated and characterized in the mammalian brain (Ng et al, 1982). Since it has been reported that hypophysectomy did not affect the brain β-endorphin level, the brain peptides were suggested to be of extrapituitary origin (Rossier et al, 1977). In order to extend this hypothesis to non-mammalian vertebrates, we studied the brains of two species of sea snake, Hydrophis cyanocinatus and Lapemis hardwickii. However, owing to the fact that only a very small amount of materials was obtained, assays

were performed using the acid acetone powder (AAP) which is a crude extract of the brains.

The results of these assays demonstrated the presence of opioid peptides in snake brain AAP. This phenomemon indicated that snakes, like the mammals, may also possess opioid peptides and corticotropin-like material in their brains. Although the biosynthetic pathway of the proopiomelanocortin-derived peptides was not so concretely established in brain as in pituitary, the colocalization of these activities in the snake brain extracts might indicate their production from a common precursor in this organ.

4.4 GENERAL DISCUSSION AND SCOPE OF FURTHER STUDIES

Since it was reported that corticotropin-like immunoreactivity (Saito et al, 1983) and opioid immunoreactivity (Spampinato and Goldstein, 1983) are widespread in a large number of rat tissues, pro-opiomelanocortin-related peptides are suggested to be present and function in many tissues other than the pituitary and the brain of mammals. In order to accumulate more evidence for this hypothesis and to obtain a better understanding on the extra-pituitary function of the pro-opiomelanocortin, we have studied a number of mammalian extra-pituitary tissues. In our study, we demonstrated the presence of biologically active opioid and corticotropin-like material in pancreas, placentas, testes and brains but not in livers, kidneys and lungs. Based on this result, we can conclude that the opioid and corticotropin-like activities may be localized in the cells of the tissues rather than in blood trapped in the tissues because similar activities were not detected in liver which may house a large quantity of blood. Moreover, we can conclude that the extraction method employed was effective in extracting opioid and corticotropin -like material from tissue. Similarly, the result may also be used as an evaluation for our assay system as it was shown that even the most enzyme-riched tissues, liver and kidney, could not produce apparently positive results in our assays. So, proopiomelanocortin-related peptides may not be universally distributed in all extrapituitary tissues but localized in a number of organs in which these peptides may exert physiological functions.

To gather more information about this pro-opiomelanocortin related system in extra-pituitary tissues, we shall have to obtain pure forms of these extra-pituitary peptides, most practically by HPLC or by electrophoresis, and to identify the nature of these peptides by radioimmunoassay and eventually amino acid sequencing. To elucidate the physiological roles of such peptides in the tissues in which they are found, observations have to be made on the responses of the tissues to exogenous administration of the peptides and/or responses to the depletion or abolishment of activities of the endogenous peptides from the tissues.

Moreover, our study also adds more information to the evolutionary history of proopiomelanocortin-related peptides in the animal kingdom. In the animal kingdom, this family of peptides have been detected in mammals (Hammond et al, 1982; Scott et al, 1974), birds (Li et al, 1977), amphibians (Jegou et al, 1983) and fish (Kawauchi et al, 1979, 1980, 1982). However, no reports on the presence of these peptides in the snakes have appeared. In order to fill this missing gap in the evolutionary history of proopiomelanocortin related peptides, we chose to study the brain extracts of two sea snakes and positive results were obtained.

In most studies on non-mammalian pro-opiomelanocortin related peptides, radioimmunoassays were used e.g. Rodrigues and Sumpter (1982) and the biological activities of these peptides were seldom studied and reported. Hence we used bioassays for

detection of opioid peptides and corticotropin-like activities in snake brain extracts and fish pituitary extracts. We demonstrated opiate receptor binding activity and corticotropin-like activities in the pituitaries of carp (C. carpia) and sockeye salmon (O. nerka) and in the brain of two sea snakes (H. cyanocinctus and L. hardwickii). However, because of the small amount of materials available, the properties of the peptides could not be studied in detail. So a further study should be performed on the pure peptides which can be obtained by HPLC, TLC or electrophoresis. Then with their primary sequences elucidated and compared to the known structures of their mammalian counterparts or to those of other vertebrates, one can have an insight into the evolutionary history of this proopiomelanocortin system in the vertebrates. Studies can also be performed to study the biosynthesis for these peptides in the non-mammalian tissues. Messenger RNA for these peptides can also be isolated and characterized from the tissues. Furthermore, studies on the physiological roles of these peptides in non-mammalian vertebrates may provide a clue to their function in the mammalian system.

REFERENCES

REFERENCE

Alumets, J.; Hakanson, R. and Sundler, F. (1983) Ontogeny of endocrine cell in porcine gut and pancreas : an immunocytochemistry study. Gastroenterology. 85: 1359-1372.

Baker, B.I (1979) The evolution of ACTH, MSH and LPH-structure function and development. In: Hormones and Evolution (E.J.W. Barrington, ed.). vol. 2, pp. 643-723. (Academic Press, New York / London).

Birk, Y. and Li, C.H. (1964a) β-lipotropin. J. Biol. Chem. 239: 1048-1052.

Birk, Y. and Li, C.H. (1964) An efficient method with high yield for isolation of adrenocorticotropin from sheep pituitaries. Biochim. Biophys. Acta (Amst). 82: 430-432.

Bloom, F.E.; Rossier, J.; Battenberg, E.L.F.; Bayen, A.; French, E.; Henrikson, S.J.; Siggins, G.R.; Segal, D.; Browne, R.; Ling, N. and Guillemin, R. (1978) Beta-endorphin : cellular localization, electro-physiological and behavioral effects. Adv. Biochem. Psychopharmacol. 18: 89-109.

Bloom, F.E.; Battenberg, E.L.F.; Rossier, J.; Ling, N. and Guillemin, R. (1978) Neurons containing β-endorphin in rat brain exist separately from those containing enkephalin : immunocytochemical studies. Proc. Natl. Acad. Sci. USA. 75: 1591-1593.

Bruni, J.F.; Watkin, W.B.; Yen, S.C.C. (1979) β-endorphin in the human pancreas. J. Clin. Endocrinol. Metab. 49: 649.

Chang, A.C.Y.; Cochet, M and Cohen, S.N. (1980) Structural orgainization of human genomic DNA encoding the pro-opiomelanocortin peptide. Proc. Natl. Acad. Sci. USA. 77: 4890-4894.

Cohen, B.P. (1951) In Manometric techniques and tissue metabolism, p. 132, Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (editors) 2nd edition, Burgess Publishing Co., Minneapolis.

Cox, B.M.; Gentleman, S.; Su, T.P. and Goldstein, A. (1976) Further characterization of morphine-like peptides (endorphins) from pituitary. Brain Res. 115: 285-296.

Dores, R.M. (1982) Localization of multiple forms of ACTH- and β-endorphin-related substances in the pituitary of the reptile, Anolis carolinensis. Peptides. 3: 913-924.

Dores, R.M. (1982) Evidence for a common precursor for α-MSH and β-endorphin in the intermediate lobe of the pituitary of the reptile, Anolis carolinensis. Peptides. 3: 925-935.

Dores, R.M. (1984) Biosynthesis of multiple forms of β-endorphin in the reptile intermediate pituitary. Peptides. 4: 889-896.

Dores, R.M. (1984) Further characterization of the major forms of reptile beta-endorphin. Peptides. 4: 897-905.

DeRoos, R. and DeRoos, C. (1967) Presence of corticotropin activity in the pituitary gland of Chondrichthyean fish. Gen. Comp. Endocrinol. 9: 267-275.

Ferrara, P.; Houghten, R. and Li, C.H. (1979) β-endorphin: characteristics of binding sites in the rat brain. Biochem. Biophys. Res. Commun. 89(3): 786-792.

Follenius, E.; Dorsselafer, A.U. and Meunier, A. (1985) Separation and partial characterization by High-Performance Liquid Chromatography and radioimmunoassay of different forms of melanocyte-stimulating hormone from fish (Cyprinidae) neurointermediate lobes. Gen. Comp. Endocrinol. 57: 198-207.

Gerendai, I.; Shaha, C.; Thau, R. and Bardin, C.W. (1984) Do testicular opiates regulate Leydig cell functions. Endocrinol. 115(4): 1645-1647.

Graf, L.; Ronai, A.Z.; Bajusz, s.; Cseh, G. and Szekely, J.F. (1976) Opiate agonist activity of β-lipotropin fragments : a source of morphin-like substances in the pituitary. FEBS Lett. 64: 181-184.

Hammond, G.L.; Chung, D.; and Li, C.H. (1982) Isolation and characterization of δ-melanotropin, a new peptide from

bovine pituitary glands. Biochem. Biophys. Res. Commun. 108(1): 118:123.

Haning, R.; Tait, S.A.S. and Tait, J.F. (1970) In vitro effects of ACTH, Angiotensins, Serotonin and potassium on steroid output and conversion of corticosterone to aldosterone by isolated adrenal cells. Endocrinol. 87: 1147-1167.

Hermansen, K. (1983) Enkephalins and the secretion of pancreatic somatostatin and insulin in the dog : studies in vitro. Endocrinol. 113(3): 1149-1154.

Hodgen, G.D.; Gulgas, B.J. and Tullner, W.W. (1975) Role of the primate placenta in cortisol secretion by the maternal adrenal. Steroids. 26: 233.

Hope, J. and Lowry, P.J. (1981) Pro-opiocortin : the ACTH / LPH common precursor protein. in: Front. Horm. Res. vol. 8. pp. 44-61. (Karger, Basel).

Houck, J.C.; Chang, C. and Kimball, C. (1981) Pancreatic beta-endorphin-like polypeptides. Pharmacology. 23: 14-23.

Houck, J.C.; Kimball, C. and Chang, C. (1980) Placental β-endorphin-like peptides. Sci. 207(4): 79-80.

Hugh, P.J.B.; Lowry, P.J. and McMartin, C. (1974) Structural studies of α-melanocyte-stimulating hormone and a novel β-melanocyte stimulating hormone from the neurointermediate lobe of the dogfish, Squalus acanthias. Biochem. J. 141:439-444.

Hughes, J. (1975a) Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. Brain Res. 88: 295-308.

Hughes, J. (1975b) Search for the endogenous ligand of the opiate receptor. Neurosci. Res. Prog. Bull. 13: 55-58.

Jegou, S.; Toron, M.C.; Leroux, P.; Delarue, C.; Leboulenger, F.; Pelletier, G.; Cote, J.; Ling, N. and Vaudry, H. (1983) Immunological characterization of endorphins, adrenocorticotropin and melanotropins in frog hypothalamus. Gen. Comp. Endocrinol. 51: 246-254.

Jones, M.T.; Gillham, B.; Renzo, G.D.; Beckford, U. and Holmes, M.C. (1981) Neural control corticotropin secretion. in: Front. Horm. Res. vol. 8. pp. 12-43. (Karger, Basel).

Juniewice, P.E. and Johnson, B.H. (1984) Influence of adreanl steroids upon testosterone secretion by the boar testis. Biol. Reprod. 25: 725-733.

Kawauchi, H. and Muramoto, K. (1979) Isolation and primary structure of melanotropins from salmon pituitary glands. Int. J. Peptide Protein. Res. 14: 373-374.

Kawauchi, H.; Kitasato, M.; Kanezawa, A. and Kitagawa, H. (1980) Occurrence of two different endorphins in the salmon pituitary. Biochem. Biophys. Res. Commun. 92(4): 1278-1288.

Kawauchi, H. (1981) Biochemistry of salmon pro-opiocortin-related peptides : Bihormonal hypothesis. Tanpakushitsu Katasan Koso, 26: 138-150.

Kawauchi, H.; Takahashi, A. and Abe, K. (1982) Occurrence of a new corticotropin-like intermediate lobe peptide in salmon pituitary gland. Arch. Biochem. Biophys. 213(2): 680-688.

Kawauchi, H.; Kawazoe, I; Adachi, Y.; Buckley, D.I. and Ramachandran, J. (1984) Chemical and Biological characterization of salmon melanocyte-stimulating hormone. Gen. Comp. Endocrinol. 53: 37-48.

Kendall, J.W.; Orwoll, E. (1980) Anterior pituitary hormones in brain and other extrapituitary sites. in: Frontiers in Neuroendocrinology (Martin, L and Ganong, W.F., eds) vol. 6, pp. 33-65 (Raven Press, New York).

Kimura, S.; Lewis, R.U.; Stern, A.S.; Rossier, J.; Stein, S. and Udenfriend, S. (1980) Probable precursors of [Leu]enkephalin and [Met]enkephalin in adrenal medulla : peptides of 3-5 kilodaltons. Proc. Natl. Acad. Sci. USA. 77(3): 1681-1685.

Kloppenborg, W.C.; Island, D.P.; Liddle, G.W.; Michelakis, A.S. and Nicholson (1968) A method of preparing adrenal cell suspensions and its applicability to the in vitro study of adrenal metabolism. Endocrinol. 82: 1053-1058.

Knobil, E. and Briggs, F.N. (1955) Fetal-maternal endocrine interrelations : the hypophyseal-adrenal system. Endocrinol. 57: 147.

Knudtzon, J. (1983) Alpha-melanocyte stimulating hormone increases plasma levels of glucagon and insulin in rabbits. Life Sci. 34: 547-554.

Korn, E.D. (1955) Cleaning factor, a heparin-activated lipolrotein lipase. I. Isolation and characterization of the enzyme for normal rat heart. J. Biol. Chem. 215: 1-14.

Kosterlitz, H.W. (1979) Multiple receptors for opiates and opioid peptides. in: Brain peptides : A new endocrinology (North-Holland, Biochemical press).

Kraicer, J.; Elliot, N.L.; Zimmerman, A.E. (1978) In vitro release of ACTH from dispersed rat pars intermediate cells. 3. Multiple forms of ACTH biological activity. Neuroendocrinology. 27: 86-96.

Krieger, D.T.; Liotta, A.S.; Suda, T.; Palkovits, M. and Brownstein, M.I. (1977) Presence of immunoassayable β-lipotropin in bovine brain and spinal cord : lack of concordance with ACTH concentrations. Biochem. Biophys. Res. Commun. 76: 930-936.

Lambert, M. and Neish, A.C. (1950) Rapid method for estimation of glycerol in fermentation solutions. Can. J. Research. 28B: 83-89.

Lee, C.Y.; McPherson, M.; Loko, V. and Ramachandran, J. (1980) Pituitary corticotropin-inhibiting peptide : properties and use in study of corticotropin action. Arch. Biochem. Biophys. 201: 411-419.

LeRoith, D.; Liotta, A.S.; Roth, J.; Shiloach, J.; Lewis, M.E.; Pert, C.B. and Kreiger, D.T. (1982) Corticotropin and β-endorphin-like materials are native to unicellular orgainism. Proc. Natl. Acad. Sci. USA. 79: 2086-2090.

Li, C.H. (1952) Preparation and properties of a highly active adrenocorticotropic hormone preparation. J. Am. Chem. 74:

2134.

Li, C.H.; Schnabel, E.; Chung, D. and Lo, T.B. (1961) Synthesis of L-Methionyl-L-Glutamyl-L-Histidyl-L-phenylalanyl-L-Arginyl-L-Tryptophyl-Glycine and its melanocyte-stimulating and corticotropin-releasing activity. Nature (London). 189: 143.

Li, C.H. and Chung, D. (1976) Primary structure of human β-lipotropin. Nature (London). 260: 622-624.

Li, C.H. and Chung, D. (1976) Isolation and structure of an untriakontapeptide with opiate activity from camel pituitary glands. Proc. Natl. Acad. Sci. USA. 73: 1145-1148.

Li, C.H.; Chung, D. and Doneen, B.A. (1976) Isolation, characterization and opiate activity of β-endorphin from human pituitary glands. Biochem. Biophys. Res. Commun. 72: 1542-1547.

Li, C.H.; Tan, L. and Chung, D. (1977) isolation and primary structure of beta-endorphin and beta-lipotropin from bovine pituitary gland. Biochem. Biophys. Res. Commun. 77: 1088-1093.

Li, C.H. (1978) Isolation, characterization and synthesis of a corticotropin-inhibiting peptide from human pituitary glands. Proc. Natl. Acad. Sci. USA. 75(9): 4306-4309.

Li, C.H.; Tseng, L.F.; Jibson, M.D.; Hammonds, R.G.; Yamashiro, D. and Zaoral, M. (1980) β-endorphin-(1-27) : acetylation of alpha-amino groups enhances immunoreactivity but diminished analgesic and receptor binding activities with no change in circular dichroism spectra. Biochem. Biophys. Res. Commun. 97(3): 932-938.

Li, C.H.; Ng, T.B.; Yamashiro, D.; Chung, D.; Hamonds, R.G. and Tseng, L.F. (1981) β-endorphin : isolation, amino acid sequence and synthesis of the hormone from horse pituitary gland. Int. J. Peptide Protein Res. 18: 242-248.

Li, C.H.; Ng, T.B. and Cheng, C. (1982) Melanotropins : aldosteone- and corticosterone-stimulating activity in isolated rat adrenal cells. Int. J. Peptide Protein Res. 19: 361-365.

Liotta, A.S.; Houghten, R.; Krieger, D.T. (1982) Identification of a β-endorphin-like peptides in cultured human placental cells. Nature. 295: 593.

Liotta, A.S. and Krieger, D.T. (1980) In vitro biosynthesis and comparative post-translational processing of immunoreactive precursor corticotropin / β-endorphin by human placental and pituitary cells. Endocrinol. 106: 1504.

Liotta, A.S.; Loudes, S.; Mckelwy, J.F. and Krieger, D.T. (1980) Biosynthesis of precursor corticotropin / endorphin-, corticotropin-, β-melanotropin-, β-lipotropin-, and β-endorphin-like materials by cultured neonatal rat hypothalamic neurons. Proc. Natl. Acad. Sci. USA. 77: 1880.

Liotta, A.S.; Osathanodh, R.; Ryan, K.J. and Krieger, D.T. (1977) Presence of corticotropin in human placenta : demonstration of in vitro synthesis. Endocrinol. 101: 1552-1558.

Liptrap, R.M. and Raeside, J.I. (1975) Increase in plasma testosterone concentration after injection of adrenocorticotropin into the boar. J. Endocrinol. 66: 123-131.

Loh, H.H.; Tseng, L.F.; Wei, E. and Li, C.H. (1976) β-endorphin is a potent analgesic agent. Proc. Natl. Acad. Sci. USA. 73(8): 2895-2898.

Lowry, P.J.; Bennett, H.P.J. and McMartin, C. (1974) The isolation and amino acid sequence of an adrenocorticotrophin from the Pars distalis and corticotrophin-like intermediate-lobe peptide from the neurointermediate lobe of the pituitary of the Dogfish, Squalus acanthias. Biochem. J. 141: 427-437.

Lowry, P.J. and Chadwick, A. (1970) Purification and amino acid sequence of melanocyte stimulating hormone from the dogfish, Squalus acanthias. Biochem. J. 118: 713-718.

Lowry, P.J.; Hope, J.; Silman, R.E. (1976) The evolution of corticotropin, melanotropin and lipotropin in: James, Proc. 5th Int. Congr. Endocrinol. vol. 1, pp. 77-76 (Excerpta Medica, Amsterdem, 1976).

Lowry, P.J.; Silman, R.E.; Hope, J. and Scott, A.P. (1977) Structure and biosynthesis of peptides related to cortico-

tropins and β-melanotropins. Ann. N. Y. Acad. Sci. 297: 49-62.

Mains, R.E.; Eipper, B.A. and Ling, A (1977) Commom precursor to corticotropins and endorphins. Proc. Natl. Acad. Sci. USA. 74: 3016-3018.

Margioris, A.N.; Liotta, A.S.; Vaudry, H.; Bardin, C.W. and Krieger, D.T. (1983) Characterization of immunoreactive proopiomelanocortin-related peptides in rat testes. Endocrinol. 113(2): 663-671.

Martin, A.R. (1967) Opioid antagonist. Pharmacol. Rev. 19:463-521.

Martin, W.R.; Eades, C.G.; Thompson, J.A.; happier, R.E. and Gilbert, P.E. (1976) The effects of morphine and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. J. Pharmacol. Exp. Ther. 197: 517-532.

Mather, J.P. (1980) Establishment and characterization of two distinct mouse testicular epithelial cell lines. Biol. Reprod. 23: 243.

Matsukura, S.; Yoshimi, H.; Sueoka, S.; Kataoka, K.; Ono, T. and Ohgushi, N. (1978) The regional distribution of immunoreactive β-endorphin in the monkey brain. Brain Res. 159-228.

Nakanishi, S.; Inoue, A.; Taii, S. and Numa, S. (1977) Cell free translation product containing corticotropin and β-endorphin encoded by messenger RNA from anterior lobe and intermediate lobe of bovine pituitary. FEBS Lett, 84: 105-109.

Nakanishi, S.; Inoue, A.; Kita, T.; Nakamura, M.; Chang, A.C.Y.; Cohen, S.N. and Numa, S. (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin-,β-lipotropin precursor. Nature (London). 279: 423-427.

Ng, T.B.; Chung, D. and Li, C.H. (1982) Isolation and properties of β-endorphin-(1-27)-like peptide from bovine brains. Int. J. Peptides Proteins Res. 19: 343-347.

Odagiri, E.; Sherrell, B.J.; Mount, C.D.; Nicholsm, W.E. and Orth, D.N. (1979) Human placental innunoreactive

corticotropin, lipotropin, and β-endorphin : evidence for a common precursor. Proc. Natl. Acad. Sci. USA. 76: 2027-2031.

Osborne, H.; Holt, V. and Herz, A. (1978) Potassium-induced release of enkephalins from rat striatal slices. Europ. J. Pharmacol. 48: 219-221.

Osborne, H.; Przewlocki, R.; Holt, V. and Herz, A. (1979) Release of β-endorphin from rat hypothalamus in vitro. European J. Pharmacol. 55: 425-428.

Patten, L.R. (1970) The reciprocal regulation of lipoprotein lipase activity and hormone-sensitive lipase activity in rat adipocytes. J. Biol. Chem. 245(21): 5577-5584.

Pfeiffer, A.; Pasi, A.; Mehraein, P. and Herz, A. (1982) Opiate receptor binding sites in human brain. Brain Res. 248: 87-96.

Polak, J.; Bloom, S.; Sullivan, S.; Facer. P. and Pearse, A. (1977) Enkephalin-like immunoreactivity in the human gastrointestinal tract. Lancet i. : 972-974.

Pitzel, L.; Sander, B.A. and Konig, A. (1982) Insulin-induced elevation of plasma conticosteroid and testosterone levels in male rabbits. Acta. Endocrinol. 99 (suppl. 246): 106-107 (Abstr.).

Pemalla, P.D.; Clarke, W.C.; Lis, M., Seidah, N.G. and Chretien, M. (1978) immunological characterization of β-lipotrophin fragment (endorphin, β-MSH and N-fragment) from fish pituitary. Gen. Comp. Endocrinol. 34: 163-168.

Rafferty, B.; Zanelli, J.M.; Rosenblatt, M. and Schulster, D. (1983) Corticosteroidogenesis and adenosine 3',5'-monophosphate production by the amino-terminal (1-34) fragment of human parathyroid hormone in rat adrenocortical cell. Endocrinol. 113(3): 1036-1041.

Ramachandran, J.; Farmer, S.W.; Liles, S. and Li, C.H. (1976) Comparison of the steroidogenesis and melanotropic activities of corticotropin, β-melanotropin and analogs with their lipolytic activities in rat and rabbit adipocytes. Biochimica et Biophysica Acta. 428: 347-354.

Rao, A.J.; Long, J.A. and Ramachandran, J (1978) Effects of antiserum to adrenocorticotropin on adrenal growth and function. Endocrinol. 102(2): 371-378.

Ree, J.M.V. and De Wied, D. (1981) Behavioral effects of β-endorphin fragments. in: Front. Horm. Res. vol. 8. pp. 178-192. (Karger, Basel).

Rees, L.H.; Burke, C.W.; Chard, T.; Evans, S.W. and Letchworth, A.T. (1975) Possible placental origin of ACTH in normal human pregnancy. Nature (London) 254, 620-622.

Reid, R.L.; Sandler, J.A. and Yen, S.S.C. (1984) β-endorphin stimulates the secretion of insulin adn glucagon in Diabetes Mellitus. Metabolism. 33(3): 197-199.

Roberts, J.L and Herbert, E. (1977) Characterization of a common precursor to corticotropin and β-lipotropin : cell free synthesis of the precursor and identification of corticotropin peptides in the molecules. Proc. Natl. Acad. Sci. USA. 74: 4826-4830.

Rodbell, M. (1964) Metabolism of isolated fat cells. J. Biol. Chem. 239:375-380.

Rodrigues, K.T. and Sumpter, J.P. (1982) The distribution of some pro-opiomelanocortin-related peptides in the pituitary gland of rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 51: 454-459.

Rodrigues, K.T. and Sumpter, J.P. (1984) The radioimmunoassay of β-melanocyte stimulating hormone and endorphin in trout (Salmo gairdneri) and the effect of blinding on the plasma levels of these peptides. Gen. Comp. Endocrinol. 54: 69-75.

Rossier, J.; Vargo, T.M.; Minick, S.; Ling, N.; Bloom, F.E. and Guillemin. R. (1977) Regional dissociation of β-endorphin and enkephalin contents in rat brain and pituitary. Proc. Natl. Acad. Sci. USA. 74: 5162-5165.

Rudman, D.; Berry, C.J.; Riedebury, C.H.; Hollins, B.M.; Kutner, M.H.; Lynn, M.J. and Chawla, R.K. (1983) Effects of opioid peptides and opiate alkaloids on insulin secretion in the rabbit. Endocrinol. 112(5): 1702-1710.

Roberts, J.L. and Herbert, E. (1977) Characterization of a common precursor to corticotropin and β-lipotropin : identification of β-lipotropin in the precursor synthesized in a cell-free system. Proc. Natl. Acad. Sci. USA. 74: 5300-5304.

Saito, E.; Iwasa, S. and Odell, W.D. (1983) Widespread presence of large molecular weight adrenocorticotropin-like substance in normal rat extrapituitary tissues. Endocrinol. 113(3): 1010-1019.

Schusdziarra, V.; Specht, J.; Schick, R. and Pfeiffer, E.F. (1983) Effect of opiate-active substances on basal somatostatin release. Horm. Metab. Res. 15: 407-408.

Scott, A.P.; Lowry, P.J.; Ratcliffe, J.G.; Rees, L.H. and Landon, J. (1974) Corticotrophin-like peptides in the rat pituitary. J. Endocrinol. 61: 355-367.

Sharp, B.A. and Pekary A.E. (1981) β-endorphin and other β-endorphin-immunoreactive peptides in human semen. J. Clin. Endorcrinol. Metab. 52: 586.

Sharp, B.A.; Pekary, A.E.; Meyer, N.V. and Hershman, J.M (1980) β-endorphin in male rat reproductive organs. Biochem. Biophys. Res. Commun. 95: 618.

Simantov, R. and Snyder, S.M. (1976) Isolation and structure identification of a morphine-like peptide "encephalin" in bovine brain. Life Sci. 18: 781-785.

Sjolund, K.; Sanden, G.; Hakanson, R. and Sundler, F. (1983) Endocrine cells in human intestine : an immunocytocheical study. Gastroenterology. 85: 1120-1130.

Spampinato, S. and Goldstein, A. (1983) Immunoreactive dynorphin in rat tissues and plasma. Neuropeptides. 3: 193-212.

Stern, A.S.; Wurzburger, R.J.; Barkey, R. and Spector, S. (1982) Opioid polypeptides in guinea pig pancreas. Proc. Natl. Acad. Sci. USA. 79: 6703-6706.

Swann, R.W. and Li, C.H. (1980) Isolation and characterization

of β-endorphin-like peptides from bovine brain. Proc. Natl. Acad. Sci. USA. 77(1): 230-233.

Takahashi, A.; Kawauchi, H.; Mouri, T. and Sasaki, A. (1984) Chemical and immunological characterization of salmon endorphin. Gen. Comp. Endocrinol. 53: 381-388.

Terenius, L. (1981) Endorphin and pain. in: Front. Horm. Res. vol. 8. pp. 167-177. (Karger, Basel).

Tseng, L.F.; Loh, H.H. and Li, C.H. (1976) β-endorphin as a potent analgesic by intravenous injection. Nature (London). 263: 239-240.

Tsong, S.D.; Phillips, D.M.; Bardin, C.W.; Halmi, N.; Liotta, A.S.; Margioris, A. and Krieger, D.T. (1982) ACTH and β-endorphin-related peptides are present in multiple sites in the reproductive tract of the rat. Endocrinol. 110: 2204-2206.

Tsong, S.D.; Phillips, D.M.; Halmi, N.; Krieger, D. and Bardin, C.W. (1982) β-endorphin is present in the male reproductive tract of 5 species. Biol. Reprod. 27: 755-764.

Uhl, G.R.; Childers, S.R. and Snyder, S.H. (1978) Opioid peptides and the opiate receptors. Front. Neuroendocrinol. 5: 289-322.

Watkin, W.B.; Bruni, J.F. and Yen, S.C.C. (1980) β-endorphin and somatostatin in the pancreatic D-cells : colocalization by immunocytochemistry. J. Histochem. Cytochem. 28(1): 1170-

Wolstencroft, J.H.; West, D.C. and Gent, J.P. (1978) in: Iontophoresis and transmitter mechanism in the mammalian nervous system (Ryall, R.W. and Kelly, J.S., eds) pp. 349-343. (Elsevier / North-Holland Biochemical Press, Amsterdam).