Purification, Characterization, and Biological Action of Corticostatic Peptides

By

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Dedicated to my wife Jing

and to my son Jonathan

Abstract

A family of four low molecular weight peptides, that are rich in arginine and cysteine and which have the ability to inhibit ACTH stimulated adrenocortical steroidogenesis have been purified from rabbit lung and neutrophil extracts and sequenced. They are called corticostatin I (CSI), II (CSII), III (CSIII), and IV (CSIV) respectively. Among them CSI is the most potent with ED_{50} of 25 nM against a concentration of 150 pg/ml ACTH stimulation. The mechanism of action of CSI has been studied extensively. CSI shows a high degree of specificity in that it does not inhibit the action of angiotensin II in the adrenal cortex. CSI does not inhibit dbcAMP induced steroidogenesis, however it does inhibit the accumulation of cAMP in response to ACTH in the rat adrenal cell suspension. CSI acts by competing with the basic $-Gly_{14}-Lys_{15}-Lys_{16}-Arg_{17}-Arg_{18}-$ sequence of ACTH for its binding site.

Abrégé

Une famille de quatre peptides à faible poids moléculaire a été purifiée d'extraits de poumons et de neutrophiles de lapin et leurs séquences ont été déterminées. Ces peptides, qui sont riches en arginine et cystéine, peuvent inhiber l'action de l'ACTH dans la synthese de steroides provenant du cortex surrénalien, et ils sont nommés corticostatin I (CSI), II (CSII), III (CSIII), et IV (CSIV). De ces quatre peptides, CSI est celui qui est le plus actif ayant un ED_{50} de 25 mM face à une stimulation d'ACTH d'une concentration de 150 pg/ml. Le mécanisme d'action de CSI a été étudie a fond. CSI démontre un très haut degre de spécificité car il n'inhibe pas l'action de Angiotensin II sur le cortex surrénalien. L'action de l'AMP cyclique di-butyl sur la stimulation de stéroides ne fût pas affectée par CSI, qui cependant peut inhiber l'accumulation de l'AMP cyclique dûe à l'action de l'ACTH dans le bioessai de cellules de surrénale de rat. Aussi CSI agit de façon compétitive avec la séquence - Gly_{14} - Lys_{15} - Lys_{16} - Arg_{17} - Arg_{18} - de l'ACTH pour son site de liaison.

Preface

In the course of trying to isolate and sequence ACTH from rabbit fetal lung by RP-HPLC, It was noted that in the dispersed rat adrenocortical cell bioassay there was a dip in corticosterone response which was a reproducible phenomenon. The work presented in this thesis is the consequences of this finding.

Most of the studies described in this thesis have been published or prepared for publication in the following original articles and abstracts.

Zhu, Q., and Solomon, S.
Mode of action of corticostatin peptides in the adrenal gland in preparation for Endocrinology

Zhu, Q., Bateman, A., Singh, A., and Solomon, S.
Isolation and biological activity of corticostatic peptides (anti-ACTH).
Endocrine Research. U.S.A. 15:129-149 (1989).

Singh, A., Bateman, A., Zhu, Q., , Shimasaki, S., Esch, F. and Solomon, S.
Structure of a novel human granulocyte peptide with anti-ACTH activity.
Biochemical. Biophysical Research Communications 155 524-529 (1988).

Bateman, A., Singh, A., Zhu, Q., and Solomon, S.
The isolation and identification of a novel human neutrophil anti-ACTH peptide.
Program and Abstract of the Endocrine Society 70th Annual Meeting, (abstract 330) (1988).

Zhu, Q., and Solomon, S.
Isolation and bioactivity of naturally occurring corticostatic (anti-ACTH) peptides.
Program and Abstract of the Endocrine Society 70th Annual Meeting, (abstract 1142) (1988).

Zhu, Q., Hu, J., Mulay, S., Esch, F., Shimasaki, S. and Solomon, S.
Isolation and structure of corticostatin peptides from

rabbit fetal and adult lung
Proc. Natl. Acad. Sci. USA 85 592-596 (1988)

Zhu, Q. and Solomon, S.
Isolation and Identification of a 34 amino acid peptide from rabbit fetal and adult lung with corticostatin activity.
Program and Abstract of the Endocrine Society 69th Annual Meeting,(abstract 501) (1987).

Zhu, Q., Singh, A. V., Bateman, A., Esch, F. and Solomon, S.
The corticostatin (anti-ACTH) and cytotoxic activity of peptides isolated from fetal, adult and tumor bearing lung.
J. Steroid Biochem. 27 1017-1022 (1987).

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ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone; α-melanotropin
3ß-HSD	3ß-hydroxy-5-ene steroid dehydrogenase
A	alanine
AII	angiotensin II
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
Ala	alanine
AP	acute phase protein
Arg	arginine
Asn	asparagine
Asp	aspartic acid
AtT20	a clonal mouse anterior pituitary corticotroph tumor cell line
AVP	arginine vasopressin
B	corticosterone
BBB	blood-brain barrier
BSA	bovine serum albumin
BSF-2	B-cell stimulatory factor-2
C	cysteine
cAMP	cyclic adenosine 3':5' monophosphate
CCK	cholecytokinin
cDNA	complementory deoxyribonucleic acid
cGMP	cyclic guanosine 3':5' monophosphate
CLIP	corticotropin-like intermediate lobe peptide
CM	carboxymethyl
Con A	concanavalin A
CRF	corticotropin-releasing factor
CRFs	corticotropin-releasing factors
CSI	rabbit corticostatin I
CSIa	synthetic rabbit corticostatin I
CSIb	isoform b of synthetic rabbit corticostatin I
CSIc	isoform c of synthetic rabbit corticostatin I
CSII	rabbit corticostatin II
CSIII	rabbit corticostatin III
CSIV	rabbit corticostatin IV
Cys	cysteine
D	aspartic acid
dbcAMP	dibutyryl cyclic adenosine 3':5' monophosphate
DHEA	dehydro-epiandrosterone

DNA	deosyribonucleic acid
DNase	deoxyribonuclease I
E	glutamic acid
ED_{50}	fifty percent effective dose
F	phenylalanine
FSHRH	follicle stimulating hormone releasing factor
FSH	follicle stimulating hormone
G	glycine
GH	growth hormone
Gi	inhibitory GTP-binding proteins
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GPCS	guinea pig corticostatin peptide
GRF	growth hormone releasing factor
G_s	stimulatory GTP-binding proteins
GTP	guanosine triphosphate
H	histidine
HDL	high density lipoproteins
HFBA	heptofluorobutyric acid
HGF	hybridoma growth factor
His	histidine
HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A
hnRNA	primary transcript of RNA
HP-4	human corticostatic peptide 4
HPA	hypothalamic-pituitary-adrenal
HPGF	hybridoma plasmacytom growth factor
HPLC	high performance liquid chromatography
HSF	hepatocyte stimulating factor (IL-6)
I	isoleucine
IFN-γ	interferon-γ
IFN-ß2	interferon-ß2
IGF-I	insulin-like growth factor I
IGF-II	insulin like growth factor II
IL-1	interleukin 1
IL-2	interleukin 2
IL-3	interleukin 3
IL-6	interleukin 6
Ile	isoleucine
IR-ACTH	immunoreactive ACTH
IR-oCRF	immunoreactive ovine CRF
K	lysine
Kd	apparent dissociation constant
L	leucine

LDL	low density lipoproteins
Leu	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LPS	bacterial lipopolysaccharide
Lys	lysine
M	methionine
ME	median eminence
MeCN	acetonitrile
N	asparagine
NAD	nicotinamine adenine dinucleotide
NADPH	nicotinamine adenine dinucleotide phosphate (reduced form)
P	proline
P_{450c11}	11ß-hydroxylase, 18-hydroxylase and 18-oxidase
P_{450c17}	17-α-hydroxylase and 17,20 lyase
P_{450c21}	21-hydroxylase
P_{450scc}	cholesterol side -chain cleavage enzyme
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PHA	phytohemaglutanin
Phe	phenylalanine
PKI	cAMP-dependent protein kinase inhibitor, the Walsh inhibitor
Pro	proline
PTH	phenylthiohydantion
PVN	hypothalamic paraventricular nucleus
PVNmpd	the dorsal medial parvicellular part of hypothalamic paraventricular nucleus
Q	glutamine
R	arginine
R-1	rat arginine-rich cysteine-rich peptide 1
R-2	rat arginine-rich cysteine-rich peptide 2
R-3	rat arginine-rich cysteine-rich peptide 3
R-4	rat arginine-rich cysteine-rich peptide 4 with corticostatic activity
R-5	rat arginine-rich cysteine-rich peptide 5 with corticostatic activity
RP-HPLC	reversed-phase high performance liquid chromatogrphy
S	serine

ß-EP	ß-endorphin
SCP-2	sterol carrier protein-2
Ser	serine
T	threonine
TFA	trifluoracetic acid
TGF-ß	transforming growth factor ß
TNF	tumor necrosis factor
TRF	thyroid stimulating hormone releasing factor
TSH	thyroid stimulating hormone
Tyr	tyrosine
V	valine
Val	valine
W	tryptophan
Y	tyrosine
Y-1	mouse tumor adrenocortical cell line

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

This chapter concerns itself with the interactions of the nervous system with the endocrine system and the endocrine system with the immune system. It also attempts to integrate the immune system interaction with the nervous and endocrine systems. This is very broad subject and the literature in this field has become so voluminous, that it is not possible to detail it in an introduction to a Ph. D. thesis. As a result only a small selection of representative references will be given and mention will be made of current reviews.

The recognition that the endocrine system is controlled and regulated by central nervous system was first made by the pioneering work of E. Scharrer (1) during the 1930's on the hypothalamic "glandular neurons" in ertebrate and from the work of Harris (2), Rasmussen (3), Scharrer (4) and Szentagothai (5) among others on the hypophyseal portal circulatory system. In contrast, the immune system was considered as a completely autonomous system until 1970's when Besedovsky, Sorkin, and del Ray and colleagues (reviewed by Besedovsky and Sorkin, see ref. 6) extensively explored the relation between the immune-defence and endocrine systems. In addition the elegant review of Munck

et al. (7) on the physiological functions of glucocorticoids greatly contributed to the current knowledge of the interaction of nervous, endocrine and immune systems.

The direct control of the immune system by the nervous system has not as yet been established. The immune system more likely is controlled to a lesser or greater extent by nervous system through hypothalamic-pituitary-adrenal (HPA) axis. The physiological significance of the neuronal control of the immune system via the HPA axis or other endocrine axis is not obvious. A relatively autonomous immune-defence system with a latency of neuronal control might be necessary for the body to survive from extremely complex and dangerous surroundings.

A detailed discussion concerning the regulations within the immune-defence system and within the nervous system will not be found in this thesis, and historical development of current knowledge will also be omitted in most cases. Since substantial progress has been made in mapping pathways of the information flow, (although most of them still remain largely unidentified) this chapter will briefly cover pathways of information flow from the neuronal to the hypothalamic to the pituitary to the adrenal then to the immune and back to the neuronal systems.

1.2. The Hypothalamus: The Link Between the Nervous and Endocrine Systems

During the so called, "the highly productive days" of the 1940s and 1950s, a number of gifted neuroscientists around world, such as F.O. Schmitt, J. Z. Young. J. Szentagothai, D. Hebb among others made a series of important discoveries which greatly contributed to our body of modern neuroscience. Their stories have been vividly recorded in the book, "The Neuroscience: Paths of Discovery" (8). The recognition of the high degree of functional interdependence between nervous and endocrine system was also made in that period, but the phenomena that the hypothalamus might play a fundamental role in the maintenance of the normal function of the anterior pituitary, were noticed long before that time. In 1912, Aschner (9) reported that lesions in the hypothalamus result in gonadal atrophy. Similar observation were made by Camus and Roussy (10), Bailey and Bremer (11) and Smith (12). The question as to how the information flow passes from the central brain to the endocrine system became a burning issue.

One way in which this was deemed feasible at least at that time was a direct nervous control of endocrine cells by conventional synaptic transmission as in the control of the muscular contraction. In order to result in appropriate

responses, but unlike regular interneuronal or neuromuscular communication, simultaneous and continuous signals would have to be directed via these synaptic complexes. This would have to be directed to each or at least to most of the endocrine effector cells over periods of time. This kind of neuron effector junction with endocrine cells has never been demonstrated.

In 1930's, based on the examination of 200 human specimens, E. Scharrer described unusual nerve cells which contain granules and droplets of protein, which exhibit staining reactions and physical properties resembling those of colloid in organs such as the thyroid (1). These unusual nerve cells were found in the supraoptic nuclei and parventricular nuclei of the human hypothalamus late which was called the neurosecretory neurons. The neurosecretory neurons are neither ordinary nervous elements nor glandular cells, but a combination of both. The contents of these protein-rich droplets and the physiological significance of their existence in the neurosecretory neuron were unknown at that time.

The other piece of information available at that time was a knowledge of the hypophyseal circulation. Although the pituitary gland does not receive a major innervation from the hypothalamus, it is supplied by blood that comes directly from the hypothalamus via the portal-hypophyseal vessels. These

vessels begin in capillary loops that penetrate the median eminence of the hypothalamus and pass down the pituitary stalk to the anterior pituitary where they end in the sinusoids supplying the anterior pituitary cells (for review see ref. 13). Taking into consideration of these facts Harris (2, 14) and Green and Harris (15) proposed that the link between the hypothalamus and the anterior pituitary might be neurovascular. It had been assumed that the neurons of the hypothalamus produce some hypothetical substances essential for the pituitary, which might be carried by the portal circulation to the cells of the anterior pituitary. Later on, this hypothesis was proven to be correct by numerous experiments in particular the observations that extracts of hypothalamic origin have a direct effect on pituitary hormone secretion. Such an action was first shown for ACTH secretion and the active substance was called Corticotropic Hormone Releasing Factor, CRF (16-20). Further experiments revealed that beside CRF, hypothalamic extracts also contain other substances which stimulate the secretion of other pituitary hormones such as TSH (TRF) (21-25). LH (LHRH) (26-29), FSH (FRF) (30), and GH (GRF) (31, 32).

Thus, the linkage of the two systems, neuronal and endocrine systems was established. The concept that information from other brain centres is relayed to hypothalamic parvicellular secretory neurons which then secrete their hypophysiotropins into the

pituitary portal vasculature of the median eminence (ME) which in turn act on the pituitary gland to release pituitary hormones, has been firmly established (for review see ref. 5).

After the first full characterization of the thyrotropin-releasing hormone (33, 34), a number of anterior pituitary hormone-releasing factors have been isolated from the hypothalamus and characterized. They are all small polypeptides, consisting of 3 to 44 amino acids (for a review see ref. 35).

Although corticotrophin-releasing factor(s) (CRF) was the first hypothalamic substance to be detected in the hypothalamic extracts in 1955, the discovery of 41-amino acid residue ovine CRF was made more than two decades later by Vale and co-workers (36). Familari *et al.* (37) recently demonstrated that arginine vasopressin was a much more likely candidate CRF in the ovine than the 41 amino acid ovine CRF. Nevertheless, a large body of evidence supports the concept that the secretion of ACTH is regulated by a constellation of hypothalamic factors secreted into the hypophysial portal circulation. Among them, CRF and arginine vasopressin play predominant roles in response to a variety of stressors (for review see ref. 38). The control and regulation of ACTH secretion by neuron and hypothalamic factors will be discussed in the following sections.

1.3. Neural Control of ACTH Secretion

Much work, during the past several decades, has been devoted to the elucidation of the neural pathways, neurotransmitters, hypothalamic factors and control mechanisms involved in the regulation of pituitary-adrenocortical responses to stress. However, many controversies still remain, which have arisen from the use of pharmacological tools for investigating the nature of the neurotransmitters involved in the control of ACTH secretion. The use of different forms of stress, each being, not only possibly but also likely, mediated by distinct neuroanatomical pathways or structures. The use of different species of animals, some of them being quite discrete from others, has given rise to numerous inconsistencies in the results obtained. The absence of a proper method to investigate the electrophysiological properties of secretory neurons and to see how the signals from different afferent inputs that are activated by stressful stimuli are processed with reasonable efficiency, has made the research more difficult and slower. The existence of different time-related feedback mechanisms might have been at the origin of some of these inconsistencies in studies using different duration of stress.

Most of the information available so far was obtained by surgical lesioning or electrical stimulation of anatomically

defined pathways to the ME, immuno-neutralization of CRFs *in vivo*, or administration of receptor antagonists of CRFs, or measurement of ACTH in plasma or measurement of CRFs in pituitary portal blood, or immunocytochemical studies together with various systemic and neurogenic stressful paradigms (39). The stressful paradigms used have been emotional immobilization stress (40-42), audiovisual stimulation (43-45), insulin induced-hypoglycemia, (42, 45-50), hemorrhage (51-56), hypothermia (57, 58), ether stress (59-62), ether laparotomy (63), formalin injection (57), trauma (64-67) and immuno challenge, (6, 68, 69).

Among the pathways currently under investigating, those responsible for hemorrhage-induced ACTH secretion have been mapped in the greatest detail especially in cats by Gann and co-workers. (For a detail discussion of these pathways, the reader is referred to the references listed above).

Although it has been speculated for a long time that neurons in the hypothalamic paraventricular nucleus (PVN) mediate the stress-induced ACTH secretion (70, 71), technically neither electrolytic lesions nor electrical stimulation exclude the possibility that fibres that pass through the PVN, but not the cell bodies in the PVN may mediate the stress-induced ACTH secretion. Motton *et al* (72) with the aid of ibotenic acid, a neurotoxin analogue of glutamate, which has the ability to

selectively destroy cell bodies while leaving fibers of passage intact, demonstrated that neuronal cell bodies in the hypothalamic paraventricular nucleus mediate stress induced increases of corticosterone in rats by scrambled current (0.8 mA DC) shock.

1.3.1 CRF

Immunohistochemical studies indicate that the PVN contains of the order of 2,000 CRF cells (73), and that lesions of this nucleus virtually eliminate CRF-immunoreactive terminals from the median eminence. CRF neurons are found in all eight major parts of the PVN, but a large proportion are concentrated in the dorsal medial parvicellular part (PVNmpd) (73). The CRF peptides are also present in widespread regions of the brain as well as some non-brain tissues (74-80).

In contrast to the immunocytochemical studies, RNA blot analysis (81) revealed that the highest concentration of CRF mRNA is present in the brain stem of rat brain but not in the hypothalamus. CRF mRNA can also be detected in the cerebral cortex, midbrain, striatum and hippocampus and other non-brain tissues, such as adrenal gland, spinal cord, testis, and pituitary gland, although it cannot be found in the cerebellum. Although the concentration of CRF mRNA does not correspond to the levels of CRF immunoreactivity, in general, the presence of CRF mRNA correspond to the presence of immunoreactive CRF.

Nevertheless, the numerous CRF-reactive cells in other hypothalamic regions and in tissues other than PVN are apparently not hypophysiotrophic, and their possible functions remain unknown. In supporting the suggestion that neurons in the parvicellular subdivisions of the PVN mediate the stress-induced ACTH secretion (70, 71), the studies on adrenalectomized rats show that adrenalectomy markedly enhances the staining for CRF in the parvicellular subdivisions of the PVN, but not elsewhere in the brain (82-86). In rats, CRF is the major physiological mediator of the hypothalamic control of ACTH secretion (87, 88).

There are two types of parvicellular CRF neurosecretory cells projecting into the portal capillary plexus in the external zone of the median eminence; the vasopressin-containing and vasopressin-deficient cells (89-91). The vasopressin but not CRF neurons were also found but were less than 1% of the parvicellular axons (90). The perikarya of the vasopressin-containing CRF neurons, expressing vasopressin precursor, possess different topographical distribution from the vasopressin precursor-deficient cells, and in rats they are approximately equal in number (92). Vasopressin is copackaged with CRF in the same secretory vesicles in vasopressin-containing CRF cells (89, 91-93). Very recently Whitnall (42) using a functional ultrastructural assay, demonstrated that one hour immobilization of the rats resulted in 47% depletion of

secretory vesicles from the vasopressin-containing CRF axons in the external zone of the median eminence but had no effect on the vasopressin-deficient CRF varicosities. Insulin-induced hypoglycemia caused a 48% depletion of secretory vesicles from vasopressin-containing CRF axons also had no significant effects on vasopressin-deficient CRF axons, but the injection of 100 μg colchicine (a axonal transport blocker which will activate hypothalamo-pituitary-adrenal axis at high doses) into the lateral ventricle caused 51% and 55% depletion of secretory vesicles in both vasopressin-containing and vasopressin-deficient axons respectively. These results suggested that vasopressin-containing CRF neurons and vasopressin-deficient CRF neurons are regulated by different afferent axonal projections. Differential activation of these components modulates the ratio of CRF and vasopressin in portal blood during stress. The beauty of this kind of functional ultrastructural assay is that it avoids any surgical operation that in itself might be stressful, and this was the first unambiguously demonstration of the sources of the measured peptides in the portal blood circulation. It is worth mentioning that the results obtained by Plotsky (49, 94) and cited in several influential reviews (38, 95) showing rises in portal vasopressin but no change in portal CRF during insulin-induced hypoglycemia are inconsistent with the recent observation obtained by Whitnall, and also

inconsistent with the results of Berkenbosch *et al.* (96) and Guillaume *et al.* (50). The main point which Plotsky tried to emphasize is correct, that is, the CRF containing neurons and vasopressin containing neurons are under different control. Sometimes significant difference can even be observed in the same experiment on individual animals. This is the case in the recent report from Engler, *et al.* (45). Their studies were undertaken to characterize the secretion of CRF and vasopressin into the hypophysial-portal circulation of the conscious sheep. In the experiment, five sheep were used and each of them had their own response pattern to the audiovisual and insulin stress (Fig. 1-1), though the overall tendency in increasing the secretion of vasopressin and CRF under the stress were same. Ultrastructural studies in sheep are not available. The marked alteration of the CRF:vasopressin molar ratio during stress in these five sheep suggest that the percentage of vasopressin containing CRF neurons or the amount of vasopressin copacked with CRF in the secretory vesicles is greater in general than that in rats and varies in individual animals. These results support the idea that these two types of CRF neurons are regulated by different afferent axonal projections.

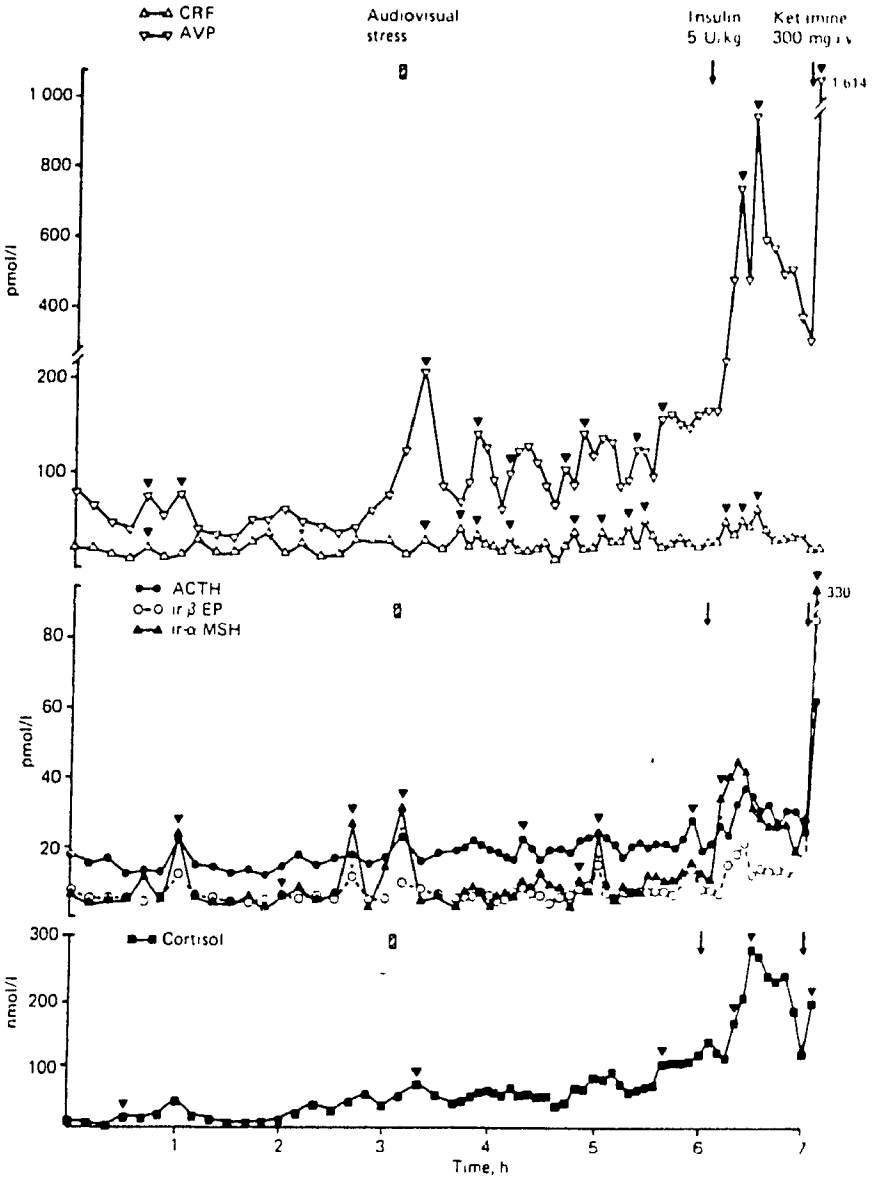

CRF
AVP
Audiovisual stress
Insulin 5 U/kg
Ketamine 300 mg i.v.
1 614
1 000
800
600
400
200
100
pmol/l
ACTH
ir β EP
ir α MSH
330
80
60
40
20
pmol/l
Cortisol
300
200
100
nmol/l
1
2
3
4
5
6
7
Time, h

Fig. 1-1a

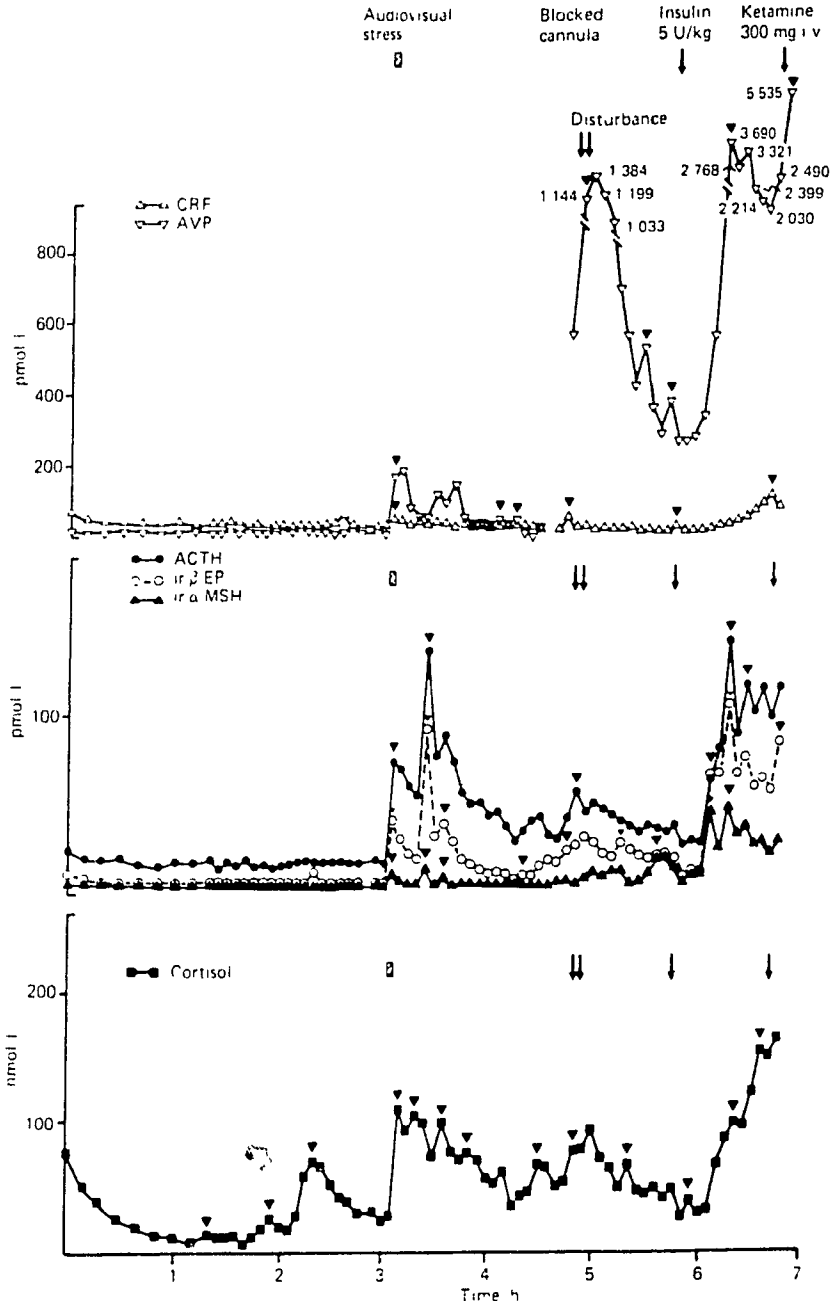

Audiovisual stress
Blocked cannula
Insulin 5 U/kg
Ketamine 300 mg i v
Disturbance
5 535
3 690
3 321
2 768
2 490
2 399
2 214
2 030
1 384
1 199
1 144
1 033
CRF
AVP
800
600
400
200
pmol l
ACTH
ir β EP
ir α MSH
100
pmol l
Cortisol
200
100
nmol l
1
2
3
4
5
6
7
Time h

Fig. 1-1b

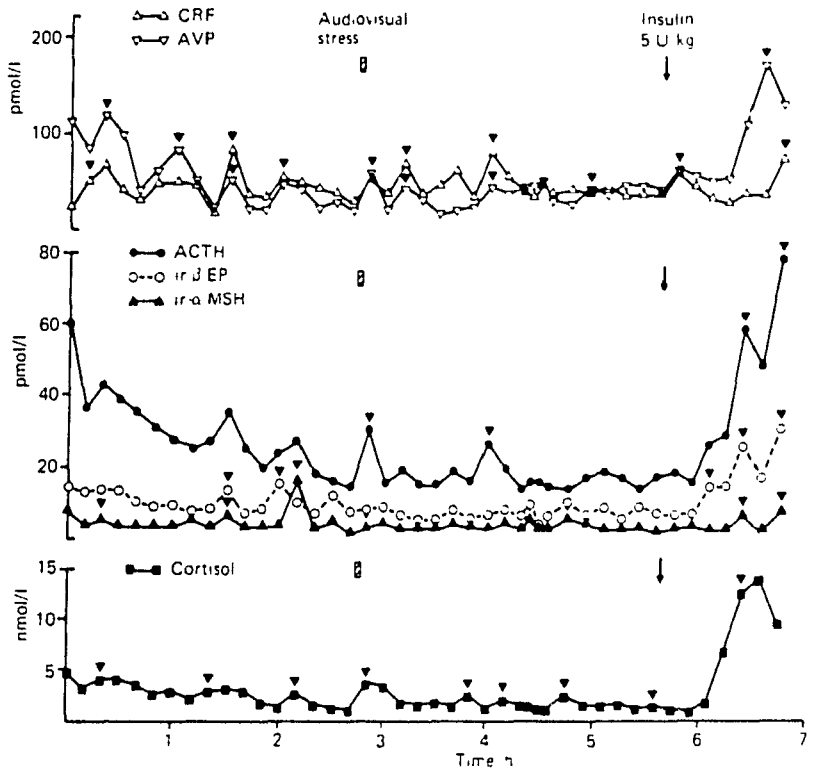

CRF
AVP
Audiovisual stress
Insulin 5 U kg
200
100
pmol/l
ACTH
ir-β EP
ir-α MSH
80
60
40
20
pmol/l
Cortisol
15
10
5
nmol/l
1
2
3
4
5
6
7

Fig. 1-1c

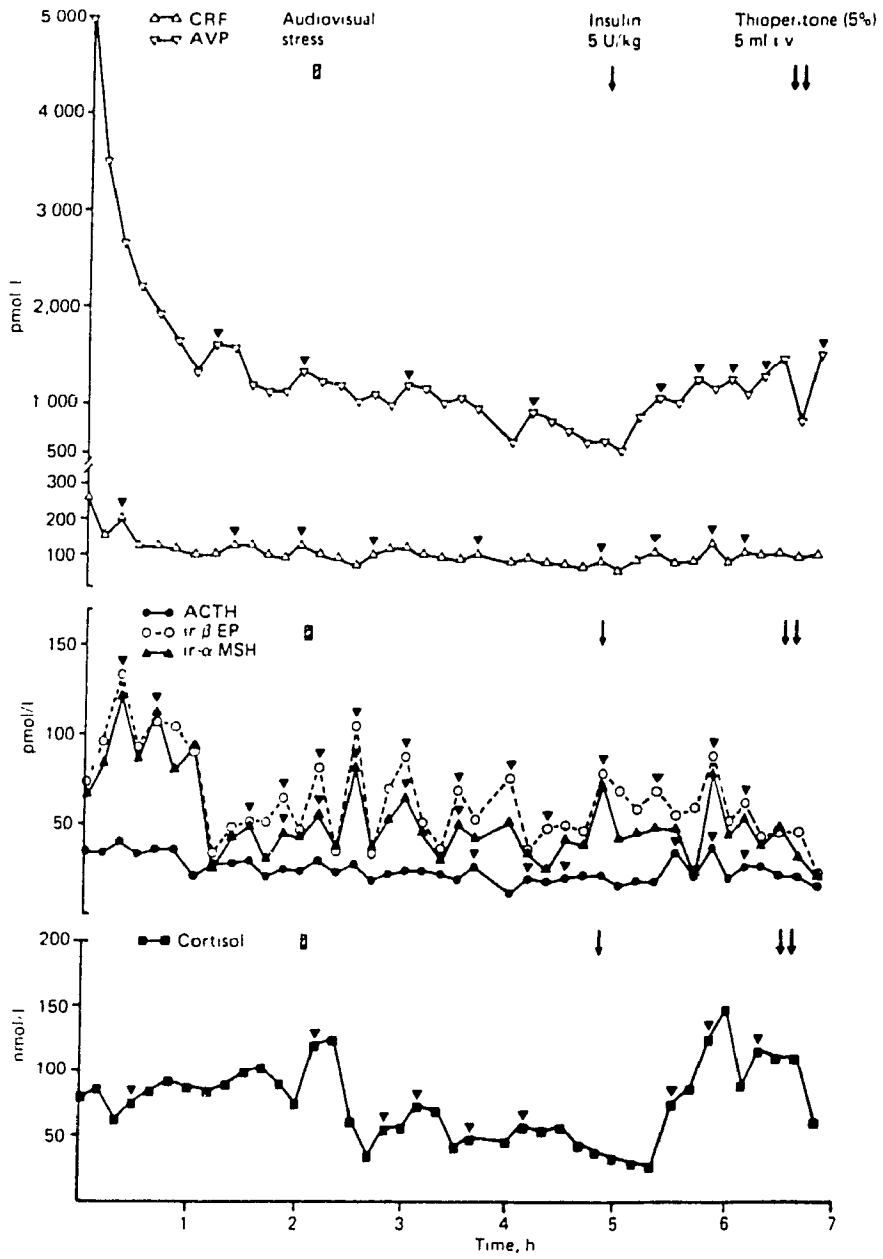

CRF
AVP
Audiovisual stress
Insulin 5 U/kg
Thiopentone (5%) 5 ml i.v
5 000
4 000
3 000
2,000
1 000
500
300
200
100
pmol l
ACTH
ir-β EP
ir-α MSH
150
100
50
pmol/l
Cortisol
200
150
100
50
nmol·l
1
2
3
4
5
6
7
Time, h

Fig. 1-1d

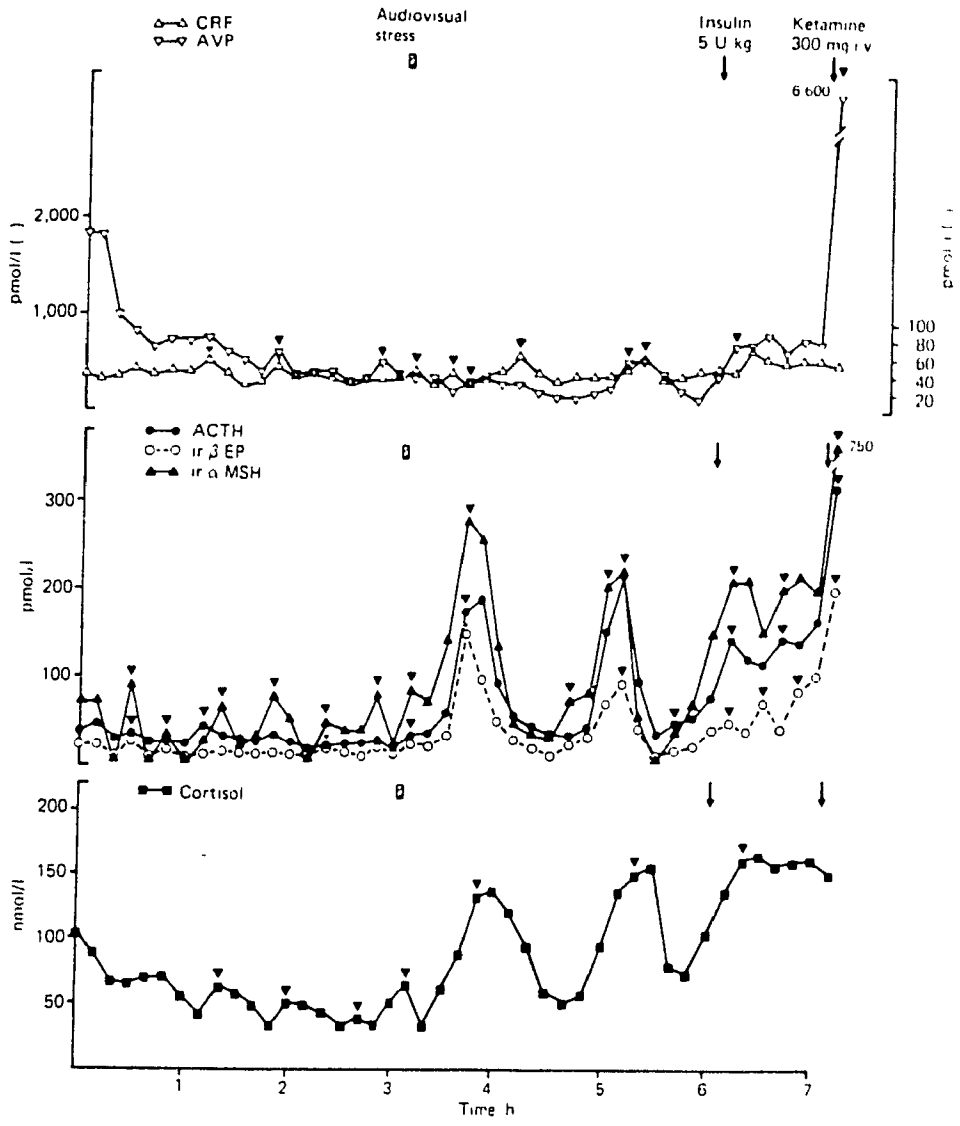

CRF
AVP
Audiovisual stress
Insulin 5 U kg
Ketamine 300 mg i v
6 600
2,000
1,000
pmol/l
100
80
60
40
20
ACTH
ir β EP
ir α MSH
300
200
100
pmol/l
750
Cortisol
200
150
100
50
nmol/l
1
2
3
4
5
6
7
Time h

Fig. 1-1e

Fig. 1-1. The secretion patterns of CRF, AVP, three POMC peptides (ACTH, ß-EP, and α-MSH), and cortisol in 5 conscious sheep (a-e). Shown also are the secretory responses to a 3-min audiovisual emotional stress (hatched area) and insulin-induced hypoglycemia (arrow). (copied from reference 45)

1.3.2 CRF and Vasopressin

One recent important observation is that CRF may not be the predominant stimulus to ACTH release (or synthesis) in all species. In sheep, arginine vasopressin is a more potent stimulus to ACTH release than ovine CRF both *in vitro* (37) and *in vivo* (98). In humans arginine vasopressin but not CRF is a potent stimulator of ACTH following electroconvulsive treatment (99). In bovine pituitary cell preparations, CRF and vasopressin appear to have equal potency (100). In duck anterior pituitary cells, neither vasopressin nor CRF but vasotosin function as the principal secretagogue (101).

As mentioned previously adrenalectomy stimulates CRF synthesis in the parvicellular subdivisions of the PVN. The vasopressin-deficient subset of CRF neurons expresses vasopressin after adrenalectomy (92). Interestingly, both CRF and vasopressin show biphasic changes after adrenalectomy, a marked depletion of CRF and vasopressin from the fibres in the external lamina of the ME by about 24 h after surgery (102), and a replenishment of stores after a few days (103). However the replenishment of vasopressin occurs faster and to a greater degree than that of CRF (104). The studies on the isolated ME demonstrate that the release of CRF from the isolated ME decreased by 40%, 4 days after adrenalectomy and increased by 60% at 4 weeks, while the release of vasopressin rose by 150%

and 500% at 4 days and 4 weeks after surgery, respectively. The ratio of vasopressin to CRF release by the ME, increased from approximately 2:1 in control animals to 9:1 in adrenalectomized rats (104). It appears that CRF functions in a permissive role, while vasopressin is the dynamic mediator of ACTH secretion (58, 49).

CRF and vasopressin serve as primary regulators of ACTH secretion, but there are several other factors (for review see ref. 38, 105) including serotonin (106), adrenalin (107, 108), oxytocin (109), angiotensin II (110-112) which play a role. These factors are less effective than CRF and vasopressin in term of stimulation of ACTH release, but they show synergism with CRF. Vasopressin and CRF potentiate each other's actions. The mechanism of action of the secretagogues involved in the control of ACTH secretion will be discussed in the pituitary section.

1.3.3. Regulation of CRF Neurons

No matter which secretagogue, CRF or vasopressin, is the dominant component in stress-induced release of ACTH, the hypothesis that CRF neurons in the PVN play predominant roles in the process has been wildly accepted. There is a large variety of kinds and sources of fibres that may influence the activity of CRF cells in the PVN (113). The exact role of limbic

structures and low brain stem and the nature of the neurotransmitter used in each of the central nervous system nuclei participating in the control of CRF neurons or ACTH secretion is still to be resolved. The majority of afferents arises either from the limbic system (subiculum, amygdala, septum) or from the lower brain (raphe nuclei, locus caeruleus, parabrachial nucleus, nucleus of the solitary tract, reticular formation (114). The amygdala, particularly the central amygdaloid nucleus has been shown to be important for the hypothalamic-pituitary-adrenal axis (41, 44, 115-120). Interestingly, Beaulieu *et al* (121) recently demonstrated that a lesion of the central nucleus of the amygdala provoked a striking decrease of the content of CRF-like immunoreactive material at both anterior and posterior levels of the external zone of the ME. Very recently, the direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus was found by the antero-grade tracing method (122) using the *Phaseolus vulgaris* leucoagglutinin lectin. Besides these, there are two other important series of evidence to support the hypothesis that the amygdaloid central nucleus is one of the major limbic structures controlling CRF neurones of the PVN. First, the amygdala is one of few sites in the brain in which corticosteroids down-regulate their own receptors (123), and second the central nucleus of the amygdala of ovine brain

contains a high concentration of CRF-like material (124). CRF has been demonstrated as a brain neurotransmitter involved in mediating the response of locus ceruleus to hemodynamic stress (125), and CRF neurons have also been shown to interact with other CRF neurons and with certain parvi- and magnocellular neurons in the PVN (86, 126). Therefore it is possible that CRF neurons within the amygdala could directly innervate CRF neurons within the PVN.

Some other neurotransmitters have been directly or indirectly implicated in the regulation of CRF neurons within the PVN. These are the catecholamines (62, 127-129) , histamine (130, 131), serotonin (132, 133), ACTH (134, 135), neuropeptide Y (136), angiotensin II (137) dopamine (138), Noradrenaline (139-142), adrenaline (142, 143), serotonin (144, 145).

CRF cells are also under peripheral control, mainly by the final products of hypothalami-pituitary-adrenal axis, the glucocorticoids, and possibly by some factors from the immune defence system such as IL-1. These will be discussed later in the section 1.6.2. on the immunoregulatory feedback of the HPA axis.

Glucocorticoids have very potent negative feedback effects on the hypothalamo-pituitary-adrenal axis. Pharmacological studies suggest that these feedback mechanisms have about three recognisable different time domains, fast-feedback (few min),

early (1-2 h) and late (≤ 24 h) delayed-feedback inhibition (for a review see ref. 146). Fast feedback appears to be controlled by a different receptor mechanism than the early or late delayed-feedback systems. Of the corticosteroids that have been tested, only corticosterone and cortisol and the synthetic analog, dexamethasone, show a fast feedback effect (147, 148).

There are probably three or more sites at which glucocorticoids act to modulate ACTH secretion, namely the limbic structure (the hippocampus and amygdala), the hypothalamus, and the anterior pituitary (for a review see ref, 149). The hypothesis that glucocorticoids preferentially act at the CRF neurons of the PVN is supported by the following. First the glucocorticoid feedback inhibition still remained when the hypothalamus was surgically isolated from the rest of the brain (150); second, graded doses of corticosterone will, within 30 min of intraperitoneal injection in conscious, freely moving rats, decrease multiple-unit activity in the parvicellular part of the PVN in response to stimuli of photic and acoustic stimulation (151); third after lesioning the paraventricular nucleus (60) or using Halasz knife lesions to destroy CRF and vasopressin input to the anterior pituitary (152), POMC mRNA levels in anterior pituitary decrease in the adrenalectomized rats; fourth, adding back increasing levels of corticosterone to lesioned adrenalectomized rats had no significant effect on

the POMC mRNA levels in the anterior pituitary (152), and finally only at very high doses do corticosteroids have significant feedback effects at the pituitary level (153). However, this evidence does not rule out the possibility that the inhibitory effects of glucocorticoids on anterior pituitary *in vivo* require the presence of some unknown hypothalamic factor(s). However CRF neurons in the PVN do contain glucocorticoid receptors (154-157), and withdrawal of endogenous glucocorticoids after adrenalectomy has been shown to increase hypothalamic CRF activity (158) and hypophyseal-portal concentrations of CRF (159-162), whereas exogenous corticosteroids inhibit CRF release from the hypothalamus *in vitro* (163) and into the hypophyseal-portal blood *in vivo* (161-162). Similarly, adrenalectomy increases hypothalamic CRF mRNA (164, 165).

The synthesis of CRF and vasopressin appear to be segregated in different functional states, being regulated by both neuronal input and glucocorticoids. As mentioned previously, vasopressin expression is suppressed in vasopressin-deficient CRF neurons in the presence of normal circulating levels of glucocorticoids, and at high concentrations of glucocorticoids, CRF and vasopressin expression is suppressed in both subsets of CRF cells. It is important to note that the lesion of the central nucleus of the amygdala caused a significant decrease of CRF in

the ME. However the regulation of CRF (or vasopressin ?) by neuronal input and glucocorticoids are accomplished by independent mechanisms. Using *in situ* hybridization histochemistry technique, Lightman *et al.* (166) recently demonstrated that after adrenalectomy, even when CRF mRNA had reached peak levels, stress still further increased CRF mRNA, and large doses of dexamethasone administration in the fast-feedback or early delayed-feedback time domains had no effect on CRF mRNA response to stress. In the late delayed-feedback time domain dexamethasone reduced basal CRF mRNA levels, however it had no or slight effect on CRF mRNA response to stress. The exact mechanisms of the control of CRF synthesis and release remain unknown.

1.4. Mechanism of Pituitary ACTH Secretion

The pituitary in contrast to the adrenal stores the hormone formed therein and requires hypothalamic releasing hormones to make the pituitary hormone available to the circulation on demand. The anterior pituitary of the adenohypophysis is the source of several peptides and protein hormones. ACTH-containing cells (corticotrophs) account for 2 to 3% of the cell population in normal rats, and 8-10% of cell population in adrenalectomized rats (167, 168). It is not clear whether there is any similarity

in the mechanisms of the regulation of ACTH expression by glucocorticoids in the pituitary and the mechanisms that regulate vasopressin expression in the CRF cells in the PVN of the hypothalamus. The pituitary appears to be a common pathway for all hypothalamic secretagogues affecting glucocorticoid secretion. However, the population of ACTH cells still appears to be heterogeneous. Using the reverse haemolytic plaque assay technique on single cell studies Leong (169) demonstrated that each corticotrope was endowed with its own activation threshold for CRF. That is, each individual ACTH cell requires specific minimal concentrations of CRF to release ACTH. A prominent feature of concentration-response curves is the recruitment of ACTH cells into the secretory pool. The mechanism underlying the phenomenon is unknown.

Specific, high affinity ($K_d \approx 10^{-9}$ M) receptors for CRF have been identified in the anterior pituitary (170-175). The carboxyl-terminus of CRF is critical for its biological activity (36). The minimal sequence required to retain full CRF activity is 15-41, however, the ED_{50} of CRF_{15-41} is much higher than CRF_{1-41} (170). The binding of CRF to its receptors activates adenylate cyclase (176) through G_s (stimulatory GTP-binding proteins) (172, 175, 177, 178) causing an increase in the intracellular concentration of cAMP (178, 179) and thereby enhancing the activity of cAMP dependent protein kinase. The requirement for

cAMP-dependent protein kinase in mediating the effects of CRH has been directly demonstrated by Reisine and co-workers (180) by incorporation of cAMP-dependent protein kinase inhibitor (PKI, the Walsh inhibitor) into AtT20 cells (a clonal mouse anterior pituitary corticotroph tumor cell line) by liposome fusion. Cells receiving PKI did not respond to CRF or 8-bromo-cAMP with increased ACTH secretion. The substrates for the cAMP-dependent protein kinase have not been characterized, but there is some evidence suggesting that the CRF-induced cAMP signal must be transduced into a Ca^{2+} signal to initiate ACTH release. Since calcium ions enter corticotrophs via channels it is possible that voltage-gated calcium channels (151, 181, 182) are required for ACTH release. Therefore the calcium channel of the plasma membrane might be one of the substrates for the cAMP-dependent protein kinase (169, 183, 184). In support of this hypothesis, Leong demonstrated, with the single cell studies, that CRF or Forskolin or 8 bromo-cAMP treatment rapidly (3-50 seconds) stimulated an increase of cytosolic calcium concentration, and high concentrations of the calcium channel antagonist, Verapamil, can completely abolish both CRF- and Forskolin-induced calcium rises and ACTH secretion (169).

The receptor mediating vasopressin action in the pituitary gland (185, for review see ref. 186) is a different type of receptor from the pressor V_1 type vasopressin receptor of the

vascular smooth muscle or hepatocyte, but both receptors have many properties in common (187). One of the recognizable differences between these two kinds of receptors is their binding characteristic and consequential biological response to some vasopressin analogs. Some pure, potent antagonists of vasopressin action on the pressor V_1 receptor, are not antagonists but weak agonists in stimulating ACTH release (185, 188-190). Binding studies indicated the presence of a vasopressin receptor (V_3) with a single site with high affinity ($K_d \approx 10^{-10}$ to 10^{-9}) and low capacity (V_{max} = 100-300 fmol/mg protein) (87, 191-193). Vasopressin enhances the breakdown of inositol phospholipids in primary cultures of pituitary cells (194) and has no effect on cAMP accumulation, so it appears to stimulate ACTH release via the cAMP-independent protein kinase-C system with mobilization of intracellular calcium and activation of protein kinase C (194-198). There is some evidence indicating that cGMP is involved in the vasopressin-induced ACTH secretion (199).

It has been speculated that oxytocin is a corticotrophin releasing factor, but the data supporting this is controversial. In the rat, oxytocin appears to be a weak vasopressin agonist and a low affinity ligand for pituitary vasopressin receptors (185, 200). However recently, a high affinity uterine type oxytocin receptor has been identified in rat pituitary (201),

but whether this high affinity receptor of oxytocin is expressed by corticotroph cells is questionable. In fact, it appears to be unlikely that this uterine type oxytocin receptor is responsible for the ACTH secretion but more plausibly, it may be responsible for PRL secretion (201-204)

Other factors such as catecholamines, angiotensin II, cholecytokinin (CCK-8), also do not act through adenylate cyclase dependent pathways but probably through phosphotidylinositol pathways like the vasopressin pathways (194-197).

As mentioned in the previous section, there are two subsets of CRF cells in the PVN of hypothalamus, vasopressin-deficient and vasopressin-containing cells and they are under different afferent control. We also already know that the amount of CRF to vasopressin and especially, the ratio of CRF and vasopressin in the ME will change from about 1:2 in normal rats to about 2:10 in the stressed or adrenalectomized animals. The question as to how these effect the secretion and synthesis of ACTH in the corticotrophs is not known.

The inter-relationships between the CRF stimulation and vasopressin stimulation appear to be complex. Priming (205, 206), potentiation (190, 206-212), and desensitization (206) have all been documented (for a review see 146), but some of the observations are controversial. The potentiating interaction has

been repeatedly studied because of its obvious physiological significance. However the degree of potentiation claimed in individual reports differ greatly from no effect at all to several fold enhancement (206, 208, 210, 211). The mechanism(s) responsible for the potentiation is not fully established, although some investigators speculate that it might be in part related to the cytosolic calcium concentration (169), or in part related to the enhanced accumulation of cAMP caused by activation of adenylate cyclase and inhibition of phosphodiesterase (213).

The elegant detailed kinetic analysis of ACTH secretion in a microperfusion system by Watanabe and Orth (214) offer us some insight as to the mechanism of CRF and vasopressin interaction in a relatively short period time (ten min). The priming effect can only be observed when vasopressin was perifused within 30 sec after CRF was stopped (the time may be variable with doses of CRF). The priming effect of vasopressin on CRF reported previously (205, 206) might be due to contamination of vasopressin in the tissue when transferred to new incubation medium. The desensitization for vasopressin happened much faster than CRF but was only restricted to itself. Of interest is the ACTH releasing patterns upon stimulation by CRF (Fig. 1-2), or vasopressin (Fig. 1-3) alone, CRF plus vasopressin and CRF followed by vasopressin (Fig. 1-4). ACTH secretion was observed

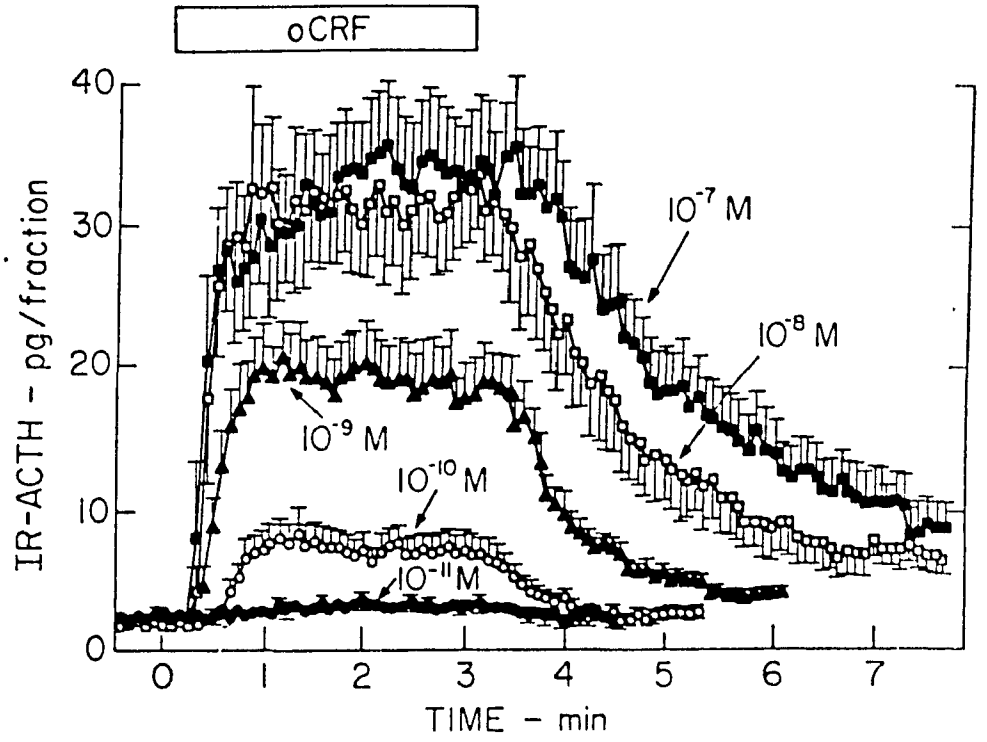


Fig. 1-2. ACTH secretory responses to increasing concentrations of oCRF. Two identical chambers were each loaded with $6x10^6$ cells and perfused with 10^{-11}-10^{-7} M oCRF for 3-min periods are 50-min intervals. Each chamber was perfused twice with each of five different concentrations of oCRF in random order. Five-second (6μl) effluent fractions were collected in tubes containing 494 μl RIA diluent. The concentrations of infused oCRF are indicated. The *box* indicates the period during which oCRF was infused into the chamber, beginning at time zero; each point represents the mean of four determinations; the brackets indicate the SEM (copied from reference 214, Fig. 6).

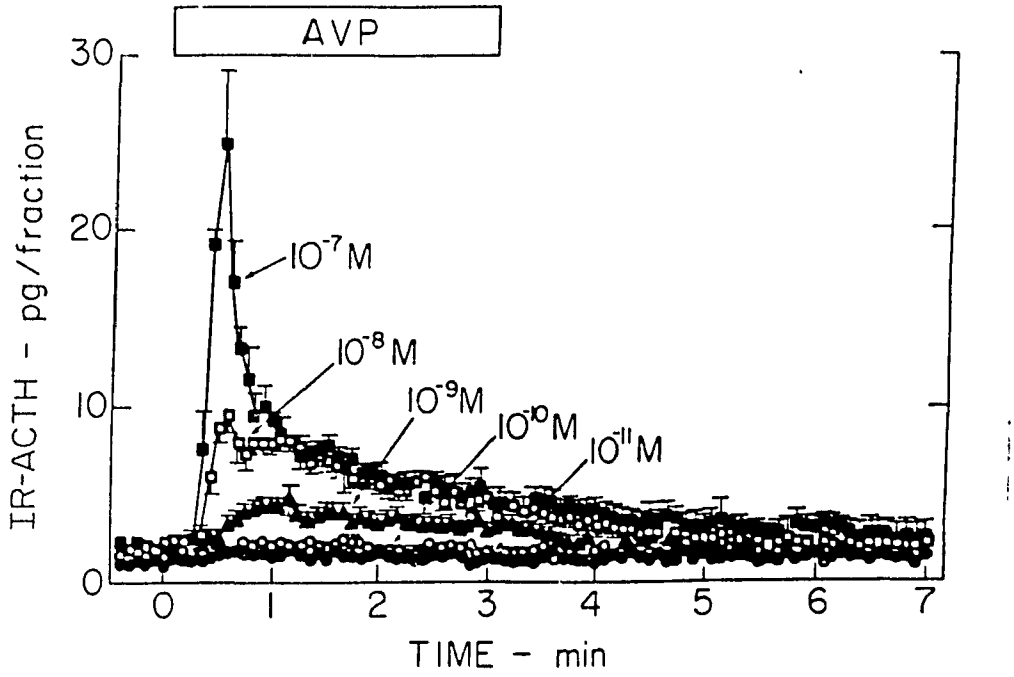


Fig. 1-3. ACTH secretory responses to increasing concentrations of AVP. Two identical chambers were each loaded with $6x10^6$ cells and perfused with 10^{-11}-10^{-7} M AVP for 3-min periods are 50-min intervals. Each chamber was perfused twice with each of five different concentrations of AVP in random order. Five-second (6μl) effluent fractions were collected in tubes containing 494 μl RIA diluent. The concentrations of infused AVP are indicated. The *box* indicates the period during which AVP was infused into the chamber, beginning at time zero; each point represents the mean of four determinations; the brackets indicate the SEM (copied from reference 214 Fig. 7).

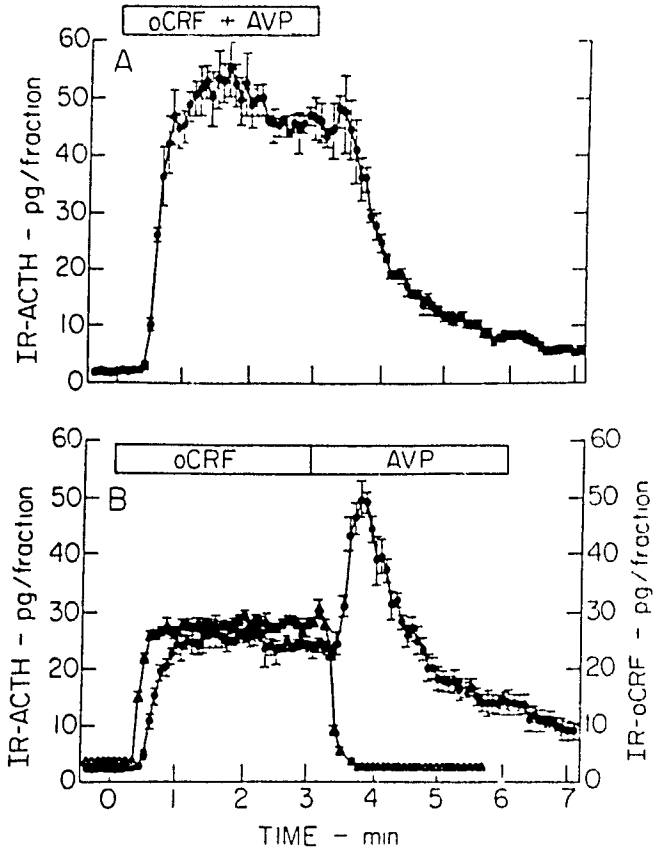


Fig. 1-4. Kinetics of the synergistic effect on ACTH secretion of oCRF and AVP. Two identical chambers were each loaded with $6x10^6$ cells and perfused for 3-min periods with 10^{-9} M oCRF and AVP simultaneously (A) and consecutively (B) at 50-min intervals in random order. Five-second (6μl) effluent fractions were collected in tubes containing 494 μl RIA diluent. o IR-ACTH; ▲ IR-oCRF; , IR-oCRF undetectable (< 2.5 pg/fraction or < 8.9 x 10^{-11} M). Each point represents the mean of four determinations; the *brackets* indicate the SEM (copied from reference 214 Fig. 11).

within 5 sec of exposure to either secretagogues and reached a maximum within 20-40 sec. ACTH secretion remained constant for as long as CRF was perfused (3 min) and then fell gradually toward the basal level. In contrast to CRF stimulation, ACTH secretion fell progressively toward basal levels despite continued vasopressin perfusion (3 min). Simultaneous perfusion of CRF and vasopressin resulted in an CRF-like response of greater magnitude whereas sequential perfusion of CRF followed by vasopressin resulted in the usual plateau response to CRF followed by the initial spike response characteristic of very high concentrations of AVP alone and subsequent rapid decrease in secretion despite continued perfusion of vasopressin. Whether this kinetic behaviour in the rat will be the same in other species such as the ovine is unknown, even though fundamental difference in intracellular mechanisms is unlikely in these two species.

Most of the studies on the potentiation of CRF and vasopressin were carried out from a few seconds to minutes with a maximum of 3 to 4 hours. That is, these studies were concerned more with the ACTH release pattern than with ACTH synthesis. Recently Suda *et al* (215) reported that after 15 h coincubation of CRF with vasopressin and other protein kinase-C-related secretagogues neither had effect on the CRF-induced POMC mRNA synthesis nor has potentiation effects on the release

of ACTH. In all experiments, cAMP can mimic CRF action in all aspects.

According to these observations we can speculate as follows: the potentiating effects of vasopressin and other secretagogues on CRF induced ACTH release do not act primarily by increasing cytosolic cAMP levels since exogenous cAMP derivatives can only mimic CRF action and can not increase the response of anterior pituitary cells to CRF stimulation. Furthermore if modification of adenylate cyclase and inhibition of phosphodiesterase (198) by vasopressin is mainly responsible for its potentiation, the priming effect by vasopressin should be able to be detected in Watanabe and Orth's system (214). However they only detected the priming effect of CRF to subsequent vasopressin perfusing but not the priming effect of vasopressin to CRF. Therefore the increases of cytosolic cAMP levels might be the consequence of vasopressin's potentiating effect but not the main causes of its potentiation of ACTH secretion. A similar argument can be made against the cytosolic Ca^{2+} acting as the key factor in vasopressin potentiation (169).

The mechanism of vasopressin potentiating effect is more likely to be a change in the activity of the cell. Changing the activity of cells mainly has the following consequences; a decrease threshold of each corticotrope to CRF and acceleration of the exocytosis process of ACTH secretory granules but having

no effect on the biosynthesis of ACTH which is regulated by CRF through cAMP dependent processes. The biggest advantage of this hypothesis is that it can explain most of the experimental results. This hypothesis implies vasopressin is a pure dynamic secretagogue and CRF is a basic and permissive modulator of ACTH synthesis and release. The activity of a cell depends on many parameters such as the physiological condition of the animals used, the temperature and the method of handling. If the experimental protocols and animals in the individual studies are not exactly the same, a significant difference in the degree of potentiation by vasopressin will be expected. The first strong peak of response of corticotropes to vasopressin stimulation (Fig. 1-3) is caused by accelerating the process of exocytosis of ACTH secretory granules. When these readily releasable pools of secretory granules have been exhausted, ACTH release decreases very rapidly, but in the presence of CRF, the secretion of ACTH can continue for a much longer period of time. This was speculated to be the cause of the potentiating effect of CRF for vasopressin stimulation. Vasopressin decreases the threshold response of corticotropes to CRF, that is, it increases the recruitment of ACTH cells into the secretory pool resulting in enhanced cAMP formation and subsequent potentiated ACTH secretion. Since vasopressin is only a dynamic secretagogue, it has no or little effect on ACTH synthesis,

therefore it has no detectable potentiating effect during the longer incubation times.

CRF has been shown to have a direct stimulatory effects on POMC mRNA accumulation in both AtT20 cells (216, 180) and on anterior and intermediate lobe pituitary cells in dispersed primary cell culture (217, 218). Furthermore, CRF was shown to elevate the level of the POMC primary transcript (hnRNA) in AtT20 cells. Using *in situ* hybridization histochemistry techniques, Fremeau *et al* (219) demonstrated that changes in levels of primary transcript occur before changes in cyto plasmic mRNA using an IVS-A probe in the postadrenalectomy period. They also suggested that these increases in the tissue as a whole may be due to increases both in the POMC gene transcription rate per cell and in increases in the detectable number of cells transcribing the POMC gene.

ACTH and a variety of biologically active, important neuromodulators and endocrine peptides are derived from the same precursor namely POMC. The POMC molecule is post-translationally processed to different biologically active peptides in corticotroph cells and in melanotroph cells of the intermediate lobe, with ACTH ß-lipotropin, and ß-endorphin being the major end products in the corticotroph cells and αMSH, corticotrophin-like intermediate lobe peptide, and acetyl-ß-endorphin in the melanotroph (220). The POMC gene is also expressed in a variety

of tissues in the mammal, including the hypothalamus, amygdala, cerebral cortex, midbrain, cerebellum, of the brain (221), adrenal medulla (221, 222), spleen macrophages (223), ovary and testes (224-226). Genomic DNA fragments containing POMC encoding sequences have been isolated and sequenced from human (227), rat (228, 229), cow (230), and mouse (231, 232). Despite the complexity of POMC gene products and their regulation, only one functional gene has been identified per haploid genome. The mouse contains a non-expressed pseudogene, possibly a product of a reverse transcription event such as transposition (231, 232).

Although it has been proven that the hypothalamus is the major site for negative feedback action of glucocorticoids *in vivo*, there is substantial evidence, specially *in vitro*, suggesting that glucocorticoids also act at the pituitary level. The precise role of glucocorticoids in modulation POMC derived peptide release processes and POMC gene expression in the corticotroph is not understood. This is in spite of the known genomic actions of glucocorticoids through transcriptional activation in some other systems which has been extensively described (233). There is a latency of 2 hr (168) to 4 hr (234) for glucocorticoids to elicit their inhibitory effect on POMC mRNA levels. Glucocorticoid inhibition of stimulated POMC-peptide release in anterior pituitary cultures was shown to be

biphasic; acute effects on stimulated secretion and biosynthesis and the chronic inhibitory effects of longer term treatment. This biphasic inhibition may be mediated by different mechanisms (235). The rapid inhibition (10-20 min) (236) by glucocorticoids of CRF-stimulated secretion is not dependent on new protein synthesis but requires significantly higher concentrations of glucocorticoids. In the late phase inhibition of CRF-stimulated release there is a dependency on new protein synthesis (235). Corticosterone inhibition of 8-bromo-cAMP-stimulated, PMA-stimulated, and dioctanoyl glycerol-stimulated release showed the same biphasic pattern (235). However glucocorticoids (dexamethasone <9 hr) were unable to prevent secretion stimulated by the calcium ionophore A23187 but they can block secretion of ACTH stimulation in response to isoproterenol or forskolin in AtT20 cells. Interestingly, dexamethasone was unable to completely abolish ACTH release induced by the combined action of epinephrine and CRF (237). Thus, glucocorticoids have effects at some as yet undefined steps in the pathway controlling hormone release.

The secretion and synthesis of ACTH in the pituitary may also be under the control of the immune system. The regulation of ACTH synthesis and secretion by the immune system will be discussed in a separate section.

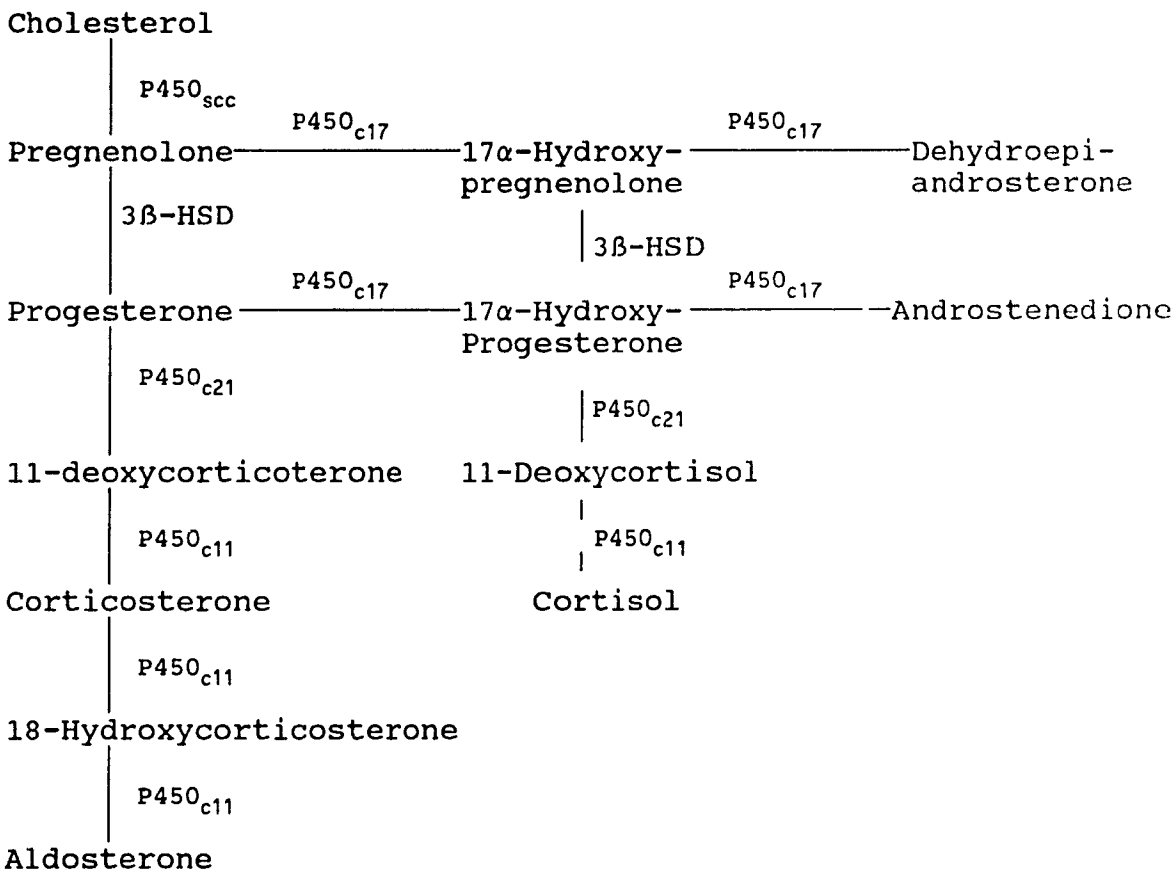
1.5. The Adrenal Glands

The adrenal gland is composed of steroidogenic (cortex) as well as chromaffin tissue (medulla). The chromaffin tissue is derived from the neural crest, while the steroidogenic tissue arises from the coelomic mesoderm in the genital ridge of the embryo. It is generally believed that in most mammals mineralocorticoids are produced in the zona glomerulosa, glucocorticoids are produced in the zona fasciculata and reticularis, and androgens in the zona reticularis (238).

ACTH acts mainly on the cells of the zona fasciculata and reticularis to stimulate glucocorticoid biosynthesis. ACTH also has some stimulatory effects especially *in vitro* on the mineralocorticoid synthesis by the cells of the zona glomerulosa, but the mineralocorticoid production is primarily regulated by renin-angiotensin system.

The structural requirement of ACTH and the ACTH receptor(s) and the interaction of ACTH with its receptor on the adrenocortical cells, is still the subject of much controversy. A detailed discussion of the possible mode of interaction of ACTH with its receptor will be presented in the discussion of this thesis. Briefly, ACTH interacts with specific ACTH receptor(s) resulting in formation of an active hormone receptor complex. The active hormone receptor complex activates adenylate

cyclase through an G_s protein, leading to the generation of intracellular cAMP, which mediates the increase in steroidogenesis. The regulation of steroidogenesis by ACTH is through its regulation of a number of enzymes and cofactors in the steroidogenesic pathway. All steroids are generated from a common precursor which is cholesterol. The current principal pathways of adrenal steroid hormone synthesis are showm in the following fig:



Steroidogenic tissues synthesize cholesterol *de novo* from

acetate (239) but human steroidogenic cells derive most of their cholesterol from plasma low density lipoproteins (LDL) (240), whereas rat steroidogenic tissues receiving most cholesterol from high density lipoproteins (HDL) (241, 242). The 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, the rate-limiting enzyme in cholesterol synthesis in man will be suppressed by adequate concentrations of LDL (243), and activated by tropic hormones (244). LDL and HDL cholesterol are taken up by adrenal cortex cells through quite different mechanisms, LDL by receptor-mediated endocytosis (240) whereas HDL does not involve internalization of the intact lipoprotein particle (246, 247). The LDL receptor has been cloned and well characterized (248).

There are two enzymes, namely, cholesterol esterase (cholesterol ester hydrolase) and cholesterol ester synthetase which control the storage of cholesterol. ACTH (249-251) stimulates the esterase and inhibits the synthetase. Cholesterol mobilized by cholesterol ester hydrolase from cytoplasmic lipid droplets may be transported into mitochondria by a sterol carrier protein-2 (SCP-2) (252-254). The flux of cholesterol across the mitochondrial membranes to the cholesterol side-chain cleavage enzyme is controlled by an unknown mechanism, but two of its elements have been defined. One element speeds the association of cholesterol with the mitochondrial cytochrome P-

450_{scc} substrate binding site (255). It can be rapidly blocked by inhibitors of protein synthesis (256-260) and by some amino acid analogs (261). Thus it is apparent that synthesis of a regulatory protein is necessary to initialize and maintain steroid production. There are several candidates proposed for this labile regulatory protein (262-269), and all of them have a short half-life and appear to be very sensitive to cycloheximide.

The first step in the biosynthetic pathway of steroidogenesis involves cleavage of the terminal six carbons of the side chain of cholesterol, resulting in the formation of pregnenolone. The conversion of cholesterol to pregnenolone is a hormonally regulated rate-limiting step, consisting of three distinct chemical reactions, 20α-hydroxylation. 22-hydroxylation, and scission of the cholesterol side chain at the bond between carbon atoms 20 and 22 to yield pregnenolone and isocaproic acid. Not long ago, these reactions were believed to be catalyzed by separate enzymes, while protein purification studies and *in vitro* reconstitution of enzymatic activity showed that a single protein, termed $P450_{scc}$ was responsible for all the steps between cholesterol and pregnenolone (271, 272, 335-338) on a single active site with an unusually low V_{max} (≈1 mol cholesterol/mol enzyme·sec) (339). This P450 was found bound to the inner mitochondrial membrane as a multimer of 16 subunits

with mass over 850,000 dalton (271, 272). Molecular biological studies of $P450_{scc}$ (273-275) supported the notion of a single $P450_{scc}$. The full length human adrenal and testis $P450_{scc}$ cDNA has been cloned and sequenced (276); it also have been cloned for bovine (273, 275) for rat (245); the gene for human $P450_{scc}$ (277) has also been cloned and much of it has been sequenced (278).

The reactions of the cholesterol side chain cleavage require three molecules of molecular oxygen (one for each reaction) and three molecules of NADPH of reducing equivalents. Electrons from NADPH are transported to $P450_{scc}$ by the common mitochondrial P450s' electron transport proteins (279-282), namely, adrenodoxin reductase (ferredoxin oxidoreductase), a flavoprotein and adrenodoxin (ferredoxin), an iron/sulfur protein. $P450_{scc}$ functions as the terminal oxidase in this mitochondrial electron transport system.

Bovine adrenodoxin has been sequenced (283). It contains 114 amino acids, including 4 cysteine residues which appear to coordinate 2 iron atoms through linkage with 2 additional noncysteine sulphur atoms. Adrenodoxin is a mitochondrial matrix protein (284-287) which forms a 1:1 complex with adrenodoxin reductase, then dissociates, then subsequently reforms an analogous 1:1 complex with $P450_{scc}$ or other P450s such as $P450_{c11}$ (288, 289), thus adrenodoxin serves as a diffusible electron

shuttle. Adrenodoxin reductase is a membrane-bound mitochondrial flavoprotein that receives electrons from NADPH. Bovine (281, 290-292), human (293) and chicken (294) adrenodoxin reductase have been cloned. The pregnenolone derived from cholesterol leaves the mitochondrion and travels to the endoplasmic reticulum, probably via passive diffusion, undergoing one of two conversions, namely, 17α-hydroxylation to 17-hydroxypregnenolone or 3ß-hydroxysteroid dehydrogenation and isomerization of the double bond (from the B ring to the A ring) to progesterone. The reactions of the conversion to progesterone from pregnenolone are relatively poorly understood. The full length cDNA structure and its deduced amino acid sequence of human 3ß-hydroxy-5-ene steroid dehydrogenase has recently been reported (295). The availability of the cDNA will lead a detailed investigation of this enzyme. (296, 297).

In man, cows, sheep, and pigs, progesterone may undergo one of two reactions 17α-hydroxylation and 21-hydroxylation; in rats, and mice only 21-hydroxylation occurs. Both pregnenolone and progesterone 17α-hydroxylations are catalyzed by the same enzyme, $P450_{c17}$, producing 17α-hydroxypregnenolone and 17α-hydroxyprogesterone respectively. The products of this reaction may then undergo scission of the C-17,20 carbon bond to yield dehydro-epiandrosterone (DHEA) and androstenedione, which are catalyzed by C-17, 20 lyase. Reconstitution of enzymatic

activity *in vitro* with purified $P450_{c17}$ (298, 299) and cells transfected with a vector expressing bovine $P450_{c17}$ cDNA (300, 301) demonstrated that both 17α-hydroxylase and 17,20-lyase activities reside in a single protein, $P450_{c17}$. The question is how these two activities can be separated since the adrenals of prepubertal children synthesize substantial cortisol but virtually no sex steroids, (only 17α-hydroxylase activity no 17,20 lyase activity) (302, 303) and patients have been described lacking of 17,20-lyase activity but retaining normal 17α-hydroxylase activity (304, 305). The detail mechanism of the control of these two enzymatic activities is not clear, but we do know several factors which are involved in this control mechanism. These are the preference for $P450_{c17}$ for a 5-ene-substrate for 17, 20-bond scission, in addition 17α-hydroxylation occurs more readily than the 17, 20-lyase reaction, and the ratio of P450 reductase to $P450_{c17}$, and P450 reductase to $P450_{c17}$. The ratio of P450 reductase to $P450_{c17}$ might be the most important factor.

The $P450_{c17}$ cDNA has been purified and sequenced entirely for human (306, 307), bovine (300), rat (308), and chicken (309). The authentic human gene for $P450_{c17}$ has also been cloned and sequenced (310) and located to chromosome 10, as a single gene (310).

$P450_{c17}$ and other P450s in the endoplasmic reticulum, such as $P450_{c21}$, receive electrons from a flavoprotein, P450 reductase distinct from the adrenodoxin reductase in mitochondria, without participation of iron/sulfur protein (311-313). P450 reductase accepts two electrons from NADPH, and transfers them to P450 one at a time (314). Cytochrome b_5 may provide the second electron to the P450 (315). Increasing the ratio of P450 reductase or cytochrome b_5 to $P450_{c17}$ *in vitro* increases the ratio of 17,20-lyase activity to 17α-hydroxylase activity (316, 317).

17α-hydroxypregnenolone can also be converted to 17α-hydroxyprogesterone before the scission of the C17,20-carbon bond by the same enzymes which catalyze the conversion of pregnenolone to progesterone. The 21 carbon of progesterone and 17α-hydroxyprogesterone will be hydroxylated, yielding 11-deoxycorticosterone and 11-deoxycortisol. 21-hydroxylation is mediated by $P450_{c21}$ which was the first P450 enzyme to be described in the steroidogenic pathway (318). $P450_{c21}$ is found in smooth endoplasmic reticulum using the same P450 reductase as that used by $P450_{c17}$. $P450_{c21}$ cDNAs (319-320) and genes (321, 322) were cloned and characterized.

The 11-deoxycorticosterone and 11-deoxycortisol leave the endoplasmic reticulum and enter the mitochondria for the final step in glucocorticoid or mineralocorticoid production, which is catalyzed by the second mitochondrial form of cytochrome P-

450 (11ß-hydroxylase cytochrome P450, or $P450_{c11}$). This single P450 has 11ß-hydroxylase, 18-hydroxylase, and 18-oxidase (aldehyde synthetase) activities. This was suggested by both immunochemical studies (323) and reconstitution of purified $P450_{c11}$ in vitro (324, 325) and confirmed by very convincing experiments (326) using purified enzyme from mitochondria from bovine adrenal zona glomerulosa and from zona fasciculata. The $P450_{c11}$ from both zones was indistinguishable enzymologically, immunologically, by amino acid composition and by N-terminal amino acid sequence. Like $P450_{scc}$, $P450_{c11}$ exists as a large multimer in vivo (327, 328) and uses adrenodoxin and adrenodoxin reductase to receive electrons from NADPH (329-331). Full length cDNA for bovine $P450_{c11}$ (332, 333, 348) and a fragment cDNA encoding the carboxyl-terminal 293 amino acids of human $P450_{c11}$ (333) were cloned and sequenced.

The enzymes of the steroidogenic pathway are regulated by ACTH. The half-life of RNA for $P450_{c11}$, $P450_{c21}$, and adrenodoxin seem to be unaffected by ACTH treatment in primary bovine adrenocortical cells (334). However, the half-life for $P450_{scc}$ RNA was increased 5-fold by ACTH treatment. ACTH treatment induces increased rates of synthesis of $P450_{scc}$ (340), $P450_{c11}$ (341), $P450_{c21}$ (342), $P450_{c17}$ (343), and adrenodoxin (345); and leads to the accumulation of mRNA specific for $P450_{scc}$ (346, 347), $P450_{c17}$ (300, 347), $P450_{cc11}$ (348), $P450_{c21}$ (349), and

adrenodoxin (290, 282). Nuclear run-on experiments have demonstrated that within 8 hours after initiation of ACTH treatment, transcription rate of RNA encoding of $P450_{scc}$, $P450_{c11}$, $P450_{c17}$ $P450_{c21}$ and adrenodoxin increased (349). All these ACTH effects can be mimiced by treatment with cAMP (350).

ACTH and cAMP can also stimulate the accumulation of IGF-II mRNA (351). There is a hypothesis that ACTH stimulates adrenal growth via stimulation of IGF-II, based on the following facts: the time course and dose-response curves for the accumulation of IGF-II mRNA and $P450_{scc}$ mRNA rose in parallel; exogenously added steroid hormones did not affect IGF-II mRNA (351) and exogenously added IGF-I or IGF-II did not affect $P450_{c17}$ and $P450_{c21}$ mRNAs in the response to ACTH but significantly reduced the ACTH-stimulated accumulation of IGF-II mRNA in the same cells (282). Further studies are needed before reaching a final conclusion.

1.6. Regulation of the Immune System and the Hypothalamic-Pituitary-Adrenal axis.

1.6.1. The Regulation of the Immune System by Glucocorticoids

The final products of the hypothalamic-pituitary-adrenal axis, the glucocorticoids will be released into the blood circulation a few minutes after the activation of the axis by

stressors. Glucocorticoids act on virtually every cell type in the body to cause alterations in differentiated function (for a review see ref. 7). The exact mechanisms of the action of glucocorticoid in each cell type is still the subject of investigation. From the forgoing, we already knew that glucocorticoids exert very profound effects on the regulation of ACTH secretion. The effects of glucocorticoids depend not only on their concentrations but also on their time domains after onset, and each cell type more likely has its own time response curve to glucocorticoid stimulation. In this way glucocorticoids segregate bioactivities of each cell type in different functional states assisting the central neuronal system to maintain the body's normal daily activities. This kind of neuronal and endocrine control becomes particularly crucial when the body is under a life threat. Under conditions of stress and in the absence of glucocorticoids there is vascular collapse which leads usually to death (352-356). The importance of glucocorticoids for survival was first noticed by Addison and later emphasized by numerous medical doctors and investigators (for reviews see 357-360).

In the body there is another critically important system for coping with certain kinds of threats and challenges namely the immune system. The immune system functions to combat microbial invasion, to provide resistance against the

development and spread of cancer, and to eliminate denatured substances from the body, that is, to distinguish self from not-self as formulated by Burnet (361). Since most immune reactions may be duplicated *in vitro* in the absence of influences emanating from other organ systems (362), the immune system is still believed to be relatively autonomous. The immune system bears a striking resemblance to both the nervous and endocrine systems. Like the nervous system, the immune system has the capability of detecting and reacting to environmental changes and of keeping the experience in its memory for later use. The molecular basis of memory is still unknown in both systems, however a cellular basis for immune memory has been suggested for both B-cells (363-365) and T-cells (for a review see 366). Moreover the immune system not only like the nervous system penetrates most of the tissues of the body and interacts by direct cell encounters, but also like the endocrine system it uses soluble hormone-like molecules (cytokines) to regulate its cell maturation, differentiation and the cellular response triggered by antigen-cellular interactions. During the last twenty years, a large number of cytokines has been discovered and characterized. With the advent of recombinant DNA technology, many of the cytokine genes have been isolated and studied to a great extent (for a review see 367). It is now clear that most of the cytokines have multiple biological

activities on different target cells, and some cytokines have antagonistic biological effects. Many cytokines are produced by a variety of cell types including monocyte/macrophage, endothelial cells, fibroblasts, stromal cells, and lymphocytes. The biosynthesis of a cytokine is influenced by other cytokines, forming the so called cytokine network (368). The biological effects of cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) are not restricted to the immune system but in fact the effects of IL-1 are manifest in nearly every tissue and organ (369). One thing should be emphasized, the immune system unlike any other system can destroy live cells or organs and improper regulation of the system will contribute to pathological processes and even death of the host.

The interaction of the endocrine system and the immune system (although the immune system is now sometimes considered as a large endocrine system) has been taken seriously only in very recent years, despite the early discovery of the anti-inflammatory effects of glucocorticoids made in the 1940s (370, 371) which has led to wide clinical use of glucocorticoids (372, 373). The importance of normal adrenal function to the immune reaction was also noticed in clinical practice. In patients with Cushing's disease, infectious illness was a major cause of death (374), however hyposecretion of glucocorticoid would also increase susceptibility to infection (375). The anti-

inflammatory effects of glucocorticoids had been excluded from physiological consideration until the 1970s when Besedovsky *et al.* deliberately investigated the interaction between immune system and neuroendocrine system (367). They found that injection of antigens into rats or mice led to a 2- to 5-fold increase in blood corticosterone levels after about 6 days when the antigenic response also reached a peak (69, 376). Later they suggested that the increase of corticosterone levels in blood could be responsible for the antigenic competition (377) based on the fact that the antigenic competition could be abolished by adrenalectomy. These authors also demonstrated that there were changes in electrical activity of neurons in the ventromedial part of the rat hypothalamus after antigenic stimulation (378) suggesting that the communication takes place at the hypothalamic level or above. They speculated that the physiological function of the delayed rise in glucocorticoid levels during the primary immune response may be acting to preserve the antigenic specificity of the response by preventing lymphocytes with little affinity for the antigen from proliferating in an unrestricted way that could lead to autoimmunity.

The molecular basis for the bidirectional communication between the immune and endocrine system was not understood at that time. It has now been shown that some cytokines from the

immune system are capable of mediating the control of certain neuroendocrine functions and their syntheses are under the regulation of the HPA axis but mainly by glucocorticoids.

In the cytokine network, the polypeptide IL-1 plays a central role in stimulating the immune and inflammatory responses (379, 380). IL-1 which also has been know as the endogenous pyrogen, the leukocytic endogenous mediator, the lymphocyte activating factor, the mononuclear cell factor, catabolin, the osteoclast activating factor, and hemopoietin-1, is a polypeptide that is produced following infection, injury, or antigenic challenge. IL-1 activates lymphocytes (381) and plays an important role in the initiation of the immune response. Although the antigenically challenged macrophage is a primary source of IL-1, epidermal, epithelial, lymphoid, and vascular tissues synthesize IL-1 (382). IL-1 stimulates the production of IL-2, a T-cell-derived cytokine (lymphokine) that stimulates the proliferation of T-cells (383, 384) and also stimulates the production of the interferons, IL-3 and other bone marrow colony stimulating factors and IL-6 (B-cell-stimulating factor-2) (385). IL-1 stimulates its own production (386). However, because IL-1 lacks a signal peptide, a considerable amount of the IL-1 that is synthesized may remain associated with the cell (387), particularly as part of the plasma membrane (388). Moreover, membrane-associated IL-1 is

biologically active, especially in its ability to participate in lymphocyte activation and mesenchymal tissue remodelling (382). There are two gene products coding for IL-1; IL-1α and IL-1ß (389). The spectrum of biologic activities of IL-1 are induced by both forms. and receptors for IL-1 recognize both forms (390, 391).

IL-1 is a highly inflammatory molecule and stimulates the production of arachidonic acid metabolites and also can modulate some of the biological processes such as tumor cell destruction, hematopoiesis, pyrogenesis, neutrophil activation, coagulation and bone and cartilage resorption (392, 386). IL-1 also acts synergistically with other cytokines, particularly tumor necrosis factor. IL-1 is one of the major cytokines regulating the acute phase response (393). The multitude of biologic responses to IL-1 is an example of the rapid adaptive changes that take place to increase the host's defensive mechanisms. However, IL-1-induced changes take place at a considerable cost to the host, and when the production and activity of IL-1 persist and escapes regulation, IL-1 itself contributes to pathologic processes and perhaps the death of the host (382). It is consistent with the biology of IL-1 to consider that the molecule is of vital importance to the host as long as its production and activity are appropriately modulated. To date, only corticosteroids block IL-1 transcription and reduce its

production (394). It is important to note that IL-1 is capable of down regulating its own receptor (282).

That glucocorticoids reduced the amount of IL-1 activity secreted from activated mouse macrophages was first reported by Snyder and Unanue (395). Physiological concentrations of dexamethasone inhibit IL-1 production by a 2-3 fold inhibition at the mRNA level (394). This is due, in part, to a reduction in the number of IL-1 synthesizing cells, which in turn is probably is due to regulation of transcription, since the induction of IL-1 by lipopolysaccaride (LPS) is mediated at this level (396). Glucocorticoid also selectively decreases the stability of interleukin-1ß mRNA (397).

The other multiple biologically active cytokine was IL-6 which also is known as hepatocyte stimulating factor (HSF), 26kDa-protein, interferon-$ß_2$ (IFN-$ß_2$), hybridoma/plasmacytoma growth factor (HGF or HPGF), or B-cell stimulatory factor-2 (BSF-2) (398, 399). IL-6 is produced by many different cell types including normal fibroblasts, endothelial cells and monocytes, as well as cell lines of T, B, sarcomatous or carcinomatous origin (400). The IL-6 gene can be transcribed in "ordinary" fibroblasts after stimulation with the double stranded RNA, poly-rI.rC (401, 402), bacterial lipopolysaccharide (LPS) (403), IL-1 (404), or TNF (405). These observations suggest that IL-6 may appear at any time or any

place in any tissue. Thus, the induction of the factor seems to be less specific than that of IL-1.

The most important biological function for IL-6 appears to be its hepatocyte stimulating factor activity. IL-6 is the major regulator of acute-phase protein synthesis (406) in the acute phase response. The acute phase response is one of the oldest and most preserved of the homeostatic responses of the body to injury and infection. This includes leukocytosis, fever, and a characteristic increase in a group of liver-derived plasma proteins called acute phase protein (AP) or reactants (407). The true physiological role of many of the acute phase proteins remain unclear, in general they act as antiproteinases, opsonins, or blood-clotting and wound healing factors, and they likely inhibit the generalized tissue destruction that is associated with the local inhibition of inflammation. More recently, evidence has been provided which suggests that some antiproteinases such as α2 macroglobulin may act as a carrier for some cytokines, such as IL-1, TGFß, platelet derived growth factor (PDGF), and IL-6 (408). IL-1, TNFα, IL-6 are the three cytokines which are responsible for the regulation of acute phase protein synthesis in the liver. They are synergistic, additive or negative to each other dependent on individual protein induction. Among these three mediators, only IL-6 is able to induce the full spectrum of acute-phase proteins (406).

Although glucocorticoids also directly regulate acute-phase protein gene expression and are required for synergistic and additive effects of IL-1 and IL-6, the production of IL-6 and TNFα are under negative control by glucocorticoids (409) as well as IL-1. Dexamethasone was found to inhibit the enhancement of IL-6 (IFN-β_2) mRNA levels in fibroblasts induced with IL-1, TNF or LPS (410, 403).

The production of a number of important T cell derived lymphokines including IL-2, IL-3, GM-CSF and IFN-τ, are also suppressed by the synthetic glucocorticoid analogue such as dexamethasone (411, 412). In common with many other glucocorticoid effects, this inhibition is manifest at the mRNA level (412).

The suppression of immune defence system by glucocorticoids has also been proposed as being associated in part with their suppressive effects on formation of leukotrienes and prostaglandins. These substances and other metabolites of arachidonic acid are potent mediators of the inflammatory response (413). Published data suggests that glucocorticoids act at the step of phospholipases, enzymes that cleave phospnolipids to release arachidonic acid (414). The following mechanism of glucocorticoid action has been speculated on. Glucocorticoids first bind to receptors in the cytosol. The glucocorticoid-receptor complexes are then transferred into the nucleus where

they regulate the expression of genes and therefore the synthesis of phospholipase inhibitory proteins, lipocortins, also called lipomodulins (415, 416). Highly purified lipocortins can mimic some of glucocorticoid actions such as inhibiting chemotaxis of neutrophils, blocking desensitization of beta-adrenergic receptors, increasing the affinity of beta-adrenergic receptors for agonist ligands, blocking the activation of phospholipase A_2 in neutrophil plasma membranes by fMetLeuPhe. Lipocortins, like glucocorticoids, can inhibit proliferative responses of T cells in response to mitogens such as phytohemaglutinin (PHA) and concanavalin A (Con A). This might contribute to the reduction in interleukin 1 production and release (417).

In summary, the inhibition of IL-1, along with the inhibition of other cytokines, and the suppression of the formation of leukotrienes and prostaglandins helps us to understand the anti-inflammatory action of glucocorticoids.

Recently, the HPA axis derived substances other than glucocorticoids especially opioid peptides have been speculated to act as mediators of the immune system (for reviews see 418, 419). This is based on the existence of opioid receptors in the cells of immune system which exert a variety of direct opioid effects (418). However, levels of active circulating opioid peptides are difficult to measure (420-423) and do not alone

constitute strong evidence for humorally mediated opioid regulation of the immune system. Although the production of the opioid peptide ß-endorphin and ACTH is not restricted to cells of the central nervous system, the presence of these POMC derived peptides has also been detected in the placenta (424), gonads (425, 426), pancreas, gut (427) and the immune system (428, 429). Results of a number of studies suggest that cells of the immune system can synthesize (430-432) and secrete (433, 434) POMC-derived peptides. Therefore circulating concentrations need not reflect opioid levels in the architecturally specialized areas where lymphocyte maturation takes place in thymus, lymph nodes, and spleen. Preferential synthesis, uptake, metabolism, and release of opioid peptide could affect local concentration but under normal circumstances the rate of synthesis and secretion of POMC derived peptides in extrapituitary sites is extremely low. Their physiological significance is still questionable.

1.6.2. Immunoregulatory Feedback of the HPA Axis.

1.6.2.1. *The positive control of the HPA axis*

As mentioned in the previous sections Besedovsky *et al.* demonstrated that the existence of bidirectional communication between the immune and endocrine systems in the 1970s'. In the 1980s, Besedovsky, *et al.* reported a series of studies demonstrating that IL-1 is the most important cytokine

responsible for the mediation of HPA axis. One of the first important observation they made was that Newcastle disease virus-activated human peripheral blood leukocytes *in vitro* produced a ACTH-like substance(s) which was not ACTH but which was able to increase glucocorticoid blood levels (435). They later reveal that the human leukocyte-derived substance was IL-1 by using a human IL-1 antibody to neutralize its capacity of increasing corticosterone and ACTH blood levels in rats (394). Injection of purified human IL-1 or recombinant human IL-1 to rats can mimic the action of human leukocyte-derived substance (394). The IL-1-stimulated ACTH and glucocorticoid increase was not the secondary action resulting from its pyrogenic effect, as at the doses used (0.25 μg/rat to 1 μg/rat), the body temperature of rats was normal in all instances (394). The possibility that the stimulatory effect of IL-1 was a consequences of lymphokine cascade was excluded by repeating the experiments in the athymic nude mice which lack functional T cells. In this experiment, IL-1 was still able to stimulate ACTH and corticosterone secretion (394). Injection of mice with IL-2, IFNγ, or TNF (tumor necrosis factor) did not induce an ACTH or glucocorticoid response (394).

An independent study conducted by Woloski (436) show that IL-1 and IL-6 had corticotropin-releasing activity on the AtT-20 cell line and the potency of IL-1's corticotropin-releasing

activity was in the same order of CRF and 3 times more potent then AVP. These results suggest that IL-1 acts at the pituitary level to stimulate ACTH secretion. However there is considerable evidence suggesting that IL-1 acts on CRF-secreting neurons. First, rats treated with anti-CRF antiserum prior to IL-1 injection did not show the expected increase in ACTH (437-439) and intracerebroventricular injection of recombinant human IL-1 can elicit a rapid increase in circulating ACTH at a much lower dose (30 ng) than the dosage required for intraperitoneal injection (1 μg) (440). Moreover, blood samples taken from the hypothalamic-hypophyseal portal system showed increased CRF levels after IL-1 injection (439). Studies *in vitro* support this point of view showing that IL-1 acutely and directly stimulates the release of CRF-41 from rat hypothalamus at doses which are unable to modify the direct pituitary release of ACTH. Direct pituitary actions of IL-1 have been observed, but all have involved prolonged incubation for several hours with cultured cells, which are unlikely to relate to the rapid pituitary-adrenal response seen in vivo. The potency of IL-1α and IL-1ß is of the same order. The maximum stimulation of the CRF production in the hypothalamic incubation by IL-1α and IL-1ß were 20 picomoles and 2.4 picomoles respectively (441).

Very recently Berkenbosch (442) demonstrated that *in vivo* IL-1 stimulated corticotropin-releasing factor (CRF) turnover

at an approximate rate of 15%/h, but did not cause a concomitant change in AVP turnover as can be observed after insulin-induced hypoglycemia. These results suggest that IL-1ß selectively stimulates the subtype of vasopressin deficient CRF neurons. As mentioned early, the vasopressin deficient CRF neurons can produce vasopressin after depleting endogenous glucocorticoids such as occurs following adrenalectomy. Of interest is that IL-1 failed to activate ACTH secretion in the adrenalectomized rat (443). These results suggest that the vasopressin deficient CRF neuron undergoes some possible reversible cell differentiation when vasopressin gene is being activated. So far the mechanism of this phenomenon has not been clarified.

Although a large body of evidence suggests that IL-1 acts at CNS levels especially at the level of the hypothalamus, the basic question as to how IL-1 crosses the blood -brain barrier (BBB) still remains to be answered. Very recently Banks *et al.* (444) presented some direct evidence showing that IL-1(α) can bidirectionally cross the BBB possibly via a saturable component and that the hypothalamus had the highest entry rate on a weight basis accounted for 2% of total entry.

IL-6 has also been shown to act as corticotropin-releasing factor (445, 446). Like IL-1, IL-6 acts at the hypothalamic CRF-containing neuron. The following factors from the immune system have also been reported to activate the HPA axis. Mitogen

stimulation of rat spleen cells produces a corticosterone-releasing activity that can act directly on the adrenal gland (447). Other factor such as thymosin fraction 5 (TF5), a complex mixture of thymic peptides, causes increased corticosterone secretion in rodents *in vivo* (448-451). TF5 has also been found to inhibit the binding of dexamethasone to thymocytes (452). Thymosins reportedly can potentiate recovery from the hydrocortisone-induced decrease in responsiveness of mouse splenic lymphocytes to the mitogen concanavalin A (Con A) (453). These results are very difficult to interpret.

1.6.2.2. ***The negative control of the HPA axis***

A functional immune system is necessary for the elimination of microbial invaders and neoplastic cells. As mentioned in the previous sections, the HPA axis can be activated by many stressful stimuli other than IL-1 and other cytokines such as hemorrhage, trauma, emotional stress, or combinations of these. However, the HPA axis final products, the glucocorticoids are potent suppressors of the immune system. Since the activation of HPA axis is much faster than the immune response, there is a period of time when HPA axis has been activated before normal immune defence reaction takes place. However, during the process an active immune system is still required for the host to survive. Therefore a negative regulation mechanism on HPA axis by the immune system appears to be not only necessary but also

important (see chapter V for further discussion).

The negative control of the HPA axis by the immune system has not been investigated as much as its positive control. However, evidence has now accumulated to suggest that some factors of the immune system can inhibit the HPA axis the level of adrenal.

Mathison et. al. (454, 455) reported that murine macrophages produce a factor that *in vitro* inhibits the action of ACTH on the adrenal cortex. The inhibitory factor has an apparent molecular weight greater than 10,000, and is relatively stable to heat and pH. Secretion of this factor is stimulated by bacterial lypopolysaccharide and enhanced by a lymphokine derived from the supernatant of a murine T-cell hybridoma. This factor also inhibits cholera toxin-induced steroidogenesis, suggesting the site of its action is somewhere after G stimulatory protein.

Transforming growth factor ß, TGF-ß secreted from macrophages and other types of tissues (456, 457) *in vitro* suppresses glucocorticoid production by adrenal glands after prolonged exposure (12-18 hours). The inhibitory effect of TGF-ß is manifest at very low concentration being detectable at 10^{-13}M (458, 459), and it inhibits both basal and ACTH-induced glucocorticoid production. The detail mechanism of its action is unknown. TGF-ß may control the converting step of cholesterol

from lipoprotein (459) or inhibit the steroid 17-α-hydroxylase activity (458, 460). TGF-ß also inhibits AII-stimulated aldosterone production (460).

In this thesis, the purification, characterization, biological functions and mechanism of action of a family of arginine-rich and cysteine-rich peptides derived from neutrophil and possibly other types of cells and which has anti-ACTH activity (461-462) will be presented.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Source of Peptides

Synthetic $hACTH_{1-39}$, $hACTH_{1-24}$, $hACTH_{6-24}$, $hACTH_{7-38}$ αMSH were purchased from Peninsula Laboratories, Inc., Belmont, California, USA. Synthetic CSI was obtained from American Peptide Company, Inc (Santa Clara, CA. USA.)

[Phe^2, Nle^4]-$ACTH_{1-38}$ was obtained from Dr. J. Ramachandran, Neurex Corporation, Menlo Park, CA, USA.

$hACTH_{1-18}$ amide was obtained from Dr. K. Inouye of Shionogi Co., Osaka, Japan.

The amino acid compositions of peptides were checked by amino acid analysis prior to use.

2.1.2. Source of Tissues

Fetal human lung tissues were obtained from human fetuses of 12-18 weeks of gestation. These fetuses were obtained at the time of therapeutic abortion at the Royal Victoria Hospital in Montreal, Quebec, Canada. Fetuses were obtained from the pathologist and permission to use human fetal tissues was approved by the Ethics Committee of the Department of Obstetrics and Gynecology, and the McGill Institutional Review Board. Fetal age was estimated by measurement of crown-rump and foot length (463).

Frozen fetal rabbit lungs and adult rabbit lungs were obtained from Pel-Freez Biologicals, Rogers, Ark USA.

Rabbit peritoneal neutrophils were obtained from peritoneal exudate which was generated as previously described (464). Exudates were collected 16 h after intraperitoneal instillation of a total of 750 ml of saline solution containing 0.1% glycogen and 5 μg of Escherichia coli lipopolysaccharide.

2.1.3. Source of Reagents and Instruments

Bovine Serum Albumin (BSA, fraction V), Charcoal, Cholera Toxin, Corticosterone, db-cAMP, DNase (deoxyribonuclease I), Escherichia Coli Lipopolysaccharide, Forskolin, Glutaraldehyde (Grade 1, 25% aqueous solution), Glycogen, Guanidine, Keyhole Limpet Haemocyanin, Lysine (free base crystalline) and Trypsin Inhibitor were purchased from Sigma, St. Louis, MO, UAS.

^{125}I (100 μCi/ml in NaOH pH 7-10) and Cyclic AMP Assay Kits were obtained from Amersham, Oakville, Ontario.

Aldosterone radioimmunoassay kits were obtained from Bio-Mega Diagnostic Inc., Montreal.

Corticosterone antiserum B3-163 was obtained form Endocrine Sciences Tarzana, CA, USA.

[1,2-^{3}H (N)]-Corticosterone 58.0 Ci/mmol in ethanol solution was purchased from New England Nuclear, Boston Mass. USA.

Borosiicate glass disposable culture tubes 12 x 75 mm and 16 x 100 mm were purchased from Fisher Scientific Co. Limited, Pittsburgh, PA USA.

Acetonitrile (HPLC grade), Methanol (HPLC grade), Methylene Chloride (HPLC grade) were obtained from Fisher Scientific Fair Lawn, New Jersey, USA.

Heptafluorobutyric Acid HPLC grade and Hydrochloric acid 6 N (Sequanal grade) were purchased from Pierce, Rockford, Illinois, USA.

Trifluoroacetic Acid, 99 +% (suitable for protein sequencing) was obtained from Aldrich Chemical Company, Inc. Craftsmen in Chemistry Milwaukee Wis USA.

Gentamicin, Ham's F-12 wigh glutamine and Medium 199 were purchased from Flow Laboratories, Mississauga Ontario.

Medium 199 with Earle's Salts & L-Glutamine was obtained from Gibco Laboratories, Life Technologies Inc. Grand Island, New York, USA.

2-Mercaptoethanol (Baker Grade) was obtained from J. T. Baker Chemical Co. Phillipsburg, N.J. USA.

Heparin Leo 1,000 iu/ml from Leo Laboratories Canada LTD, Pickering Ontario.

Collagenase was purchased from Boehringer Mannheim GmbH, Germany.

Rabbits, female New Zealand rabbit weighing about 4.5 kg were purchased from Chene Bleu.

CHAP. 2. Materials and Methods

Guinea Pigs (Hartley male) weighing 450 grams and Rats Sprague-Dawley male rats weighing from 150 to 250 grams were purchased from Charles River Breeding Laboratories.

Pertussis toxin was obtained from List Biological Laboratories, Inc. Campbell, CA USA.

Deoxyribonucleic acid (phenol extracted) from salmon testes was purchased from P-L Biochemicals, Inc (Milwaukee, Wis.)

Dextran T-70 was purchased from Pharmacia, Uppsala, Sweden Sodium Pentobarbital (for veterinary use) M.T.C. Pharmaceuticals, Mississauga, Ontario.

Ficoll-Paque for *in vitro* Lymphocyte isolation was obtained from Pharmacia LKB Biotechnologh Inc. Piscataway, New Jersey USA.

RIBI adjuvant (0.5 mg MPL, 0.5 mg TDM and 2 μl of Tween-80) was purchased from RIBI Immunochem Research Inc. Hamilton, Montana, USA.

Saline was purchased from Travenol Canada Inc. Mississauga Onatario.

Xylazine (20 mg/ml) (Rompun) was purchased from Bayvet Division Chemagro Limited Etobicake, Ontario.

Ketamine 57.5 (mg/ml) (Ketalar) was purchased from Parke-Davis Canada Inc. Scarborough, Ontario.

High performance amino acid analyzer, System 6300, was purchased from Beckman Instruments, Inc.

LKB Ultrorak Fraction Collector and LKB-WALLAC 1277 Gammamaster Automatic Gamma Counter (WALLAK Oy Turku Finland) were purchased from Fisher Scientific Co. Montreal, Quebec.

Savant Speed Vac concentrator was purchased from Savant Instruments Inc. Farmingdale N.Y. USA.

Shaking water bath, Dubnoff Metabolic Shaking incubator, was purchased from Precision Scientific Co. Chicago, USA.

Spectrophotometer: Spectronic 7000, was made by Bausch & Lomb, USA; U-2000 was made by Hitachi, Japan.

μBondapak 3.9mm x 30cm and 7.8mm x 30 cm stainless steel columns, Protein-Pak-125 (old name I-125) 7.8mm x 30 cm stainless steel columns, C_{18} Sep-Paks, CM Sep-Paks, NorganicTM Water Purification System and HPLC system were purchased from Waters Associates, Milford, MA, USA.

2.2. Isolation and Purification of Peptides.

2.2.1. Extraction of Tissues

2.2.1.1. *Acidic medium extraction*

A total of 100 pairs of frozen rabbit fetal lungs obtained at late gestation or frozen rabbit adult lungs (Pel Freez) were homogenized at 4°C in an acidic medium consisting of 1M HCl/5% formic acid/1% NaCl (wt/vol)/1% trifluoroacetic acid (465). The ratio of the tissues and the extraction medium was kept at about 1:1 (wt/vol). After centrifugation (3300 x g for

15 min), the pellet was re-extracted twice with the same amount of the extraction medium and the supernatants rich in peptide were combined for the ODS silica cartridge extraction (2.2.1.2).

2.2.1.2. *ODS silica cartridge extraction*

ODS-silica cartridges (C_{18} Sep-Pak) were prepared using a slightly modified procedure originally described by Bennett *et al.* (465). A set of five C_{18} Sep-Paks were connected in series using headless pipet tips (i.e. the bottom 1 to 1.5 inch of 2 inch of 2-200 μl polypropylene pipet tips). These were first conditioned by flushing with 20 ml of 80% acetonitrile containing 0.1% TFA followed by 30 ml of 0.1% TFA. Each 150 ml of extracts of rabbit fetal lungs or 250 ml of rabbit adult lung extract (see section 2.2.1.1.) was passed through one set of the cartridges. The cartridges were then washed with 50 ml of 0.1% TFA to remove unbound material. Peptides bound to the ODS-silica resin were eluted with 10 ml of 80% acetonitrile containing 0.1% TFA. The eluates were stored at -40°C for later use or directly subjected to cationic exchange cartridge extraction (see section 2.2.1.3.) or to reversed-phase HPLC (see section 2.2.2.2.).

2.2.1.3. *CM cation exchange cartridge extraction*

The ODS silica cartridge extracts (see section 2.2.1.2.) were diluted with 4 volumes of 150 mM Tris buffer pH 7.2

containing 20% acetonitrile (466) to bring the pH to 6-7. Each 150 ml of the diluted extract was passed six times through carboxymethyl cationic ion exchange cartridges (CM Sep-Pak). Prior to use, the CM Sep-Pak was conditioned with 10 ml of 50mM Tris pH 7.2 containing 1 M NaCl and 20% acetonitrile and then equilibrated with 50 ml of 150 mM Tris pH 7.2 containing 20% acetonitrile. After loading the eluates, the cartridges were washed with 50 ml of the 150 mM Tris and the cationic peptides bound to the resin were eluted with 4 ml of 50 mM Tris buffer containing 1 M NaCl and 20% acetonitrile. The final cation exchange eluates were then concentrated in a Savant Speed Vac concentrator and stored at -20°C prior to purification on HPLC (see section 2.2.2.2.).

2.2.2. HPLC Purification

2.2.2.1. *General methods*

HPLC separations were carried out on a Waters HPLC system consisting of two M-45 pumps and a Model 680 Automated Gradient Controller. Eluates from the column were monitored at 280 nm and 215 nm using a Waters Model 441 and a Model 481 variable wavelength detector connected in series. The UV absorbance of eluates were recorded by a recorder (model SE^R 120).

HPLC grade water was made from deionized, glass-distilled water by filtering through Norganic Water Purification

System (Waters Associates, Milford, MA). All reagents used for HPLC purification procedure were HPLC grade, except TFA which was prepared as a stock solution of 1% (v/v, 0.13M) and purified by passing through a C_{18} Sep-Pak. Prior to use, the Sep-Pak was conditioned as described in section 2.2.1.2.. Acetonitrile and water were degassed under a water vacuum for about 20 minutes immediately before use.

2.2.2.2. *Reversed-phase HPLC purification*

Reversed-phase HPLC purifications were carried out as previously described (465, 467). The method used is as follows. The Sep-Pak eluates or HPLC fractions were concentrated in the Speed Vac to evaporate acetonitrile, and loaded onto a C_{18} μBondapak column (Waters Associates, Milford, MA) via an injector (Beckman Instruments Inc.) or an HPLC pump (Milton Roy Inc.) Two solvent systems were used, one employing TFA as the hydrophobic counter-ion pairing reagent at a concentration of 0.1% and the other employing HFBA as the hydrophobic counter-ion pairing reagent at a concentration of 0.13%. In both solvent systems, 80% acetonitrile was used as the organic modifier as buffer B. Buffer A was prepared with HPLC-grade water. The concentration of the corresponding hydrophobic counter-ion pairing reagent was the same in both buffer A and buffer B. Buffer A and buffer B were delivered by separated pumps, pump A and pump B which were controlled by a automated gradient

controller (Waters Model 680). Bound peptides were eluted from the column with linear gradients of solvent B. Fractions were collected in glass or plastic 12 x 75 mm test tubes, using an LKB Ultrorak Fraction Collector (Fisher Scientific Co., Montreal, Quebec.)

2.2.2.3. *Gel permeation HPLC purification*

Gel permeation HPLC separations were performed isocratically at a flow rate of 1.0 ml/min on two I-125 Waters protein analysis columns connected in series with the solvent system of 40% acetonitrile containing 0.1% TFA (467). Samples from reversed-phase HPLC were either dried to a small volume or to complete dryness and then dissolved in 50 to 100 μl of 40% acetonitrile containing 0.1% TFA for application to the columns.

2.3. Characterization of Peptides

2.3.1. Amino Acid Analysis

2.3.1.1. *Acid hydrolysis*

A total of 100 picomoles to 1000 picomoles of aliquots taken from the samples purified by HPLC (see section 2.2.2) for amino acid analysis were dried in the Speed Vac in 6 x 50 mm KIMAX culture tube and hydrolized at 105°C for 16 hours by the vapour of constant boiling HCl containing a small amount of phenol in a gas phase of 100% nitrogen on a Waters Pico-Tag work

station. HCl was evaporated under vacuum prior to analysis as described in section 2.3.1.2..

2.3.1.2. *Amino acid analysis*

Amino acid components derived from acid hydrolysis as described in section 2.3.1.1. were separated and quantitated on a high performance amino acid analyzer (System 6300, Beckman Instruments, Inc.) consisting of an automatic sample loading turn table, ion exchange HPLC and ninhydrin colour reaction. The ninhydrin derivatives were monitored by an on line spectophotometer at 440 and 570 nm and the sum of both peak areas was integrated by a Hewlett Packard 3390A Reporting Integrator. The working procedure was set up according to instructions supplied with the machine.

2.3.2. Gas Phase Sequencing of Peptides

2.3.2.1. *Reduction and pyridylethylation*

About 40 μg of purified peptides was dissolved in 200 μl of the denaturant buffer (6M Guanidine-HCl, 0.25 M Tris, 1 mM EDTA pH 8.5) and incubated with 10 μl of 1.4 M 2-mercaptoethanol at room temperature in the dark. After 2 hours, 10 μl of 4-vinylpyridine (undiluted) was added and the mixture was incubated for another 2 hours under the same conditions. The linear pyridylethylated peptide was separated from other peptides by RP-HPLC as described in section 2.2.2.2.

2.3.2.2. *Amino acid sequence analysis*

The amino acid sequence of the purified peptides was determined by automated Edman degradation in an Applied Biosystem 470 A gas-phase sequanator with the trifluoroacetic acid conversion program. The PTH (phenylthiohydantoin) amino acids were monitored by an Applied Biosystem 120 A on-line analyzer. Some sequence analysis was carried out in the Shriner's Hospital in Montreal and some was carried out in the Laboratory for Neuroendocrinology, Salk Institute, San Diego, California, USA by Dr. Fred Esch.

2.4. Radioimmunoassays

2.4.1. Radioimmunoassays of Peptides

2.4.1.1. *Iodination of peptides*

Labelled peptides for radioimmunoassay were prepared using a modification of the chloramine-T method, as described by Browne *at al* (468). 2 μg of peptide dissolved in 10 μl of 2 M phosphate buffer pH 7.4 and 1 mCi of $^{125}I^-$ in 10 μl of NaOH solution pH 8-11 were mixed with 10 μg of chloramine T in 20 μl of PBS buffer in a 1.5 ml polypropylene microfuge tube for 15 seconds, then the reaction was terminated by adding 50 μg of sodium metabisulphite in 20 μl of PBS buffer.

The ^{125}I-peptide and a small amount of unlabelled peptide were separated from iodide by chromatography on a C_{18} reversed phase cartridge. A preconditioned ODS-silica cartridge was prepared as described previously (see section 2.2.1.2.). The mixture of the products of iodination were passed through the cartridge which was then washed with 50 to 100 ml of 0.1% TFA to get rid of unbound free iodide and eluted with 3 ml of 80% acetonitrile containing 0.1% TFA. The eluates were stored at 4°C for use as a tracer in the corresponding radioimmunoassay.

2.4.1.2. *Antisera*

Antisera to ACTH, and αMSH were generated in our laboratory by Drs. C. A. Browne and S. Mulay (468).

CSI antiserum was raised in guinea pigs with conjugates of CSI and keyhole limpet haemocyanin I.M.. A total of 5 mg of synthetic CSI and 5 mg of keyhole limpet haemocyanin dissolved in 5 ml of PBS buffer were conjugated together by using glutaraldehyde to a final concentration of 0.25% v/v. After 1 hour incubation at room temperature, the reaction was terminated by adding lysine to a final concentration of 0.2 M. A total of 0.8 ml of conjugate containing 0.8 mg CSI was added to RIBI adjuvant reconstituted in 1.2 ml of saline per vial. Booster injections contained 0.4 ml of conjugate and 1.6 ml of adjuvant in reconstituted saline. Immunization was performed in guinea pigs as follows: a total of 0.2 ml of the mixture of the

conjugate and adjuvant was injected I.M. into each side of the hind legs and 0.1 ml of the mixture was injected I.P.. Boosters were given at 3, 6, 9 and 12 weeks. The blood was taken by cardiac puncture using Xylazine (1mg/kg) and Ketamine (40 mg/kg) as anaesthetics two weeks after the booster shots. The serum was stored at -20°C.

2.1.1.3. *Specificity of antisera*

The ACTH antiserum was directed towards amino acid 17-20, and cross-reacted equally well with $ACTH_{1-24}$, $ACTH_{17-39}$, $ACTH_{16-27}$ and CLIP (468). The antiserum did not cross-react significantly (<0.05%) with αMSH (468).

The αMSH antiserum was directed toward the C-terminal amino acid residues (10-13 amide), and cross-reacted poorly (<0.1%) with $ACTH_{1-39}$, $ACTH_{1-24}$ and $ACTH_{1-18}$ (468).

The CSI antiserum cross-reacted very weakly with CSII, CSIII, and CSIV (<0.01%) and had no detectable cross-reactivity with other peptide including human corticostatin, HP-4, and rat corticostatin R-4.

2.4.1.4. *Radioimmunoassays*

0.02 M Barbitone buffer containing 1% (w/v) BSA, 0.18% (w/v) NaCl, and 0.4% mercaptoethanol pH 8.6 was used for the radioimmunoassays for ACTH and αMSH, and PBS buffer containing 0.5% BSA was used for CSI radioimmunoassay.

Standard curves were constructed using known amounts of unlabelled peptide (10 to 4,000 pg of ACTH, or 50 to 12,000 pg of αMSH, or 25 to 10,000 pg of CSI) in a volume of 100 μl of buffer. Aliquots of the samples from HPLC or ODS cartridge fractions were dried in the Speed-Vac concentrator prior to assay. In order to get accurate RIA data, samples were sometimes diluted so that the RIA reading would lie in the middle of the assay standard curve.

A total of 100 μl of appropriate tracer containing 10,000 to 20,000 cpm was added to each of the tubes, followed by the addition of 100 μl of antiserum solution to all tubes except for those used to determine total counts and non-specific binding, (100 μl of buffer was added instead). The tubes were incubated at room temperature for 2 hours for the ACTH assay, 3 hours for αMSH assay and at 4°C for 16 hours for CSI assay. At the end of incubation, 750 μl of dextran-charcoal buffer containing 0.25% charcoal and 0.05% dextran T-70) at 4°C was added to each tube except for those used to determine total counts, and vortexed. The tubes were then incubated for 6 min at 4°C and centrifuged at 2000 x g for 15 min. The supernatants were decanted into 12 x 75 mm test tubes, stoppered with corks and counted for 1 to 3 min to obtain a total number of counts of at least 10,000.

2.4.2. Radioimmunoassay of Steroids and cAMP

The radioimmunoassay for corticosterone and aldosterone were carried out according to the instructions supplied by the manufacturer (BioMeg).

The radioimmunoassay for cAMP was carried out by using a kit purchased from Amersham, and the instructions supplied with the kit.

2.5. *In Vitro* Bioassay

2.5.1. Rat Adrenal Cell Bioassay

The dispersed rat adrenal cell bioassay was a modification of method of Sayers (469-473). Prior to use, all media used in the bioas. y were prewarmed to 37°C in a shaking water bath (Dubnoff Metabolic Shaking incubator, Precision Scientific Co., Chicago, USA) in an atmosphere of 95% O_2/5% CO_2. A total of 10-26 Sprague-Dawley male rats (Charles River Breeding Laboratories) weighing 150 to 250 grams were sacrificed by decapitation, and adrenals were decapsulated removed immediately, and quartered in Ham's F-12 medium containing 0.5% BSA. The tissue was then incubated for 1 hr in 10 ml of the medium with 2 mg/ml collagenase and 250 μg/ml DNase. At the end of the incubation, the dispersal of the cells was completed by pipetting the medium up and down 50 times using a Pasteur pipette. The cell suspension was centrifuged at 150 x g for 7 min, and the cell pellet was resuspended and washed twice with

12 ml of the same medium. Finally, the cell pellet was suspended in 2 ml of the medium and filtered through prewetted nylon gauze (100 μm) and layered on top of 8 ml of Ham's F-12 medium containing 2.5% BSA prior to gradient centrifugation. After centrifugation at 170 x g for 10 min, 6 ml of the upper layer was aspirated and the cells in the lower 4 ml were diluted with 10 ml of incubation medium (Ham's F-12 medium containing 0.5% BSA and 7 mM Ca^{2+}) before determining the cell number. The viability of the cells was determined by the trypan blue exclusion method. Only the cells which contained lipid droplets and excluded trypan blue were counted. The cell suspension was adjusted to a final concentration of 400,000 cells per ml and 0.5 ml was added to the incubation tubes. The cells were preincubated for 60 min at 37°C on a shaking water bath under an atmosphere of 95% O_2/5% CO_2. Then, a 0.5 ml aliquot of incubation medium or incubation medium containing either synthetic human or synthetic porcine ACTH ($ACTH_{1-39}$) alone or ACTH plus test material was added. The mixture was incubated for an additional 2 hr. After incubation the tubes were centrifuged at 120 x g for 10 min, and the supernatant was decanted into borosilicate culture tubes. Steroids were extracted with 2 ml of methylene chloride and corticosterone and aldosterone in the extract were determined by radioimmunoassay. cAMP was determined in the aqueous phase by radioimmunoassay.

2.5.2. Rat Adrenal Zona Glomerulosa Cell Bioassay

Zona glomerulosa cells were prepared essentially as described by Douglas *et al.* (474). A total of 15-20 Sprague-Dawley male rats (Charles River Breeding Laboratories) weighing at 150 to 175 grams were sacrificed by decapitation. The adrenals were freed from fat tissues before removal and decapsulation. The capsules were minced and the mince was incubated for 45 min at 37°C in 10 ml of medium 199 with 2 mg/ml collagenase and 250 μg/ml DNase on the shaking water bath in an atmosphere of 95% O_2/5% CO_2 as described in section 2.5.1.. The tissue was mechanically dispersed and centrifuged at 120 x g for 10 min and then washed with medium 199. The resulting cells were resuspended in 2 ml of the medium and filtered through nylon gauze onto a bed of 8 ml of the medium containing 2.5% of BSA prior to gradient centrifugation as described in section 2.5.1.. The cells (400,000) contained in 1 ml of medium 199 which was supplemented with 0.5% BSA and 5 mM K^+, were incubated together with either angiotensin II alone or corticostatin I plus angiotensin II for 2 h under the same condition as previously described in section 2.5.1. Aldosterone output was measured by the radioimmunoassay following extraction of supernatant with methylene chloride as described in section 2.5.1.

2.6. ACTH Receptor Assay

2.6.1. Preparation of Labelled ACTH Ligand

The fully biologically active ACTH analogue ([Phe_2, Nle^4] $ACTH_{1-38}$) was freshly iodinated and purified for each experiment. A total of 4-12 μg of [Phe_2, Nle^4] $ACTH_{1-38}$ (a gift from Dr. Ramachandran) in 10 μl of 2 M phosphate buffer pH 7.4 and 1 mCi of $^{125}I^-$ were mixed with 2 μg of chloramine T in 20 μl of PBS buffer in a 1.5 ml polypropylene microfuge tube for 15 seconds. Then the reaction was terminated by adding 25 μg of sodium metabisulphite in 20 μl of PBS buffer. The ^{125}I-ACTH analogue was separated from iodide and non-iodinated ACTH analogue by reversed-phase HPLC. After loading, the column was first washed with 0.1% TFA for 60 min to wash away unbound free iodine and then eluted with two linear gradients of 1-24% acetonitrile (for the first 10 min) and 24-32% acetonitrile (for the next 50 min). Fractions were collected every minute, and 10 μl aliquots were taken from each fraction and counted in a LKB-WALLAC 1277 Gammamaster Automatic Gamma Counter . The efficiency of the gamma counter was estimated by known dpm standard ^{129}I supplied by LKB-WALLAC company at 55% (31865 CPM = 58300 DPM). The specific radioactivity of the ^{125}I-ACTH analogue was estimated from the ratio of the UV absorbance areas of the cold and labelled ACTH analog, the amount of cold ACTH analogue being quantitated by RIA. The specific radioactivity was approximately 2000 Ci/mmol.

2.6.2. ACTH Receptor Binding Studies

ACTH binding studies were carried out as previously reported (475, 477). Decapsulated adrenal glands from male Sprague-Dawley rats were digested with collagenase and DNase as described in section 2.5.1. but using the medium 199 rather than Ham's F-12. The cells were suspended in medium 199 containing 10% fetal bovine serum and 0.004% gentamicin and washed three more times under the sterile conditions. The cells at a concentration of 7.5×10^5 cells per ml were incubated overnight (18 hr) in sterile bacterial Petri dishes at 37°C in 5% CO_2/95% air.

Cells were removed from the Petri dishes with a pipette, centrifuged at 100 x g for 10 min, and washed once with medium 199 containing 0.5% BSA. The cells were resuspended in 2 ml of the medium and filtered through nylon gauze on the top of 8 ml of medium 199 containing 2.5% of BSA for gradient centrifugation. After centrifugation at 100 x g for 10 min, 8 ml of the upper layer was aspirated and the cells in the lower 2 ml were resuspended in the medium 199 containing 0.5% BSA and 3.5 mM $CaCl_2$ at a concentration of 2×10^6 cells per ml. 0.4 ml aliquots of the cells were incubated in polypropylene tubes in duplicate with 0.1 ml of ^{125}I-ACTH analogue (175 pM) in the presence or absence of displacing ligands for 1 hr at 23°C on the shaking water bath in an atmosphere of O_2/5% CO_2. At the end of the incubation, the tubes were centrifuged at 70 x g for 7

min at 4°C and 250 µl of supernatant was taken from each tube. After extraction by 1 ml of methylene chloride, corticosterone production was measured by RIA as described in section 2.5.1. The cells in the bottom layer were resuspended using a 100 µl micropipettor, and duplicate 100 µl aliquots were removed and layered on to the top of 300 µl of ice-cold medium 199 containing 1.5% BSA in 400 µl polypropylene microfuge tubes and centrifuged at about 9000 x g in a Beckman 152-Microfuge for 5 min at 4°C. After removing the supernatant with a Pasteur pipette, the tubes were kept inverted for 30 min. The tip containing the cell pellet was cut off and counted for ? min in the τ Counter.

For estimation of DNA content the cell pellet was then dissolved in 500 µl of 0.1 N NaOH in 16 x 100 mm Borosilicate glass disposable culture tubes, mixed with 1 ml of 6 N HCl and 500 µl of 0.04% Indole and heated in a boiling water bath for 10 min. After cooling with running water, the solution was extracted two times with 2 ml of chloroform. The intensity of the indole derivative was determined in a spectrophotometer (Spectronic 7000, Bausch & Lomb, USA, or U-2000, Hitachi, Japan) at 490 nm. Deoxyribonucleic acid (phenol extracted) from salmon was used as the standard.

CHAPTER 3: RESULTS I:

PURIFICATION AND CHARACTERIZATION OF CORTICOSTATINS

3.1. The Discovery of the Existence of Anti-ACTH Peptides in the Fetal Rabbit Lungs

My first research task upon arrival in the Endocrine Laboratory was to determine if the fetal lung contained authentic ACTH. It was interesting to know whether fetal lungs can produce ACTH or some ACTH-like substances because immunoreactive ACTH had been detected in the fetal lung. To accomplish this task a scheme was worked out for the isolation of ACTH which is shown below:

Human Fetal Lungs
↓
Homogenized in Acidic Medium
↓
Centrifugation
↓
Re-extracted Twice
↓
Supernatant Pool
↓
C_{18} Sep-Pak
↓
80% Acetonitrile Eluates
↓
HPLC Purification
↓
RIA for ACTH and αMSH

CHAP. 3. Purification

A total of 20 pairs of human fetal lungs that been collected and frozen at -40°C were processed as shown in scheme above. The results are shown in Fig. 3-2. which shows that there was a certain amount of ACTH immunoactive substance present in the fractions 26-28 of the lung extract. Because it was such small amount of material, it was important to prove that the immunoreactive materials was indeed ACTH and not artifactual contamination. It should be noted that immunoreactive αMSH was also present in fairly sizable amounts. The availability of human fetal lungs was limited at that time, and we turned to examine frozen rabbit fetal lungs because immunoreactive ACTH was shown to be present in such tissues. We turned to using ODS cartridge (Sep-Pak) to fractionate the lung extracts and used the rat adrenal cell ACTH bioassay to monitor these fractions. The experiment was carried out as follows:

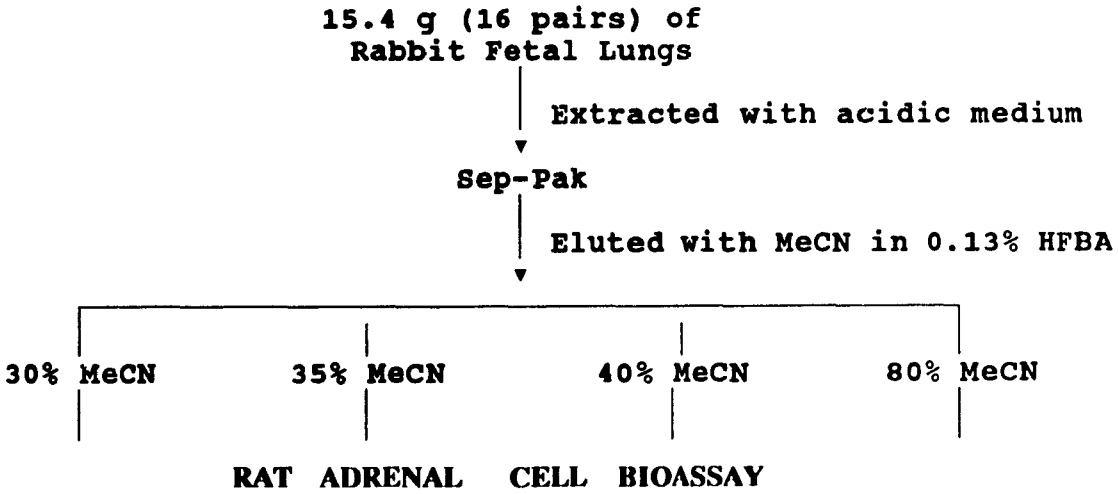


Fig. 3-2 shows that the rabbit fetal lung extracts did contain

biologically active ACTH fractions as was found for the human fetal lungs. Of interest was the fractions from 40% to 80% acetonitrile eluates which did not show any ACTH bioactivity. These results were puzzling because the resolution by ODS cartridge fractionation is usually relatively poor compared to HPLC purification and a slight contamination from fractions of 30% to 40% acetonitrile was thought to be unavoidable. These results presented several possibilities, one of which was existence of an inhibitor of corticosterone synthesis. This hypothesis was tested in the subsequent experiments. A 325 μl aliquot was taken from the fraction of 40% to 80% acetonitrile eluates (equal to 1 g of rabbit fetal lung tissues), dried in the speed-vac before co-incubating with increasing does of synthetic hACTH in the rat adrenal cell bioassay. The corticosterone response to ACTH stimulation was significantly depressed when the 40-80% acetonitrile fractions were present (Fig. 3-3). This data suggested the presence of an anti-ACTH factor(s).

Although acidic and ODS cartridge extraction procedure were designed to obtain peptide-rich fraction, the peptide nature of anti-ACTH components still needed to be confirmed. This was accomplished by trypsin digestion. After incubating with trypsin for three and half hours, the fraction lost its inhibitory activity comparing with the controls (Fig. 3-4) but it kept its

fully biologically activity after incubation for 30 min in a boiling water bath. These experiments were repeated two more times before further purification and characterization was undertaken.

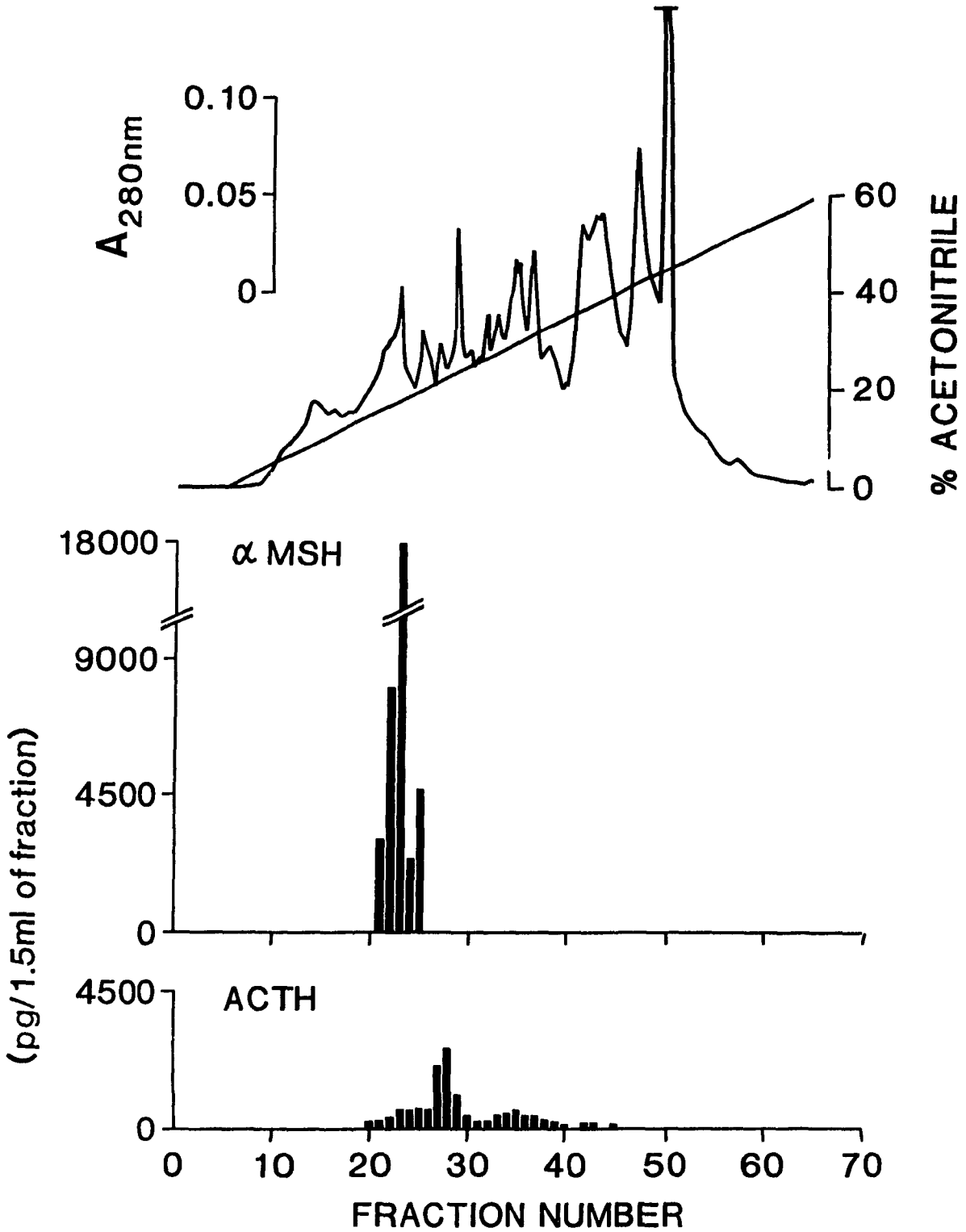

A_{280nm}
0.10
0.05
0
60
40
20
0
% ACETONITRILE
18000
α MSH
9000
4500
0
4500
ACTH
0
(pg/1.5ml of fraction)
0
10
20
30
40
50
60
70
FRACTION NUMBER

Fig. 3-1 Radioimmunoreactive ACTH in fetal human lung extracts The elution pattern of radioimmunoreactive ACTH from a reversed phase HPLC column of an extract of 20 pairs of fetal human lungs extracted as described in sections 2.2.1.1. and 2.2.1.2.. The tissue extract was dried in the speed vac to evaporate about 80% of its volume and diluted with 10 ml of 0.1% TFA and pumped directly onto a C_{18} μBondapak column that was washed with 90 ml of 0.1% TFA. The column was eluted at a flow rate of 1.5 ml/min for 1 hr with a linear gradient of 0-56% acetonitrile. Fractions were collected every min. Aliquots (100 μl) were taken from each fraction and radioimmunoassay for ACTH and αMSH were carried out as described in section 2.4.1.4.. Bars indicate the amount of radioimmunoreactive ACTH or αMSH in each fraction.

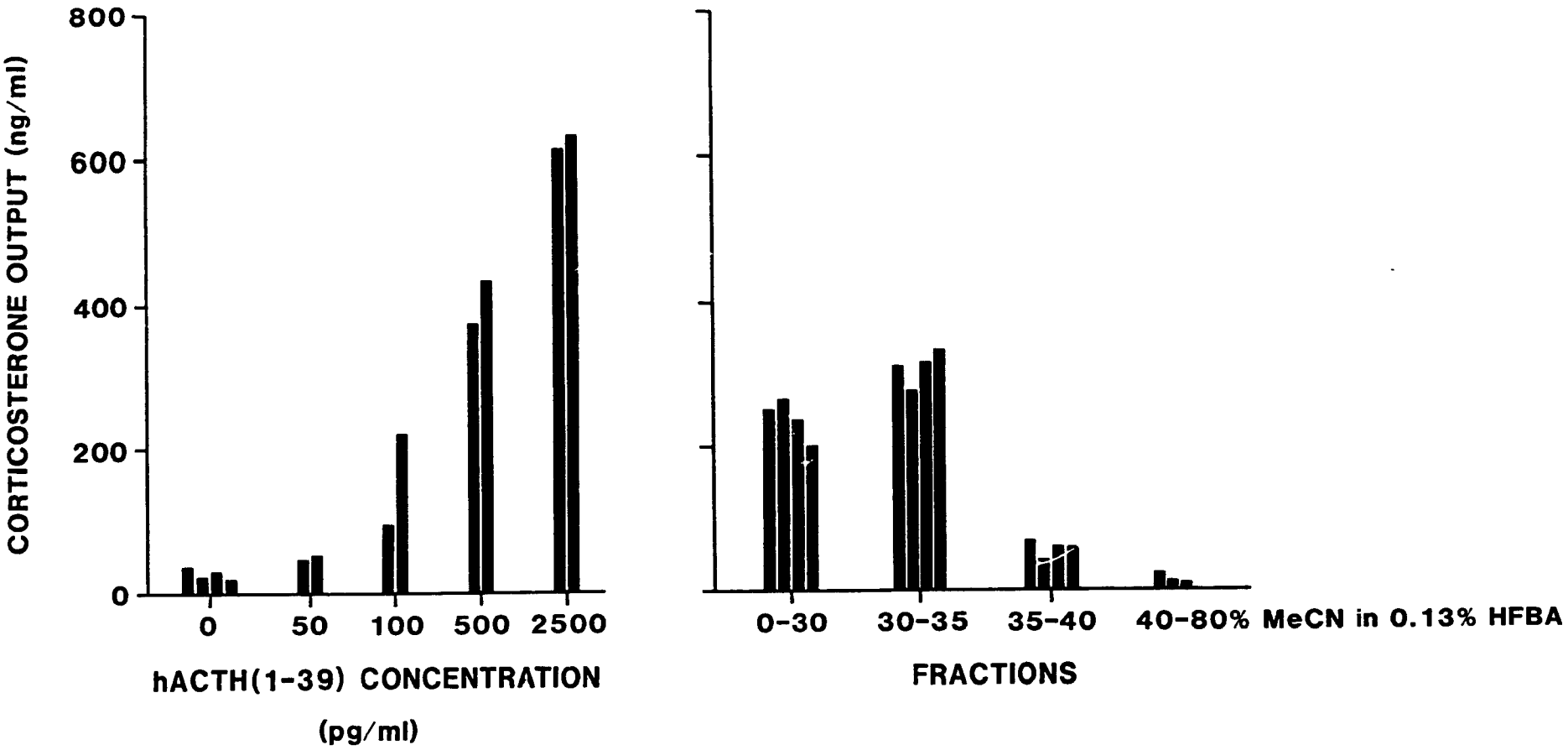

800
600
400
200
0
CORTICOSTERONE OUTPUT (ng/ml)
0
50
100
500
2500
hACTH(1-39) CONCENTRATION
(pg/ml)
0-30
30-35
35-40
40-80% MeCN in 0.13% HFBA
FRACTIONS

Fig. 3-2 Adrenocorticotropic activity in fetal rabbit lung extracts. 16 pairs of fetal rabbit lungs (15.4 g) were extracted with acidic extraction medium as described in section 2.2.1.1 . Extracts were passed through a set of five C_{18} Sep-Pak which was first conditioned by flushing with 20 ml of 80% acetonitrile containing 0.13% HFBA followed by 30 ml of 0.13% HFBA prior to use. The cartridges were then washed with 50 ml of 0.13% HFBA to remove unbound material. Peptides bound to the ODS-silica resin were fractionated first with 10 ml 30% acetonitrile containing 0.13% HFBA and then with 10 ml of 35% acetonitrile containing 0.13% HFBA, followed with 10 ml of 40% acetonitrile containing 0.13% HFBA, and finally with 10 ml of 80% acetonitrile containing 0.13% HFBA. Aliquots of 325 μl (equivalent to 1 gram tissues) were taken from each fraction and dried in the speed-vac for testing for adrenocorticotropic activity in the rat adrenal cell bioassay as previously described. Bars represent corticosterone released by dispersed rat adrenal cells in response to increasing concentrations of $hACTH_{1-39}$ and to materials contained in each fraction.

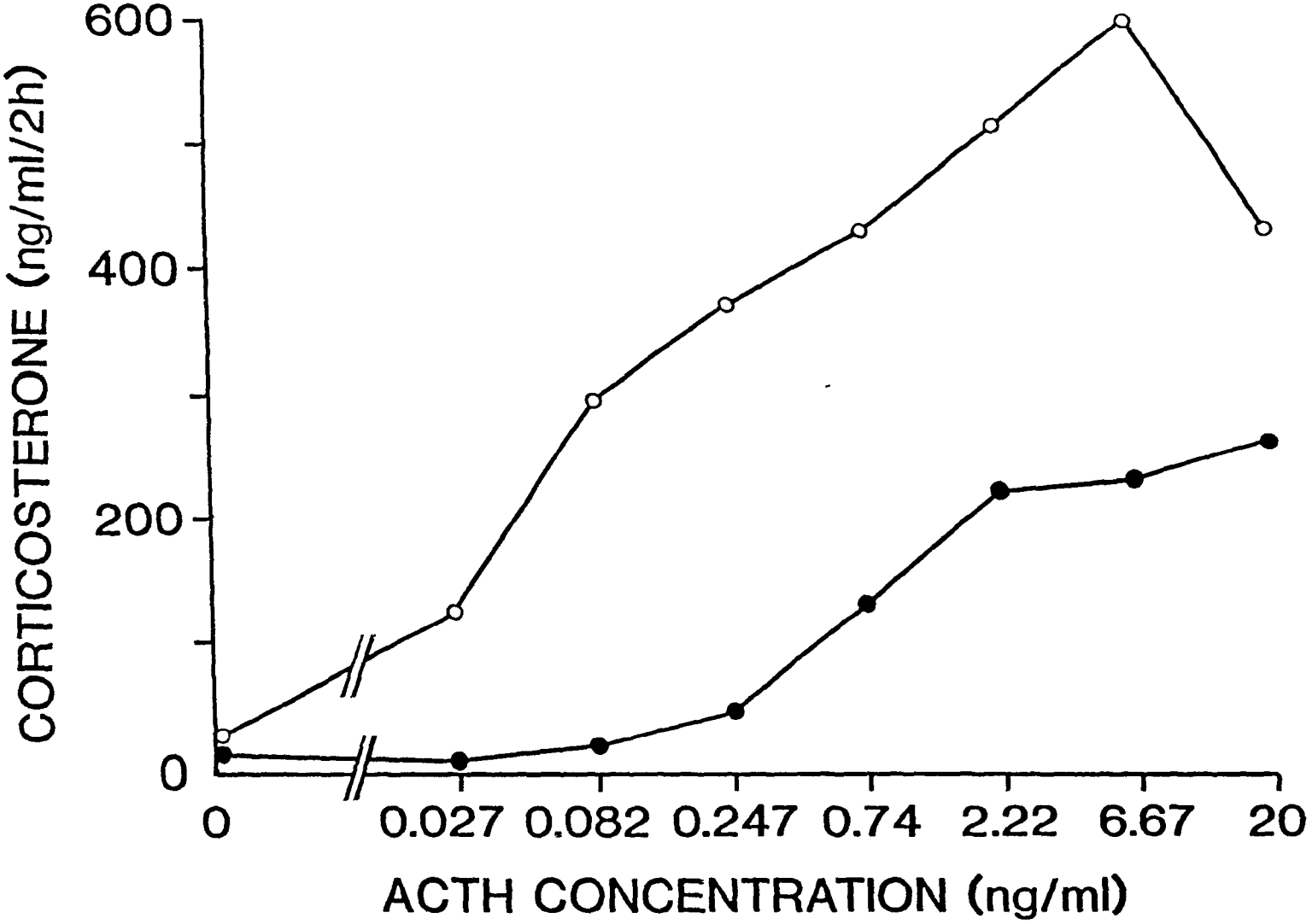

600
400
200
0
CORTICOSTERONE (ng/ml/2h)
0
0.027
0.082
0.247
0.74
2.22
6.67
20
ACTH CONCENTRATION (ng/ml)

Fig. 3-3 Inhibition of ACTH-stimulated corticosterone output by material in fraction 40-80% acetonitrile from Fig. 3-2.

Dose response of corticosterone produced by rat adrenal cells stimulated by increasing concentration of hACTH (o) and by hACTH plus aliquots (325 μl) from fractions 40-80% acetonitrile (●). The aliquots of 325μl were dried in the Speed Vac before co-incubation with ACTH and adrenal cells.

Corticosterone (ng/2x10⁵ cells/2h)
150
120
90
60
30
0

Fig. 3-4 The loss of the anti-ACTH activity following trypsin digestion. A total of 650 μl aliquots of the 40-80% acetonitrile fraction were dried in the Speed Vac and dissolved in 0.4 ml of 1% NH_4HCO_3, then divided into two. One part was co-incubated with 0.1 mg of trypsin in 0.2 ml of 1 mM HCl at 37 °C for 3.5 hr, and the other was co-incubated with 0.2 ml of 1 mM HCl first and mixed with 0.1 mg of trypsin at the end of the incubation as control. The reaction was stopped by adding 2 ml of 1% TFA. The incubation products were then passed through C_{18} Sep-Pak, which were washed with 30 ml of 0.1% TFA and eluted with 3 ml of 80% acetonitrile separately. The eluates were dried in the Speed Vac and their anti-ACTH activities were tested in the rat adrenal cell bioassay system as previously described. In this experiment 0.025% (w/v) trypsin inhibitor was added to the incubation medium and 100 pg/ml of hACTH was either added alone (first bar) or co-incubated with trypsin untreated aliquots from 40-80% acetonitrile fraction (second bar) or the controls (third bar) or with trypsinized products (fourth bar) together with the adrenal cells.

3.2. Purification and Characterization of Corticostatins

3.2.1. Purification of Corticostatins

To facilitate the isolation of ACTH-inhibitory peptides, a sensitive and reproducible bioassay was established, detailed in section 2.5.1. Fig. 3-5 shows the increase in corticosterone production on a logarithmic scale with increasing $hACTH_{1-39}$ concentrations in the rat adrenal cell preparation. The response reached a plateau at 740 pg/ml and the 50% effective dose (ED_{50}) was about 150 pg/ml (33 pM). An inhibition assay was then set up using 150 pg of ACTH per ml and adding HPLC column fraction in amounts sufficient to inhibit corticosterone production stimulated by this amount of trophic hormone. The protocol of the purification is shown as below:

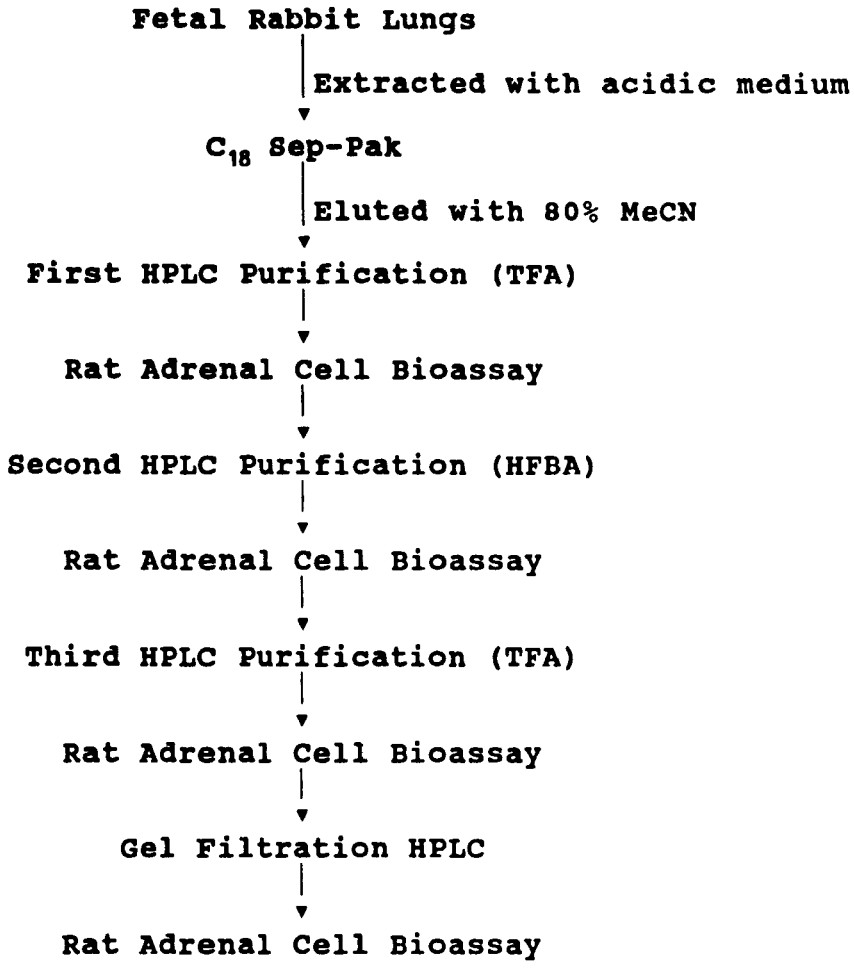


Fig. 3-6 shows that there were four areas of inhibitory materials that eluted from the column, the most abundant of which had a retention time of 72-96 min. This material was further purified on a second HPLC column using 0.13% HFBA as the counter-ion. As shown in Fig. 3-7, the peptide eluted in 38% acetonitrile and most of the proteins eluted earlier. A large degree of purification was thus achieved. The material that eluted with a retention time of 44-46 min from Fig. 3-7 was

purified by HPLC using trifluoroacetic acid as a counter ion as shown in Fig. 3-8. It is obvious from this chromatogram that the peptide was still not pure, necessitating a further purification step using gel filtration HPLC in which a uniform peak (fractions 17, 18 and 19) was obtained as shown in Fig. 3-9. Amino acid analysis of this material indicated that the peptide was homogeneous (Table 3-1).

Table 3-1. Amino acid composition of CSI after hydrolysis

	Fraction			
AMINO ACID	17		18	19
Aspartic acid	2.0		2.2	2.1
Asparagine		(2)		
Serine	2.7	(3)	2.8	2.9
Glutamic acid	1.1	(1)	1.0	1.1
Proline	1.0	(1)	1.0	1.0
Glycine	2.8	(3)	2.9	2.7
Alanine	1.9	(2)	2.0	1.7
Cysteine	5.4	(6)	5.5	5.2
Valine	2.2	(2)	2.0	2.0
Isoleucine	1.0	(1)	1.0	1.0
Tyrosine	2.0	(2)	2.0	2.1
Phenylalanine	2.0	(2)	2.3	2.0
Arginine	9.4	(9)	10.1	9.4

Numbers in parentheses are calculated from sequence analysis data (34 amino acids; M.W. 3993)

The homogeneous sample was then sent to Dr. F. Esch laboratory (Salk Institute, San Diego, CA) for sequence analysis. Gas-phase microsequencing of the peptide indicated the following sequence: **Gly-Ile-Cys-Ala-Cys-Arg-Arg-Arg-Phe-Cys-Pro-Asn-Ser-Glu-Arg-Phe-Ser-Gly-Tyr-Cys-Arg-Val-Asn-Gly-Ala-Arg-**

Tyr-Val-Arg-Cys-Cys-Ser-Arg-Arg. This peptide was named **corticostatin I** (CSI) because of its ability to inhibit ACTH and first one characterized. The ability of CSI to inhibit the secretion of corticosterone by rat adrenal cells under the stimulation of a fixed dose (33 pM) of ACTH is shown in Fig. 3-10. Total inhibition of ACTH stimulation can be achieved at high concentrations of CSI (2 μg/ml).

The ED_{50} of CSI was found to be 100 ng/ml (25 nM) and the minimum effective dose was 20 ng/ml (5 nM) against 150 pg/ml ACTH, because it is significantly different from the control value.

3.2.2. Purification of corticostatic peptides using the separation of basic peptides from neutral and acidic fractions.

In an effort to establish whether the rabbit fetal lung was the only source of corticostatic peptides, and to purify a large quantity of CSI for the further study, a simple and quantitative method for rapid scanning of tissues was developed.

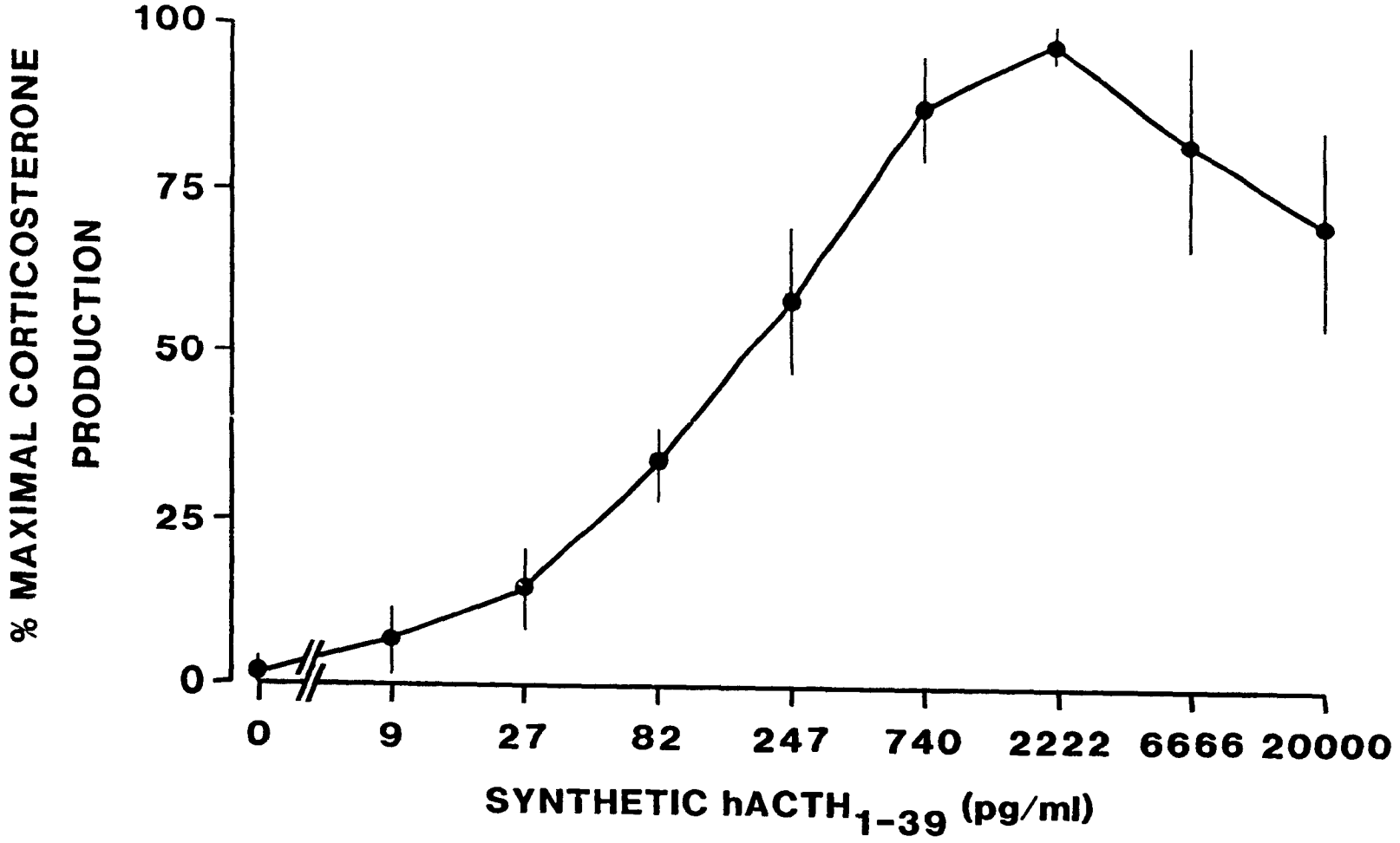

% MAXIMAL CORTICOSTERONE PRODUCTION
100
75
50
25
0
0
9
27
82
247
740
2222
6666
20000
SYNTHETIC hACTH1-39 (pg/ml)

Fig. 3-5. Dose response of corticosterone released by rat adrenal cells in response to increasing concentration of synthetic $hACTH_{1-39}$. The values shown are the means of 11 experiments done in duplicate (mean ± SD).

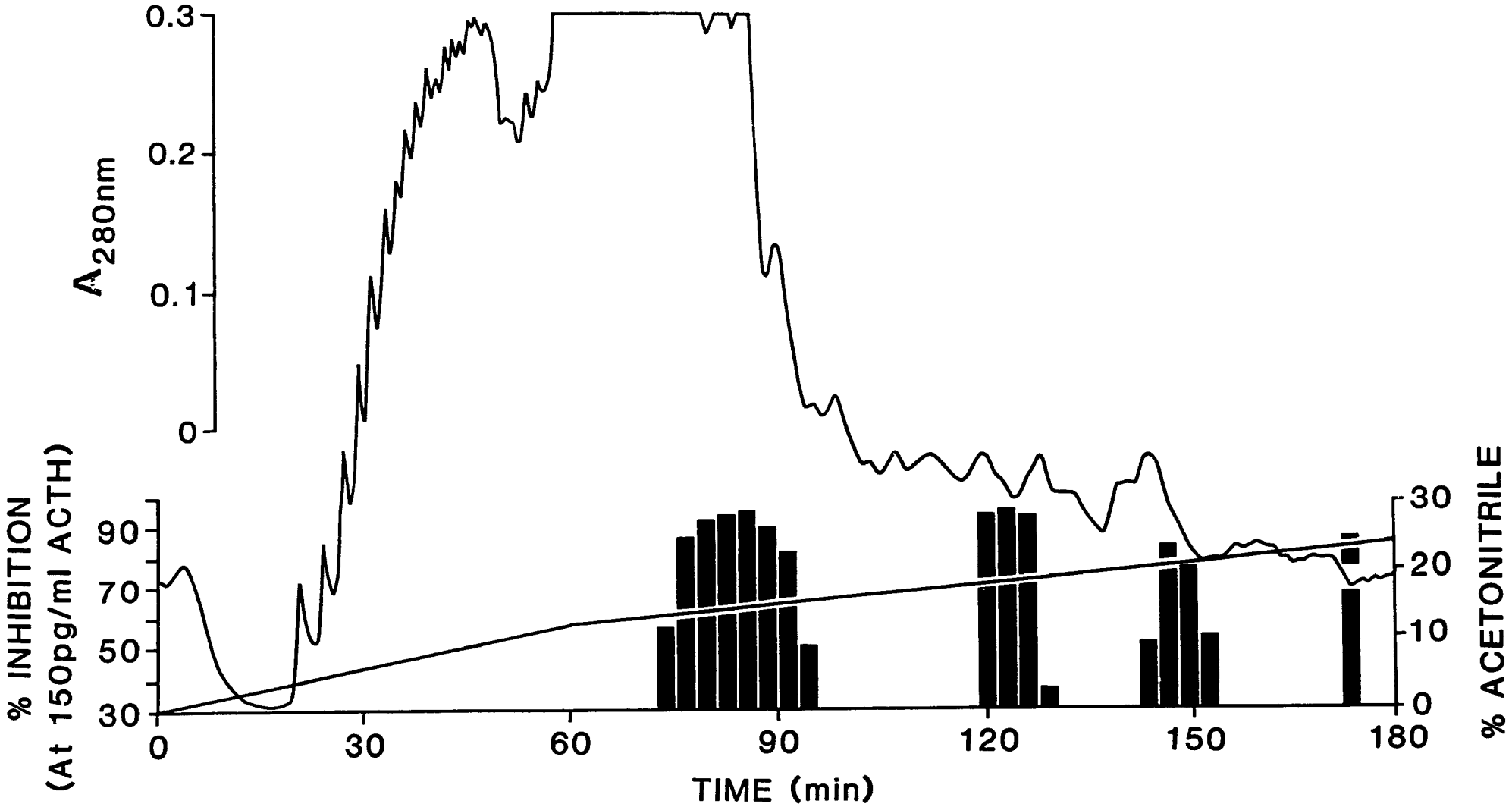

0.3
0.2
0.1
0
A_{280nm}
% INHIBITION
(At 150pg/ml ACTH)
90
70
50
30
30
20
10
0
% ACETONITRILE
0
30
60
90
120
150
180
TIME (min)

Fig. 3-6 The elution pattern of inhibitory peptides from a reverse-phase HPLC column of an extract of 100 pairs of fetal rabbit lungs The lungs were extracted as described in sections 2.2.1.1. and 2.2.1.2. The tissue extract was diluted to 20 ml with 0.1% TFA and pumped directly onto a C_{18} μBondapak column that was washed with 100 ml of 0.1% TFA. The column was eluted at a flow rate of 1.0 ml/min for 3 hr. Two linear gradients were used: 0-12% acetonitrile for 60 min followed by 12-24% acetonitrile for the next 120 min. Fractions were collected every 3 min. Aliquots (100 μl) were taken from fractions 20-60 and bioassayed as described. Bars indicate percentage inhibition of ACTH (150 pg/ml) added to the cells along with the fractions from the column. Corticosterone production was used as the end point in obtaining an ACTH titre in the inhibition reaction.

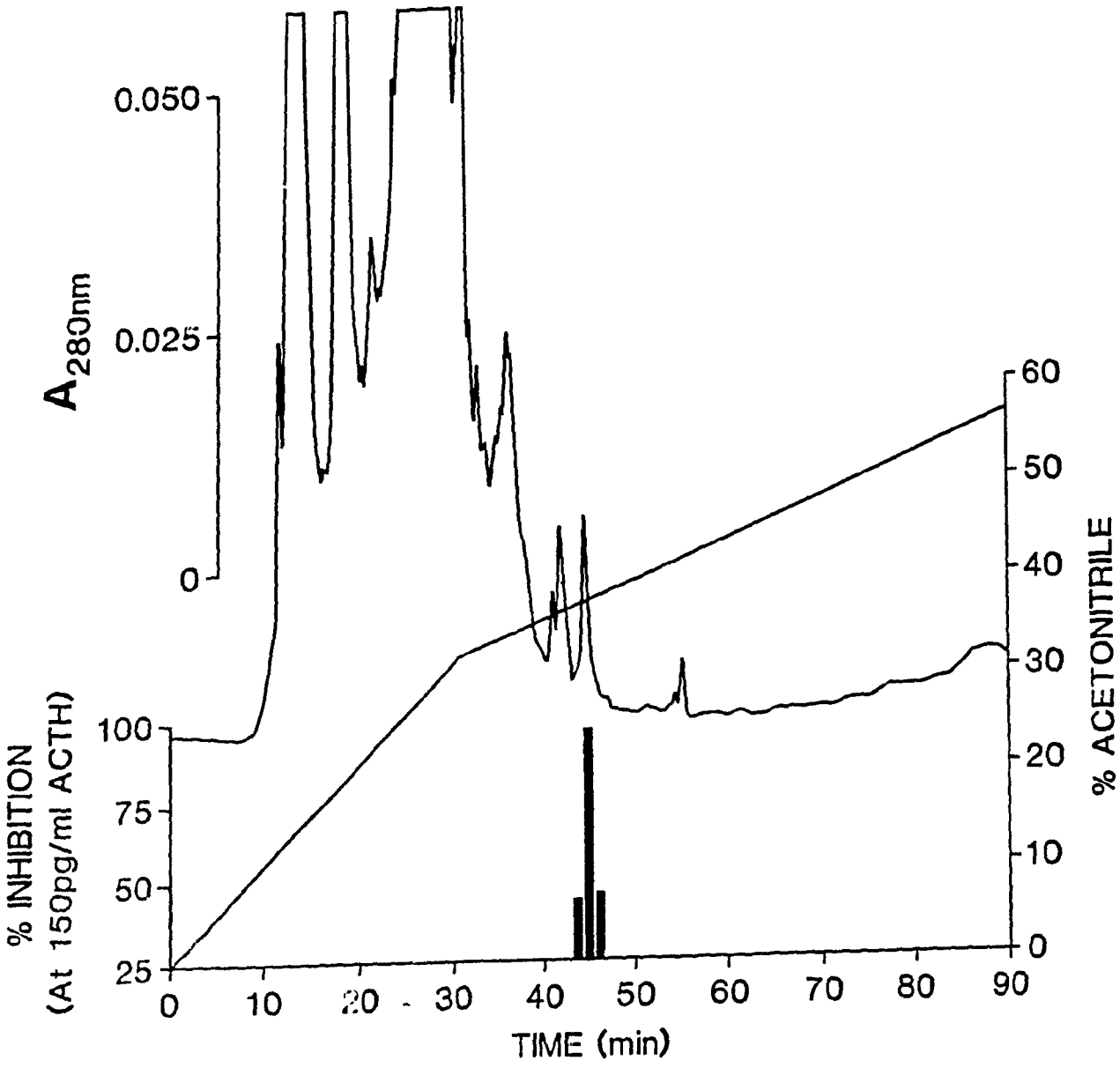

0.050
0.025
0
A_{280nm}
% INHIBITION
(At 150pg/ml ACTH)
100
75
50
25
0
10
20
30
40
50
60
70
80
90
TIME (min)
60
50
40
30
20
10
0
% ACETONITRILE

Fig. 3-7 Second Reversed-phase HPLC purification of the anti-ACTH peptide. Reversed-phase HPLC of the first peak of inhibitory activity from Fig. 3-6. Fractions with retention times of 72-96 min from Fig. 3-6 were combined, concentrated, and pumped directly onto the HPLC column, which was eluted at a flow rate of 1.5 ml/min over 90 min with a linear gradient of 0-32% acetonitrile containing 0.13% HFBA during the first 30 min and 32-56% acetonitrile containing 0.13% HFBA over the next 60 min. One minute fractions were collected and submitted to bioassay as described.

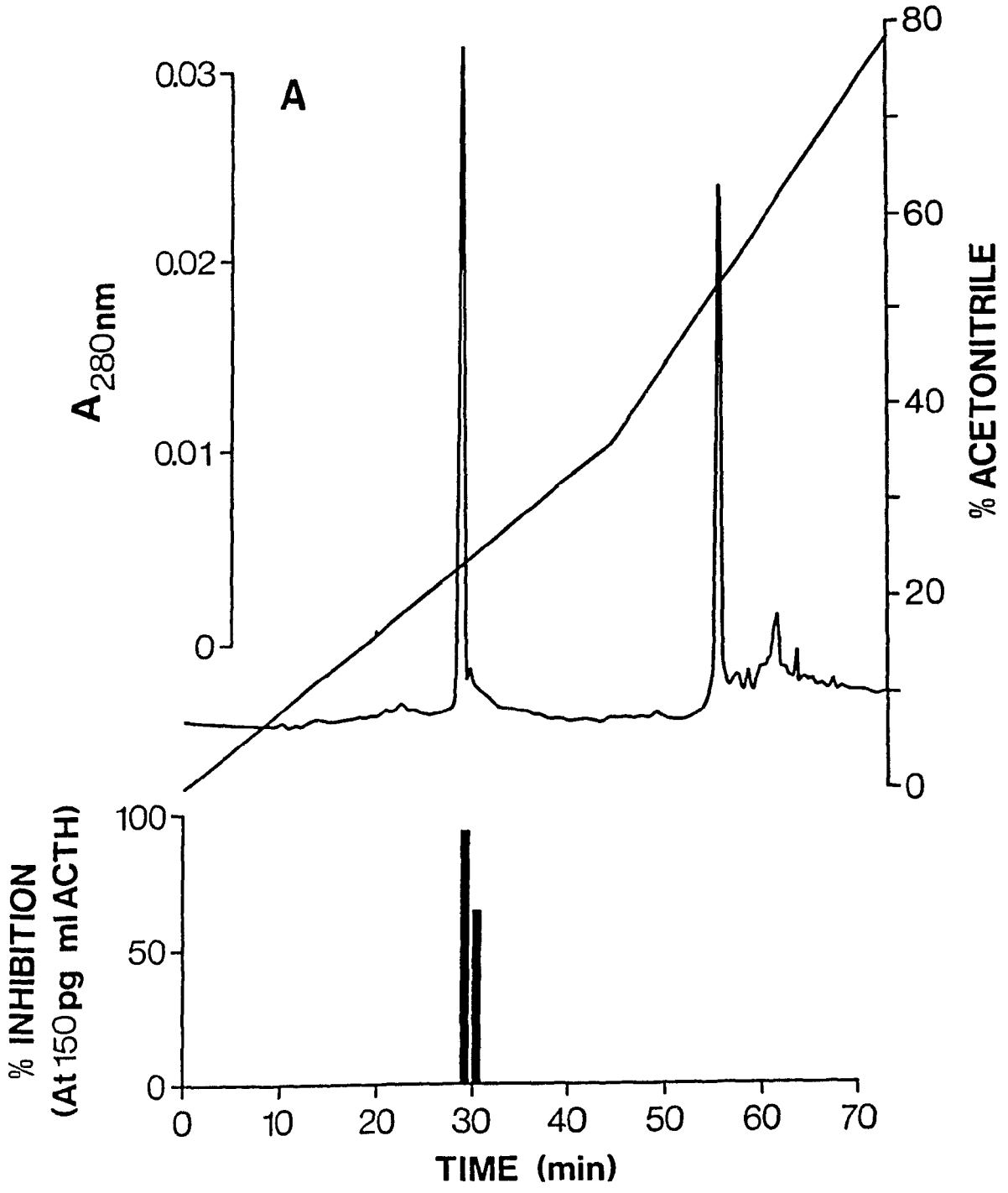

A
0.03
0.02
0.01
0
A_{280nm}
80
60
40
20
0
% ACETONITRILE
100
50
0
% INHIBITION
(At 150 pg ml ACTH)
0
10
20
30
40
50
60
70
TIME (min)

Fig. 3-8 Purification of the inhibitory material from Fig 3-7. Fractions 44-46 of Fig. 3-7 were combined, concentrated, and pumped onto an HPLC column using the same conditions described in Fig. 3-6. The linear gradient was 0-35% acetonitrile containing 0.1% TFA for the first 45 min and 36-80% acetonitrile for the next 25 min. The flow rate was 1.5 ml/min. Fraction were collected by hand and submitted to bioassay as described.

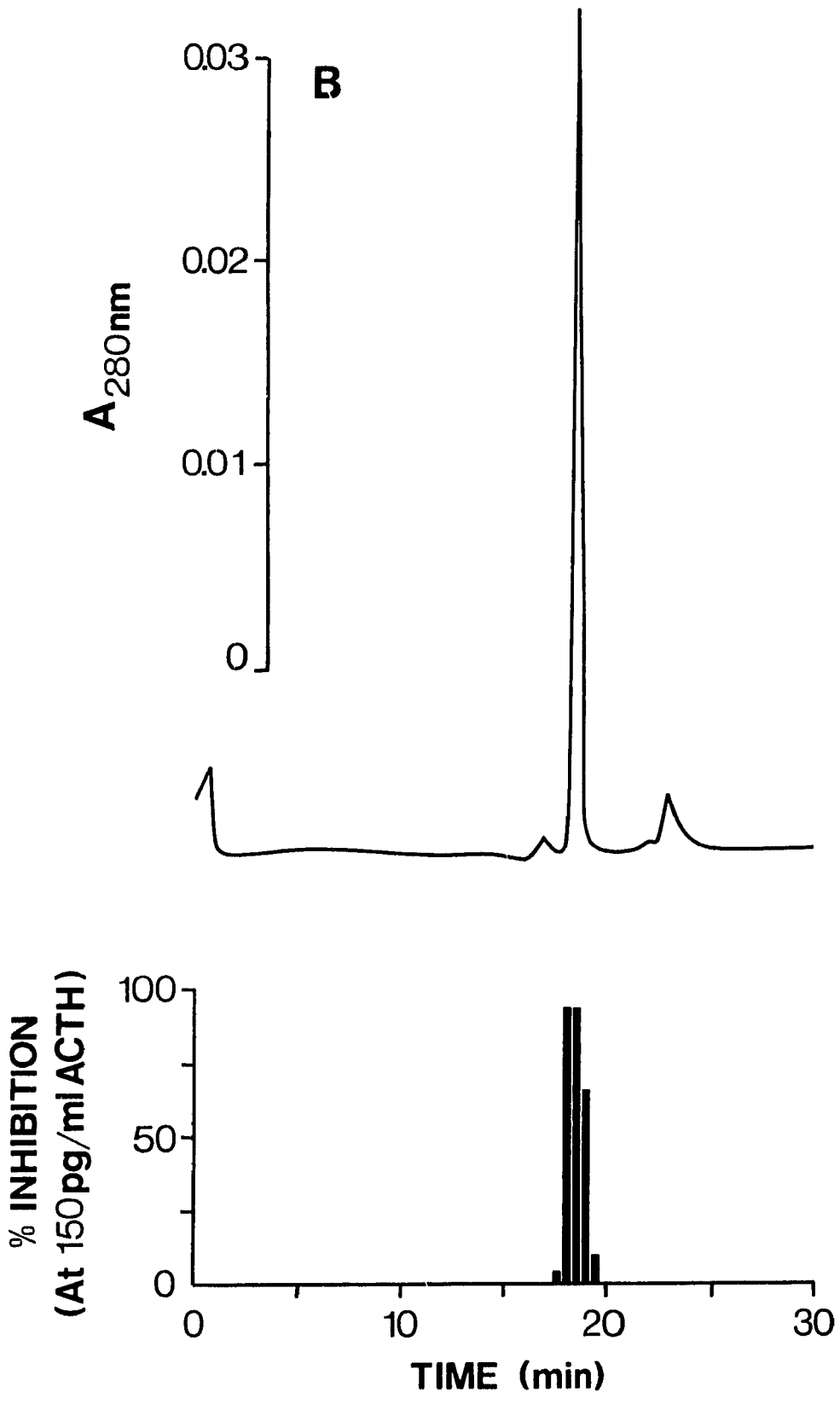
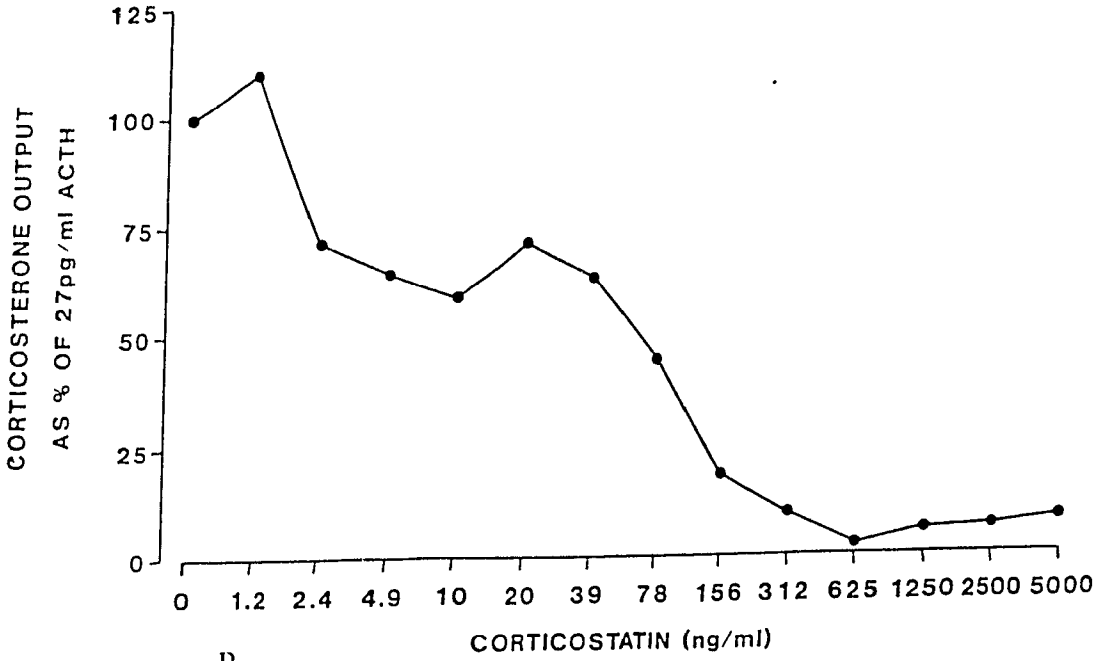
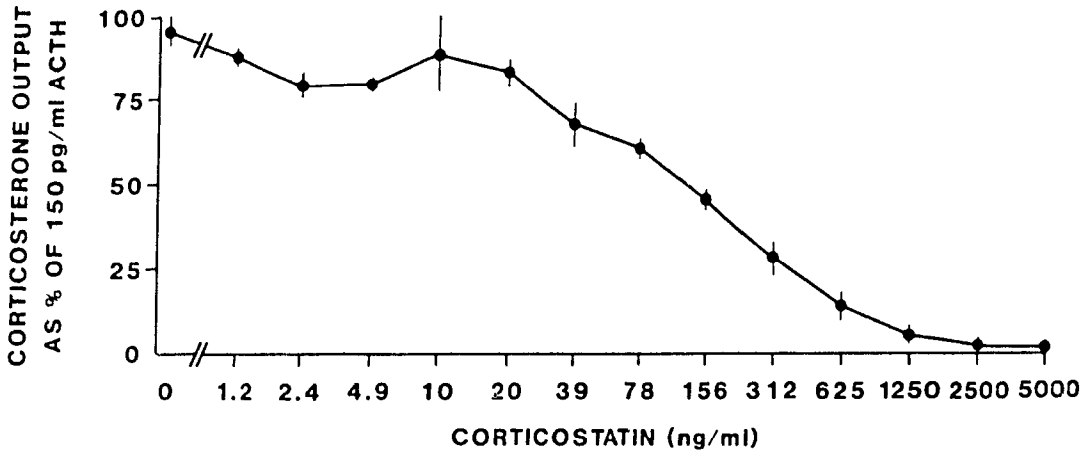

B
0.03
0.02
0.01
0
A_{280nm}
100
50
0
% INHIBITION
(At 150pg/ml ACTH)
0
10
20
30
TIME (min)

Fig. 3-9 The last purification of the anti-ACTH peptide by gel filtration HPLC. The fractions that eluted at 29 and 30 min in Fig. 3-8 were combined, concentrated in the Speed Vac to about 100 μl and injected onto two gel filtration columns using the condition as described in section 2.2.2.3. Fractions were collected every half minute and submitted to bioassay as described.

A


CORTICOSTERONE OUTPUT
AS % OF 27pg/ml ACTH
125
100
75
50
25
0
0 1.2 2.4 4.9 10 20 39 78 156 312 625 1250 2500 5000
CORTICOSTATIN (ng/ml)

B


CORTICOSTERONE OUTPUT
AS % OF 150 pg/ml ACTH
100
75
50
25
0
0 1.2 2.4 4.9 10 20 39 78 156 312 625 1250 2500 5000
CORTICOSTATIN (ng/ml)

C

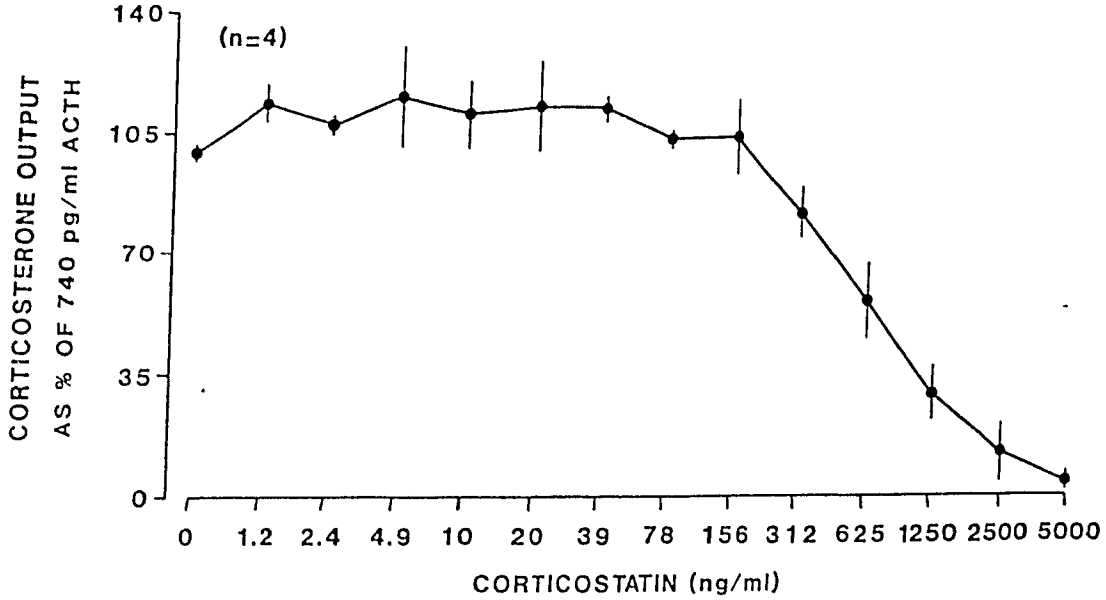

(n=4)
CORTICOSTERONE OUTPUT
AS % OF 740 pg/ml ACTH
140
105
70
35
0
0 1.2 2.4 4.9 10 20 39 78 156 312 625 1250 2500 5000
CORTICOSTATIN (ng/ml)

Fig. 3-10 Inhibition of ACTH-stimulated corticosterone output by CSI. ACTH was maintained at 27 pg/ml (A), 150 pg/ml (B), 740 pg/ml, and CSI was increased up to 5000 ng/ml. The values shown are the average of duplicate determinations in figure (A). In figures (B) and (C) are shown the mean ± SD of four separate experiments.

CHAP. 3. Purification

One of the most striking characteristics of CSI is its high content of basic amino acids. Thus cationic exchange chromatography should be one of the most suitable ways of isolating these peptides. A relatively weak cationic exchange silica based carboxymethyl (CM) cation-exchange cartridge and high ionic strength buffer (150 mM, pH 7.2 Tris buffer) were used for this purpose. The method was based on the results of pilot experiments (Fig. 3-11) using radio-iodinated CSI (see section 2.2.1.1.). Under these conditions only CSI (eight net positive changes) and a few basic peptides were retained by the cartridge. In subsequent experiments the purification procedure was simplified by including the preliminary step of cation-exchange (described in 2.2.1.3.) prior to HPLC.

Fig. 3-12 compares the HPLC profiles of the cationic fraction of fetal rabbit lung (a), adult rabbit lung (b), and rabbit peritoneal neutrophils (c). The rabbit peritoneal neutrophils were obtained as described in Materials and Methods. These results indicated that adult rabbit lung was the richest source of CSI. Several other tissues such as heart, kidney, and liver (Fig. 3-13) were also screened but CSI like peptides present in the lung and neutrophil extracts were undetectable in these tissues.

Using of the CM (carboxymethyl) cation exchange batch extraction method greatly facilitated our studies at that time

since an antiserum against CSI was not available yet. Using this technique we were able to visualize the peak fractions by using two Sep-Pak cartridge extraction steps and reversed-phase HPLC and without the use of the bioassay.

It was necessary to determine the number of CM Sep-Pak cartridges needed for efficient purification of extracts resulting from the procedure described in the materials and methods section (CHAPTER 2) of this thesis (2.2.1.3.) This was established according to the results shown in Fig. 3-14. It was also found that HFBA system gave better resolution than TFA system. Fig. 3-15 (upper panel) shows the first reversed-phase HPLC purification of the eluates of these basic peptides using 0.13% HFBA as the counter-ion. There were only four major peaks in the cationic pools and the dispersed rat adrenocortical cell bioassay revealed that they were exclusively corticostatic materials (lower panel). These four peaks were provisionally named corticostatin I, II, III, and IV in the same order as they are eluted in the HFBA system. In order to test the purity of the first peak, fractions 43 to 47 from Fig. 3-15 were rechromatographed separately on a second HPLC gradient, using 0.1% TFA as the counter-ion as shown in Fig. 3-16. In contrast to the first purification protocol discuseed in section 3.2.1., the peptide was already more than 80% pure in the first purification and reached homogeneity in the second reversed-

phase HPLC. The purities of these corticostatic peptides were confirmed later by amino acid analysis and gel filtration HPLC. The other three major peaks in the first HPLC were also homogeneous after a second reversed-phase HPLC. Their amino acid compositions are shown in the table 3-2.

Table 3-2. Amino acid composition of CSs after hydrolysis

	CORTICOSTATIC PEPTIDES							
AMINO ACID	CSI		CSII		CSIII		C S I V	
Aspartic acid	2.0	(0)	1.0	(1)				
Asparagine		(2)						
Serine	2.7	(3)	1.0	(1)				
Glutamic acid	1.1	(1)	2.1	(1)	1.1	(1)	1.1	(1)
Glutamine				(1)				
Proline	1.0	(1)	2.0	(2)	2.1	(2)	2.0	(2)
Glycine	2.8	(3)	2.9	(3)	1.9	(2)	1.8	(2)
Alanine	1.9	(2)			2.8	(3)	2.9	(3)
Cysteine	5.4	(6)	4.7	(6)	4.5	(6)	4.3	(6)
Valine	2.2	(2)	2.1	(2)	2.2	(2)	2.2	(2)
Isoleucine	1.0	(1)	2.0	(2)	2.1	(2)	2.2	(2)
Leucine			2.2	(2)	3.1	(3)	4.2	(4)
Tyrosine	2.0	(2)	1.0	(1)				
Phenylalanine	2.0	(2)	2.0	(2)	1.1	(1)	1.2	(1)
Histidine					0.9	(1)	0.9	(1)
Lysine			2.1	(2)				
Arginine	9.4	(9)	8.2	(8)	10.4	(10)	9.4	(9)
Molecular Weight	3993		4065		3887		3844	

Numbers in parentheses are calculated from sequence analysis data.

Gas-phase microsequencing of the peptides indicated the following sequence:

CSI	Gly	Ile	Cys	Ala	Cys	Arg	Arg	Arg	Phe	Cys	Pro	Asn	Ser
CSII	Gly	Arg	Cys	Val	Cys	Arg	Lys	Gln	2Leu	Cys	Ser	Tyr	Arg
CSIII	Val	Val	Cys	Ala	Cys	Arg	Arg	Ala	Leu	Cys	Leu	Pro	Arg
CSIV	Val	Val	Cys	Ala	Cys	Arg	Arg	Ala	Leu	Cys	Leu	Pro	Leu

CSI	Glu	Arg	Phe	Ser	Gly	Tyr	Cys	Arg	Val	Asn	Gly	Ala	Arg
CSII	Glu	Arg	Arg	Ile	Gly	Asp	Cys	Lys	Ile	Arg	Gly	Val	Arg
CSIII	Glu	Arg	Arg	Ala	Gly	Phe	Cys	Arg	Ile	Arg	Gly	Arg	Ile
CSIV	Glu	Arg	Arg	Ala	Gly	Phe	Cys	Arg	Ile	Arg	Gly	Arg	Ile

CSI	Tyr	Val	Arg	Cys	Cys	Ser	Arg	Arg (34)
CSII	Phe	Pro	Phe	Cys	Cys	Pro	Arg	(34)
CSIII	His	Pro	Leu	Cys	Cys	Arg	Arg	(33)
CSIV	His	Pro	Leu	Cys	Cys	Arg	Arg	(33)

It is not surprising to find out that the CSI from adult rabbit lung has the same sequence as the one from fetal rabbit lungs. By using the same procedure, CS's were also purified from peripheral leukocytes and lipopolysaccharide treated rabbit peritoneal exudate neutrophils. These tissues had almost the same peptide pattern as that from rabbit adult lungs. The abilities of the four CSs to inhibit a fixed dose (150pg/ml) of ACTH in the secretion of corticosterone by dispersed rat adrenocortical cells are shown in Fig. 3-17. CSI was the most potent inhibitor of ACTH stimulated steroidogenesis. The minimum

effective dose of CSI was 5 nM (20 ng/ml) and it reached a plateau and completely inhibited ACTH stimulation at greater than 500 nM concentrations (2 ug/ml). The ED_{50} of CSI, CSII, CSIII, CSIV were approximately 100, 500, 1500, and 2000 ng/ml, respectively.

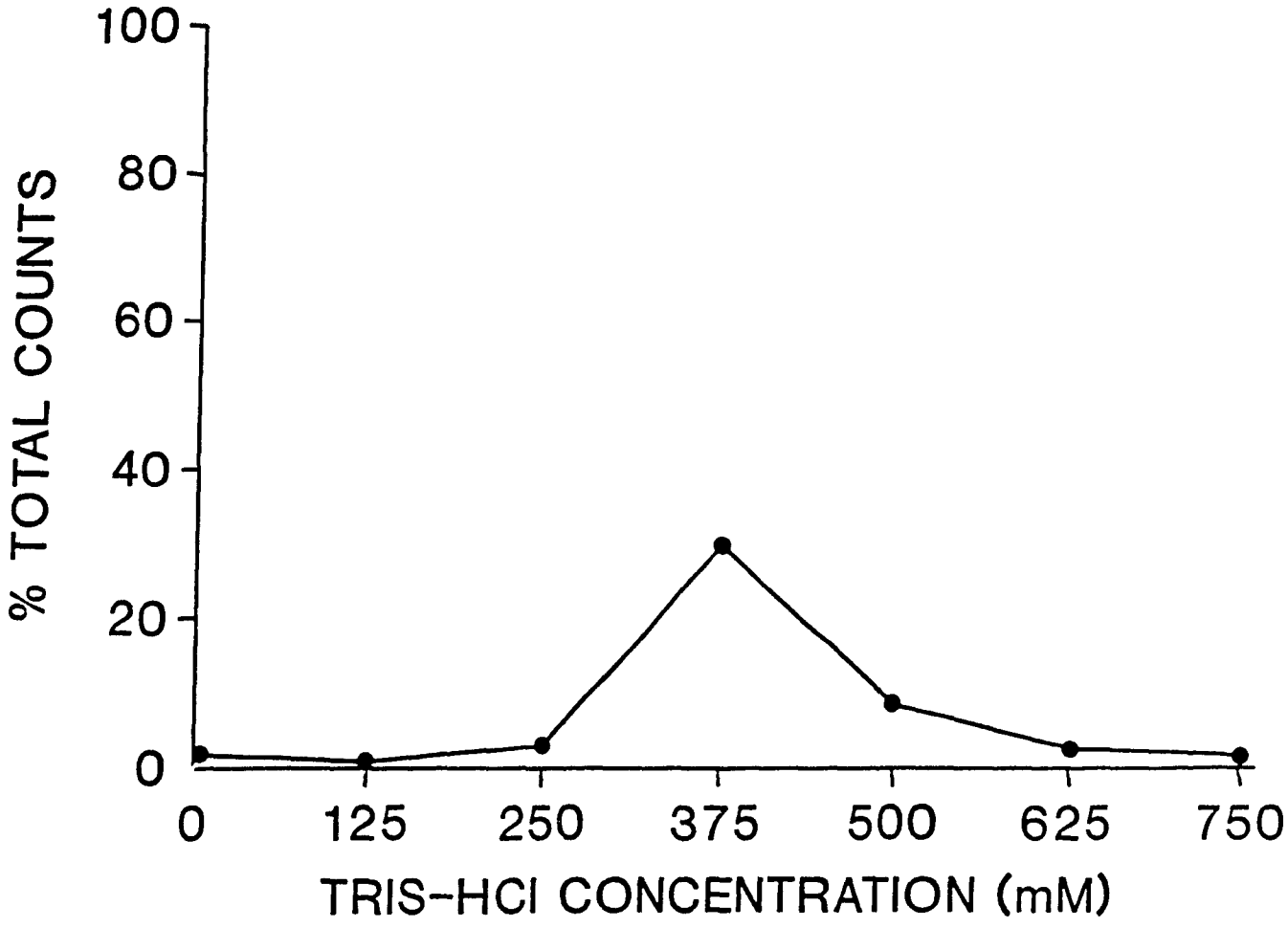

100
80
60
40
20
0
% TOTAL COUNTS
0
125
250
375
500
625
750
TRIS-HCl CONCENTRATION (mM)

Fig. 3-11 The fractionation of the radiolabelled CSI by CM cationic exchange cartridge. 2 μg of rabbit corticostatin I purified as indicated in Fig. 3-9 was radio-iodinated with I^{125} as described in section 2.4.1.1. A total of 546683 cpm in 20 ml of 5 mM Tris buffer pH 7.4 containing 20% acetonitrile was loaded onto a CM cationic exchange cartridge and fractionated by the addition of 10 ml of 50 mM, followed by 10 mls of 125 nM, 250 mM, 250 mM, 375 mM, 500 mM, 625 mM, and 750 mM finally by elution with 10 ml of 1250 mM of Tris buffer pH 7.4 (all Tris buffers used in this experiment contain 20% acetonitrile). The eluates were collected and counted in a gamma counter.

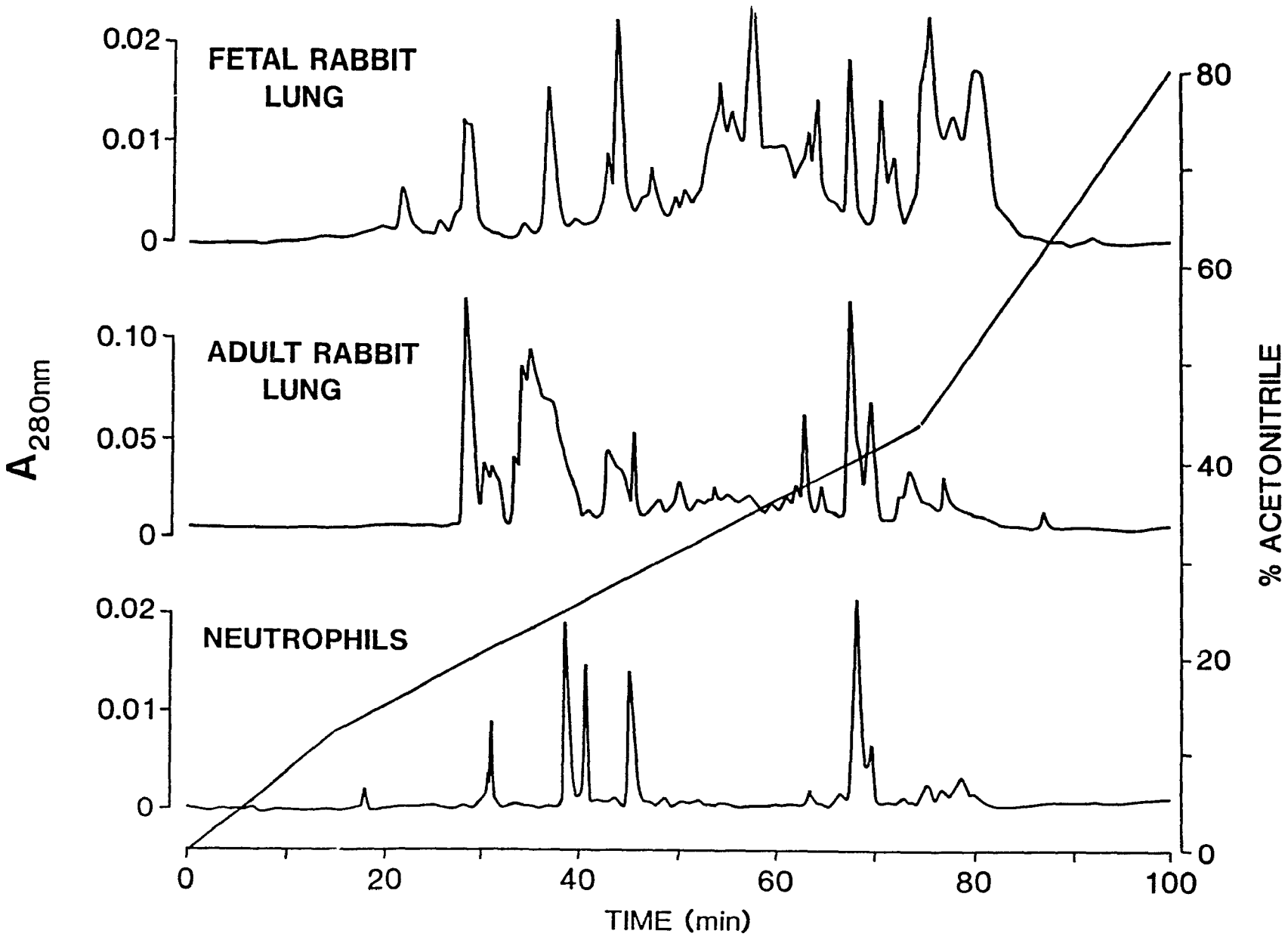

FETAL RABBIT LUNG
ADULT RABBIT LUNG
NEUTROPHILS
0.02
0.01
0
0.10
0.05
0
0.02
0.01
0
A280nm
0
20
40
60
80
100
TIME (min)
80
60
40
20
0
% ACETONITRILE

Fig. 3-12 The comparison of the amounts of cationic peptides in fetal rabbit lungs (16 pairs about 16.3 g), adult rabbit lung (one pair 17 g), and rabbit peritoneal neutrophils (from one rabbit). Tissues were extracted as described in section 2.2.1.. The linear gradient was 0-12% acetonitrile containing 0.1% TFA for the first 15 min and 12-44% acetonitrile for the next 60 min. The flow rate was 1.5 ml/min. The retention time for purified CSI had been determined and was 29 min. Therefore, CSI like materials eluted at 28 min for fetal and adult lung and at 31 min for neutrophils were submitted to the rat adrenal cell bioassay. All showed corticostatic activity.

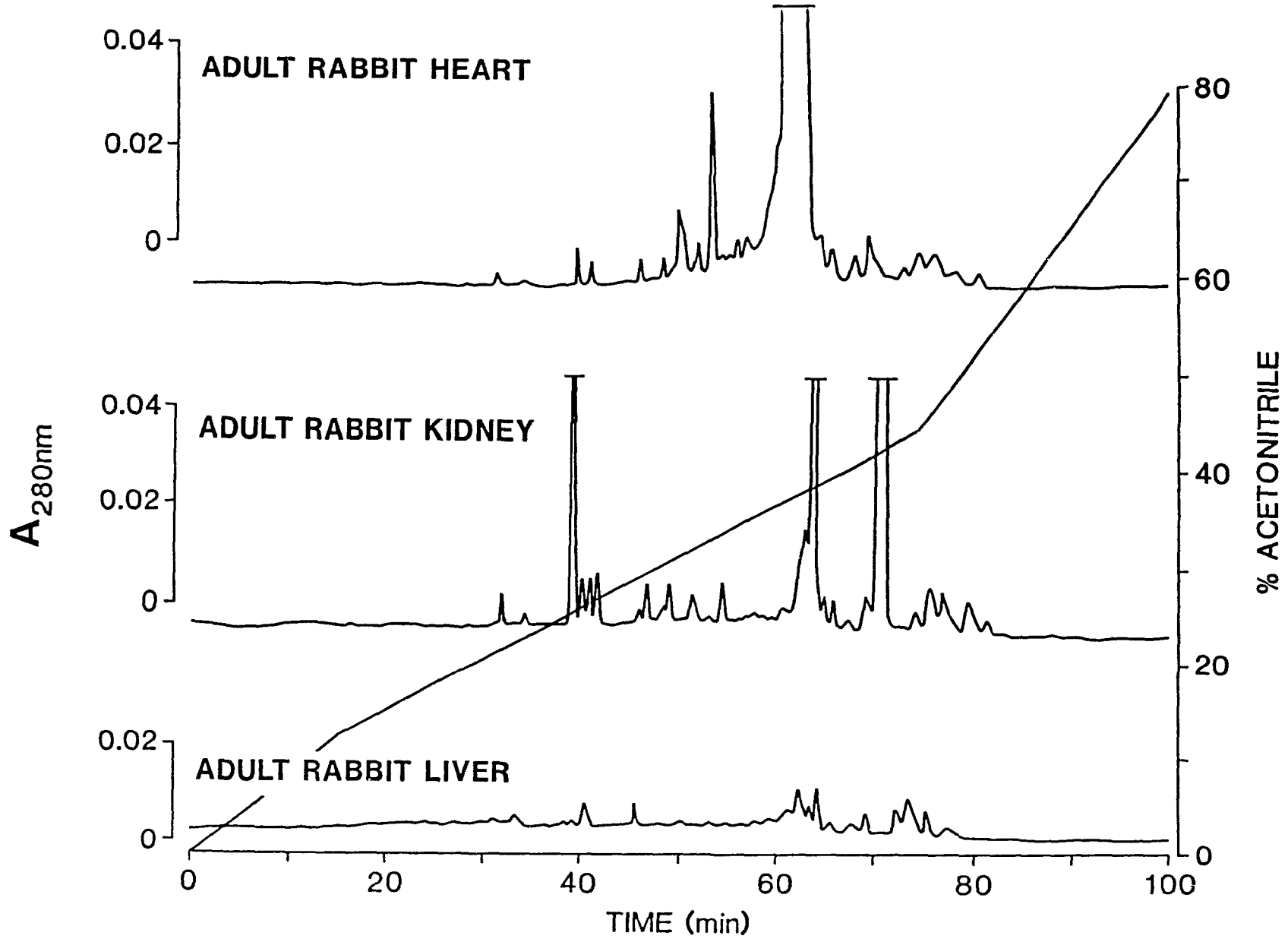

ADULT RABBIT HEART
ADULT RABBIT KIDNEY
ADULT RABBIT LIVER
0.04
0.02
0
A280nm
% ACETONITRILE
80
60
40
20
0
0
20
40
60
80
100
TIME (min)

Fig. 3-13. HPLC purification of cationic peptides from (a) adult rabbit heart (17 g), (b) kidney (17 g), (c) and liver (17 g). The conditions used were the same as those described in Fig. 3-12.

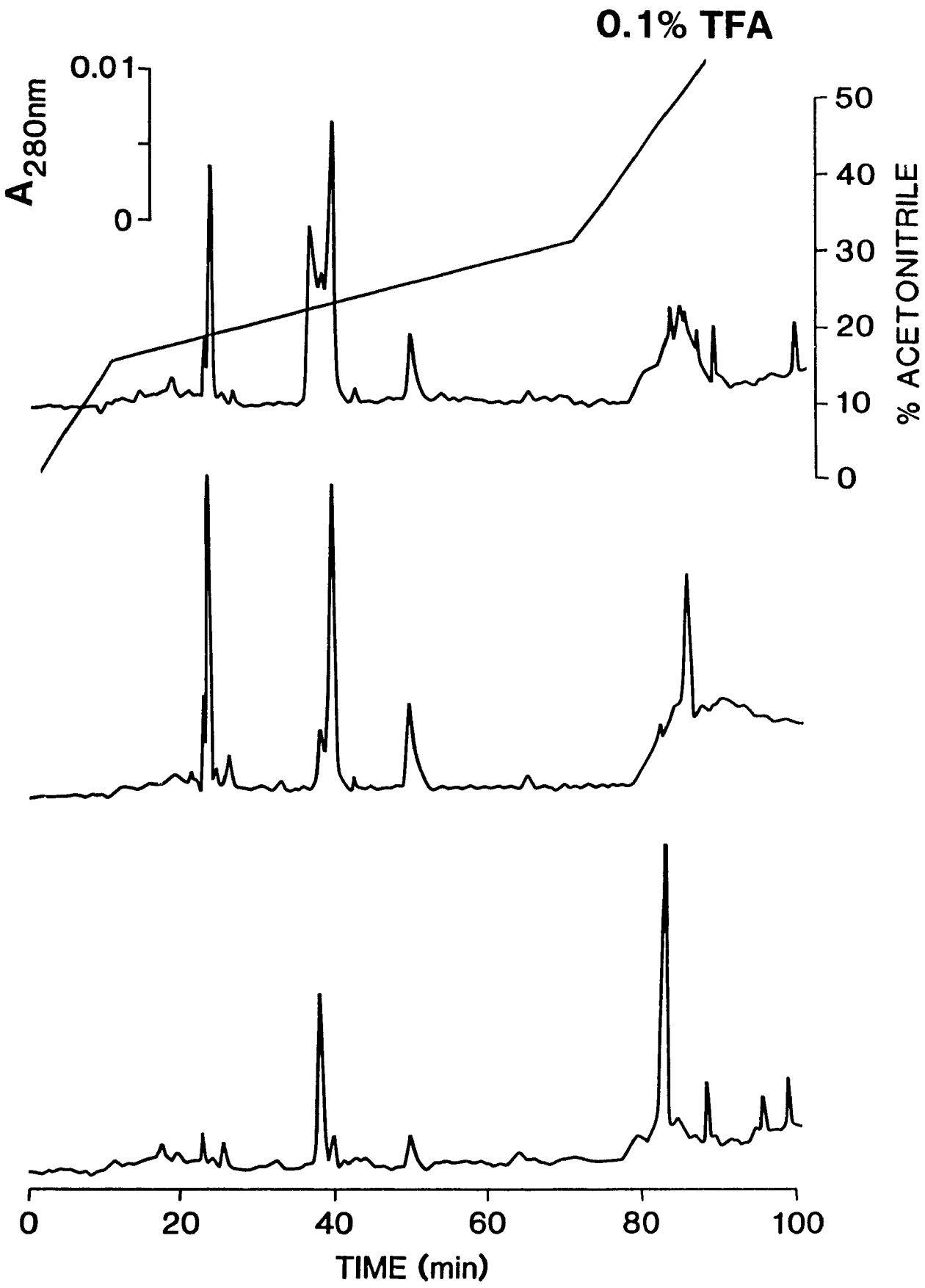

0.1% TFA
0.01
0
A_{280nm}
50
40
30
20
10
0
% ACETONITRILE
0
20
40
60
80
100
TIME (min)

Fig. 3-14 The recovery of cationic peptides after chromatography on CM-Silica cartridges. A total of 100 pairs of adult rabbit lungs were extracted using acid medium as previously described (see section 2.2.1.1.). The extracts were passed through a C_{18} Sep-Pak and eluted with 80% acetonitrile containing 0.1% TFA as described in section 2.2.1.2. A total of 30 ml of the C_{18} cartridge extracts was diluted with 4 volumes (120 ml) of 150 mM Tris buffer, pH 7.2, containing 20% acetonitrile and the mixture passed through three sets of two CM Sep-Pak connected in series by a headless pipet tip. The CM Sep-Paks were conditioned as described in section 2.2.1.3. prior to use. The aliquots equivalent to one pair of adult rabbit lungs were taken from eluates of each set CM Sep-Paks, dried in the Speed Vac and loaded onto a reversed-phase HPLC column. The gradient used was the same as described in Fig. 3-12.

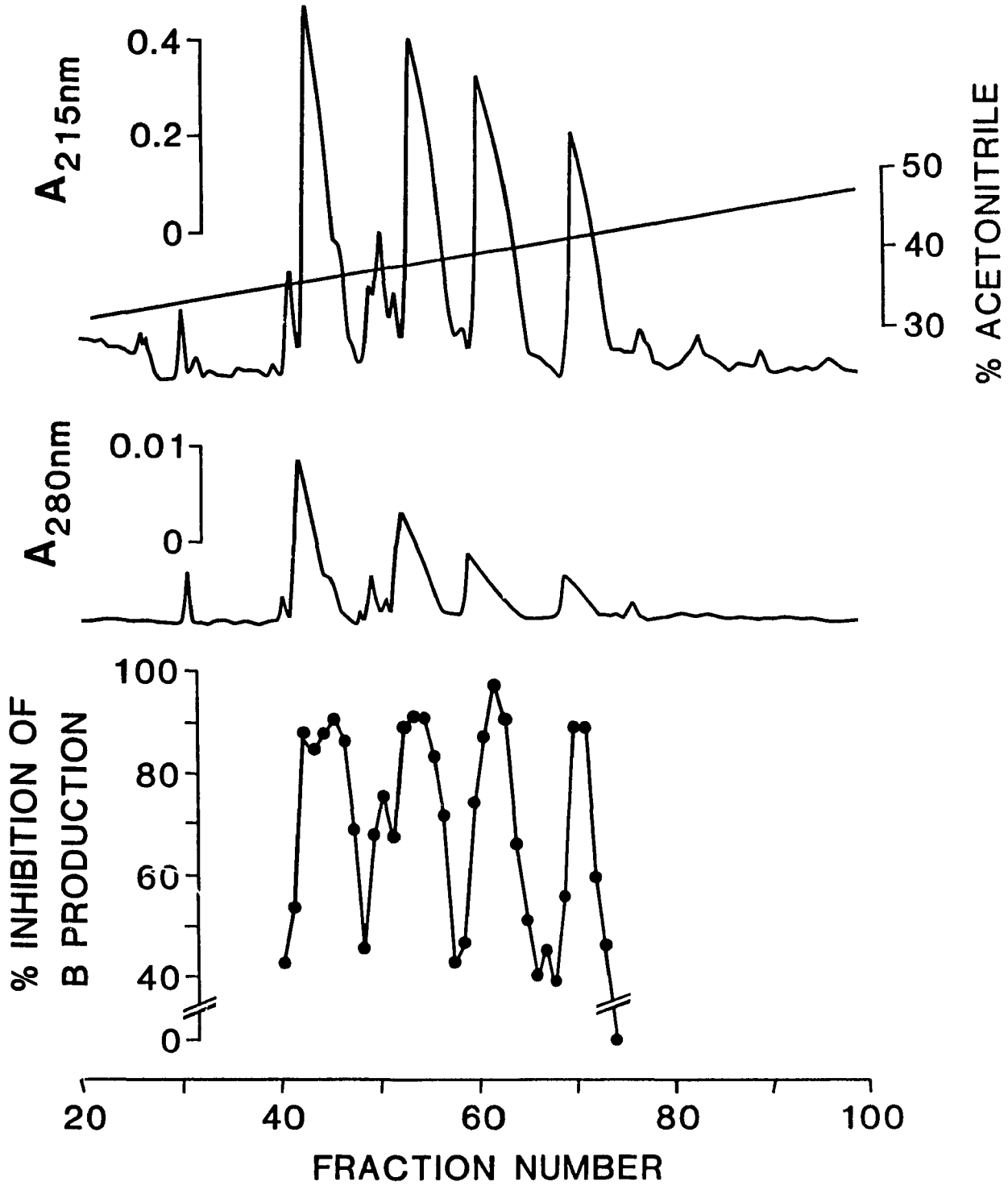

A_{215nm}
0.4
0.2
0
50
40
30
% ACETONITRILE
A_{280nm}
0.01
0
% INHIBITION OF B PRODUCTION
100
80
60
40
0
20
40
60
80
100
FRACTION NUMBER

Fig. 3-15 The purification of corticostatic peptides from adult rabbit lungs. Peptides from ten pairs of adult rabbit lungs were batch extracted using C_{18} Sep-Pak extraction then a cationic ion exchange cartridge (CM-Sep-Pak, Waters, MA), and purified by reversed-phase HPLC using a gradient of 0-32% acetonitrile over 20 minutes, followed by a linear gradient to 48% acetonitrile over 80 minutes. The flow rate was 1.5 ml/min, and 0.13% HFBA was used as the counter-ion. One minute fraction were collected. The upper two panels show UV absorbance at 215 and 280 nm. The lower panel shows the percent inhibition of corticosterone production in the presence of 150 pg/ml ACTH.

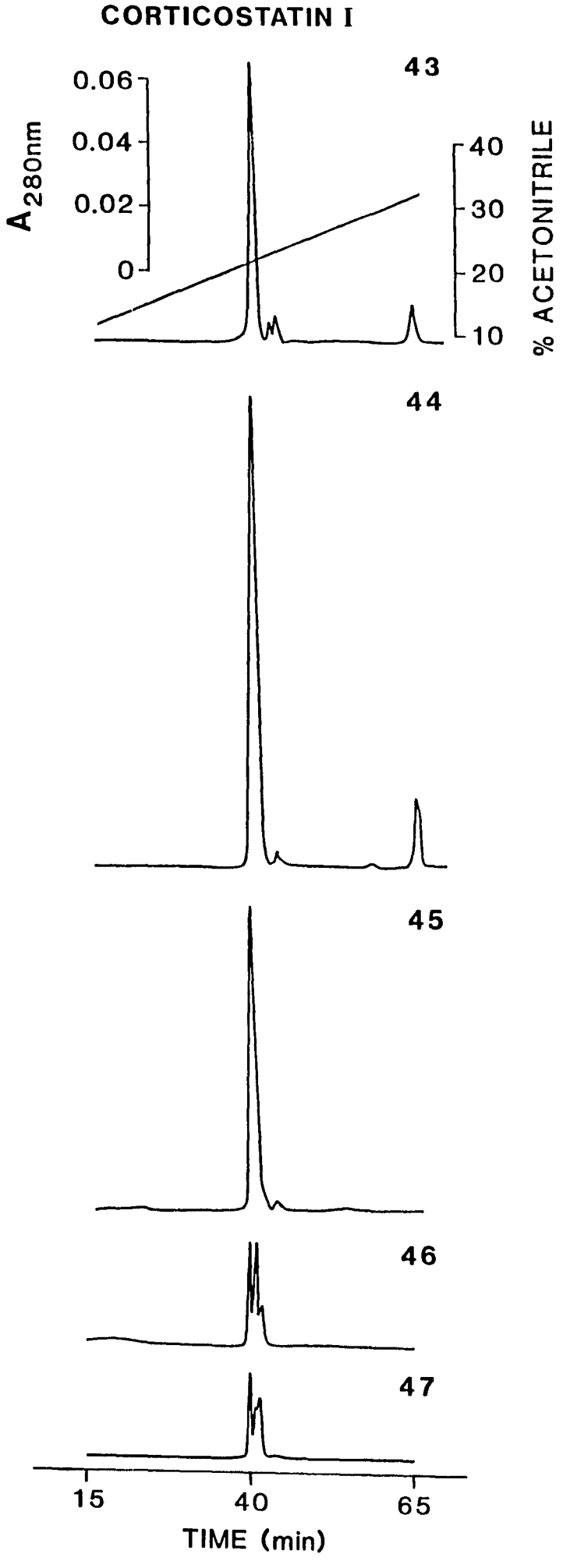

CORTICOSTATIN I
43
0.06
0.04
0.02
0
A_{280nm}
40
30
20
10
% ACETONITRILE
44
45
46
47
15
40
65
TIME (min)

Fig. 3-16 The purity of CSI fractions from the first RP-HPLC as demonstrated by a second RP-HPLC step. Fractions 43, 44, 45, 46 and 47 from Fig. 3-15 were repurified separately on a second HPLC, using 0.1% TFA as the counter-ion and a linear gradient. The gradient used was the same as those described in Fig. 3-12.

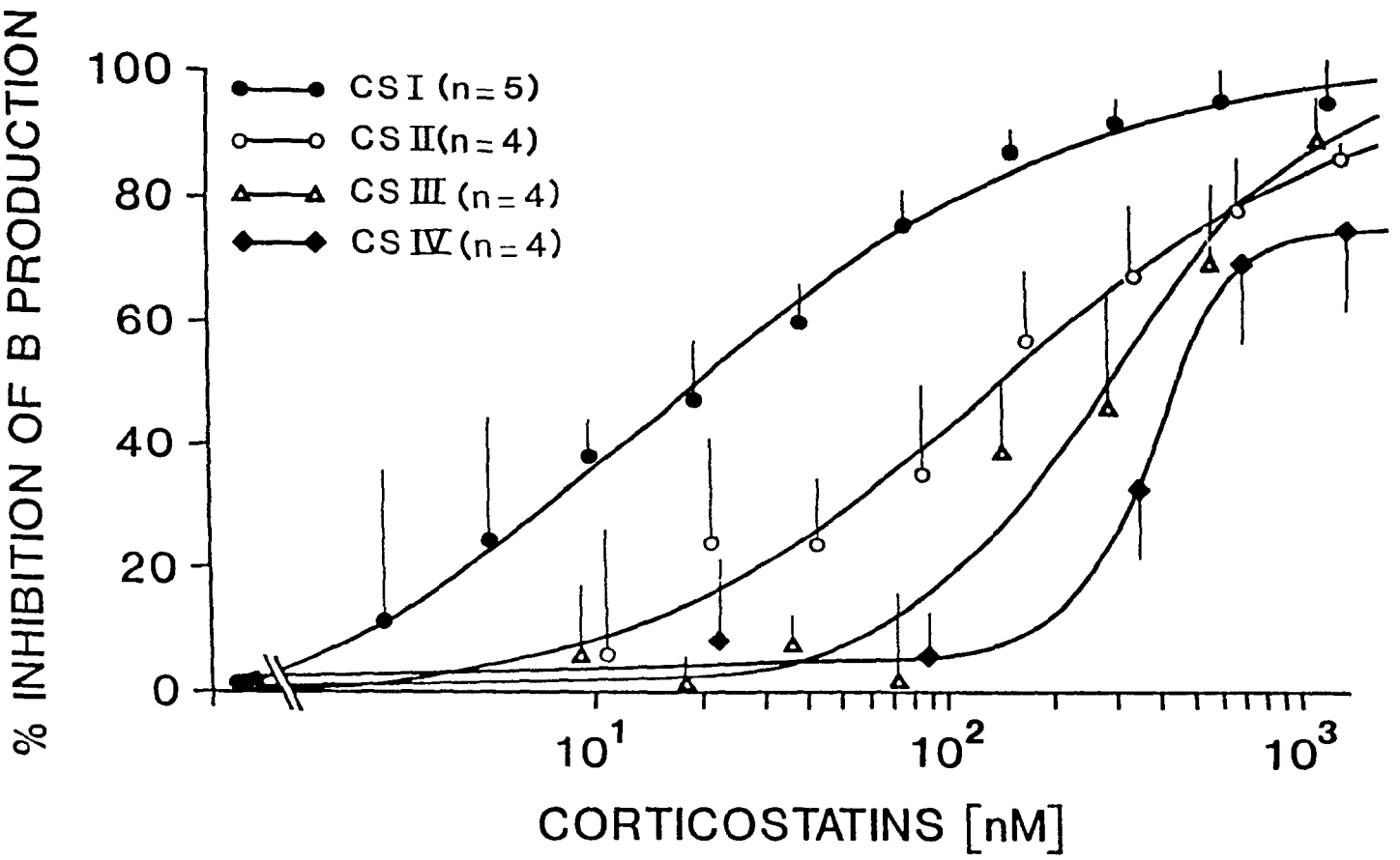

% INHIBITION OF B PRODUCTION
100
80
60
40
20
0
CS I (n=5)
CS II(n=4)
CS III (n=4)
CS IV (n=4)
10^1
10^2
10^3
CORTICOSTATINS [nM]

Fig. 3-17 A comparison of the corticostatic activity of CSI, II, III, and VI. The % inhibition of corticosterone production by isolated adrenal cells stimulated with 150 pg/ml of synthetic porcine $ACTH_{1-39}$ in the presence of increasing concentration of corticostatins (I to IV). The values are the mean of four (five for CSI) separate experiments each measured in duplicate. The vertical lines represent the standard deviation.

CHAPTER 4: RESULTS II:

THE MECHANISM OF ACTION OF CSI

4.1. The Mechanism of the Anti-ACTH Action of Corticostatin

4.1.1. The Specificity of the Action of CSI

After obtaining the CSI sequence we performed a computer search of the Protein Identification Resource of the National Biomedical Research Foundation (Georgetown University, Washington, DC) This research revealed that Selsted *et al.* (478) had found the same peptide as an antimicrobial defensin (NP-3a) previously purified from rabbit peritoneal neutrophils. The question we first wanted to answer was whether the corticostatic activity of CSI was due to its cytotoxic activity. Accordingly we performed the following study. An increasing concentration of CSI was co-incubated with isolated adrenal cells, 200,000 cells/ml for two hours in the presence of 150 pg/ml of synthetic porcine $ACTH_{1-39}$. (The experiment was performed as described in section 2.2.1.4.) At the end of the 2 hour incubation, the viability of the cells in each incubation tube was determined by the trypan blue exclusion method. The results are shown in Table 4-1. which demonstrate that the viability was affected neither by the doses nor by the presence of CSI over 2 hr period of incubation. The postulation that the corticostatic activity

of CSI is not due to its cytotoxic activity was further supported by the fact that there is no positive correlation between the corticostatic activity and antimicrobial activity. The potency of corticostatic activity of the four corticostatins is in the order CSI>CSII>CSIII≥CSIV, while the antimicrobial activity is CSIII≥CSIV>CSI≥CSII according to the report of Levitz *et al.* (479).

Table 4-1

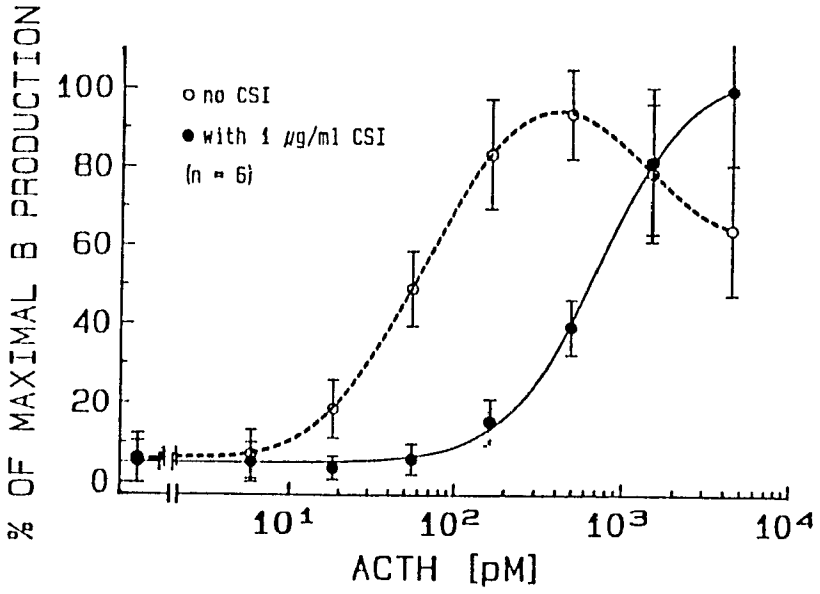
CSI ng/ml	% cells surviving ± SD
0	84.3 ± 3.5
19.5	79.4 ± 5.3
39.1	86.5 ± 7.4
78.1	85.3 ± 6.4
156.2	76.4 ± 8.5
312.5	82.4 ± 6.7
625.0	80.3 ± 4.3
1250.0	84.4 ± 8.6
2500.0	76.5 ± 16.4
5000.0	79.9 ± 6.9

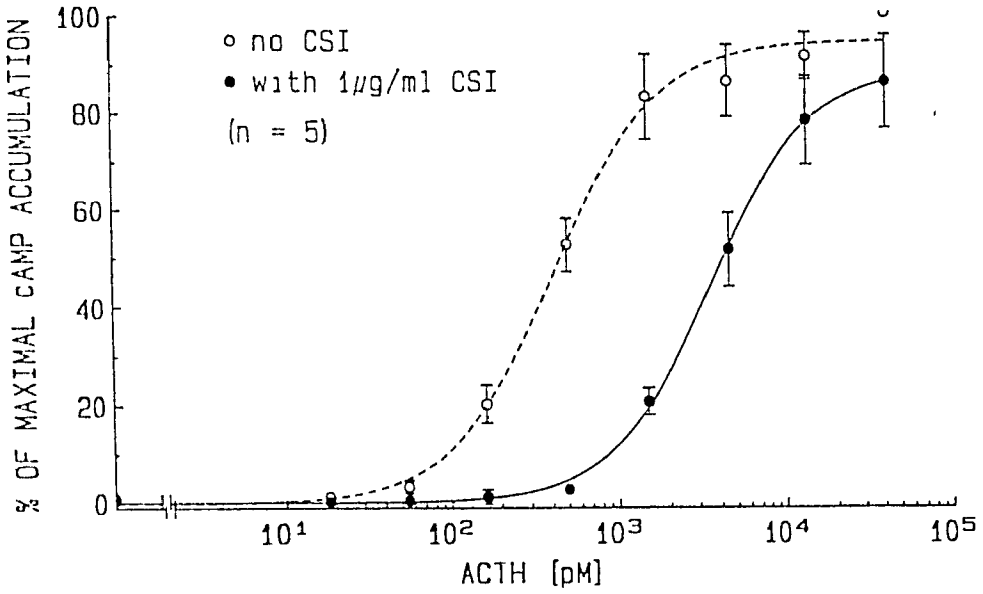
Corticostatin I was the most potent corticostatic peptide of the four, therefore it was chosen for studies of the mechanism of action of corticostatic peptides.

In the whole rat adrenal cell suspension bioassay, ACTH can

increase not only the corticosterone production and cAMP accumulation but also aldosterone output as well. The mechanism of the effect of ACTH on aldosterone secretion remains controversial (for a review see ref. 480). It could be due to the intra-adrenal ACTH-stimulated factors such as increased glucocorticoid production (481). CSI was able to suppress or even abolish all these ACTH effects (Fig. 4-1). With 1 μg/ml CSI, the dose response curves of corticosterone, cAMP, and aldosterone shift to the right about 1 order. 1 μg/ml CSI was able to suppress about 50% of steroid production in response to the stimulation of 2.2 ng/ml ACTH (Fig. 4-1). The kinetic study by adding 533 fold excess of CSI to each dose of ACTH in the rat adrenal cell bioassay suggested that the inhibition of CSI on ACTH-stimulated steroid production belong to competitive mechanism. The results in Fig. 4-2 shows that the corticosterone production was kept at half maximum level when ACTH and CSI increasing proportionally.

The concept that the CSI is a specific ACTH inhibitor arose from the fact that it did not inhibit angiotensin II-stimulated aldosterone production by rat zona glomerulosa cells as shown in Fig. 4-3.


% OF MAXIMAL B PRODUCTION
100
80
60
40
20
0
o no CSI
• with 1 µg/ml CSI
(n = 6)
10^1
10^2
10^3
10^4
ACTH [pM]


% OF MAXIMAL cAMP ACCUMULATION
100
80
60
40
20
0
o no CSI
• with 1µg/ml CSI
(n = 5)
10^1
10^2
10^3
10^4
10^5
ACTH [pM]

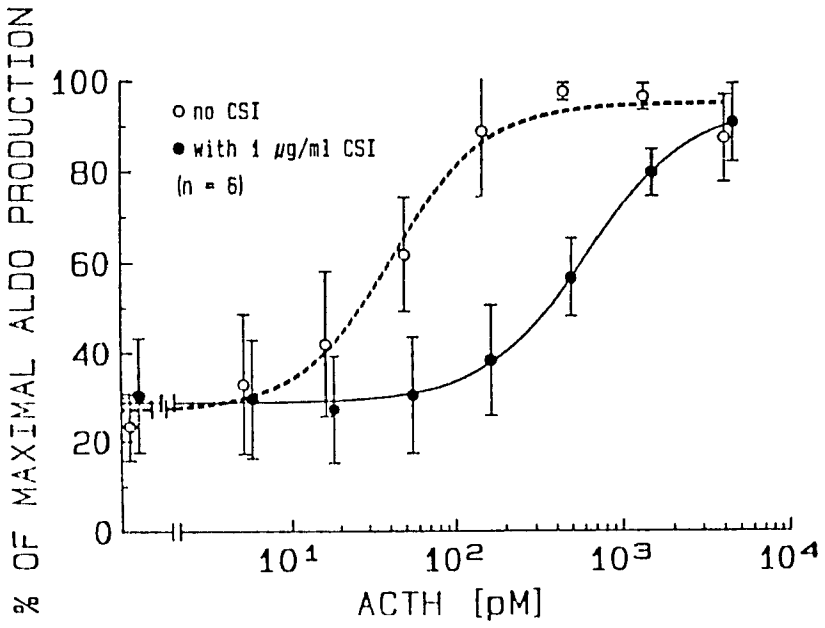
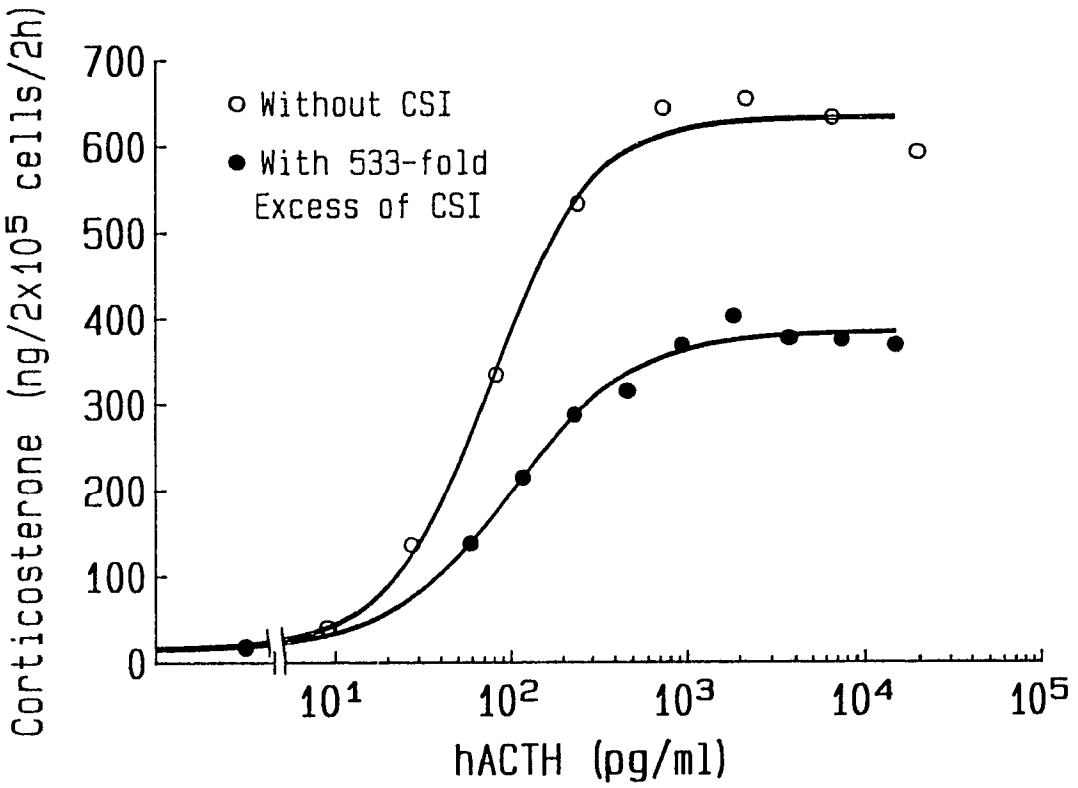

% OF MAXIMAL ALDO PRODUCTION
100
80
60
40
20
0
o no CSI
• with 1 µg/ml CSI
(n = 6)
10^1
10^2
10^3
10^4
ACTH [pM]

Fig. 4-1 The effect of CSI on the ACTH stimulated-corticosterone, aldosterone and cAMP productions in dispersed rat adrenal cell bioassay. The experiment was discussed in detail in section 2.5.1.. Corticosterone (A), cAMP (B), and aldosterone (C) log-dose response by isolated rat adrenal cells to ACTH in the absence (o) and presence (●) of 1 μg/ml CSI. The values are the means of six (A & B) or three (C) separate experiments, each measured in duplicate. The bars represent standard deviation.


Corticosterone (ng/2x10⁵ cells/2h)
700
600
500
400
300
200
100
0
Without CSI
With 533-fold Excess of CSI
10¹
10²
10³
10⁴
10⁵
hACTH (pg/ml)

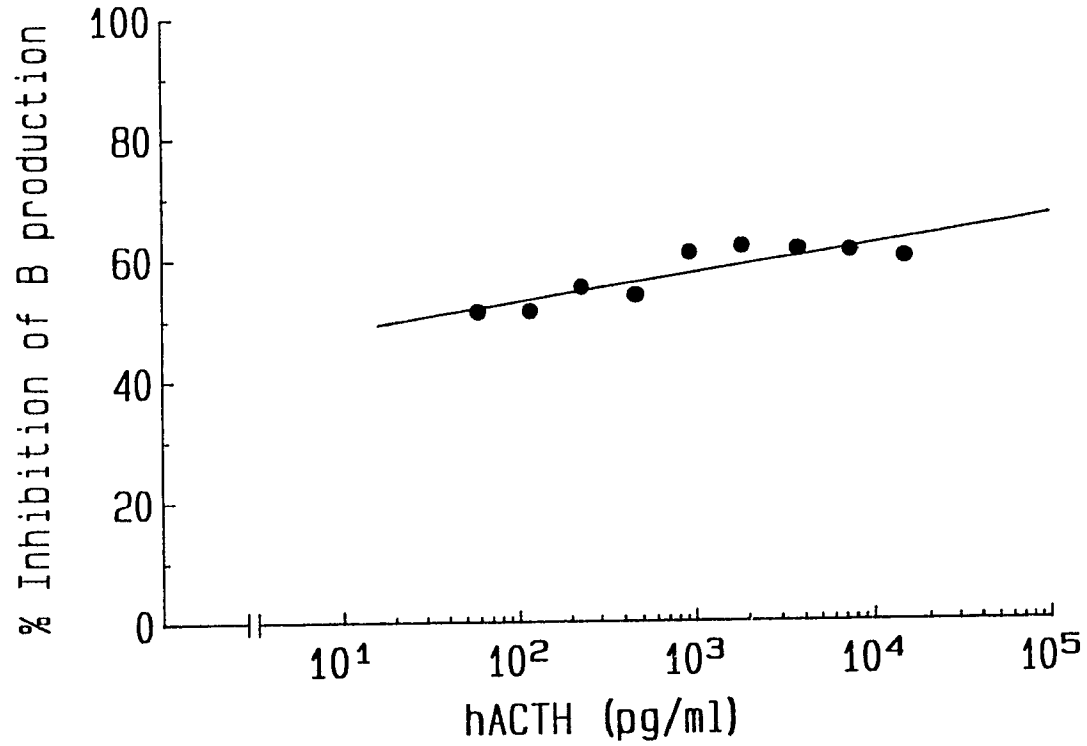

% Inhibition of B production
100
80
60
40
20
0
10¹
10²
10³
10⁴
10⁵
hACTH (pg/ml)

Fig. 4-2 The competitive inhibition pattern of CSI on ACTH stimulated corticosterone production. (a) Corticosterone log-dose-response to ACTH in the absence (o) and presence (●) of 533 fold excess of CSI (weight/weight, and 600 fold excess, molar/molar) at each dose. (b) Results from figure (a) were plotted as % inhibition which equal to corticosterone production in the presence of 600 folds of CSI divided by corticosterone production in the absence of CSI and multiplied by 100.

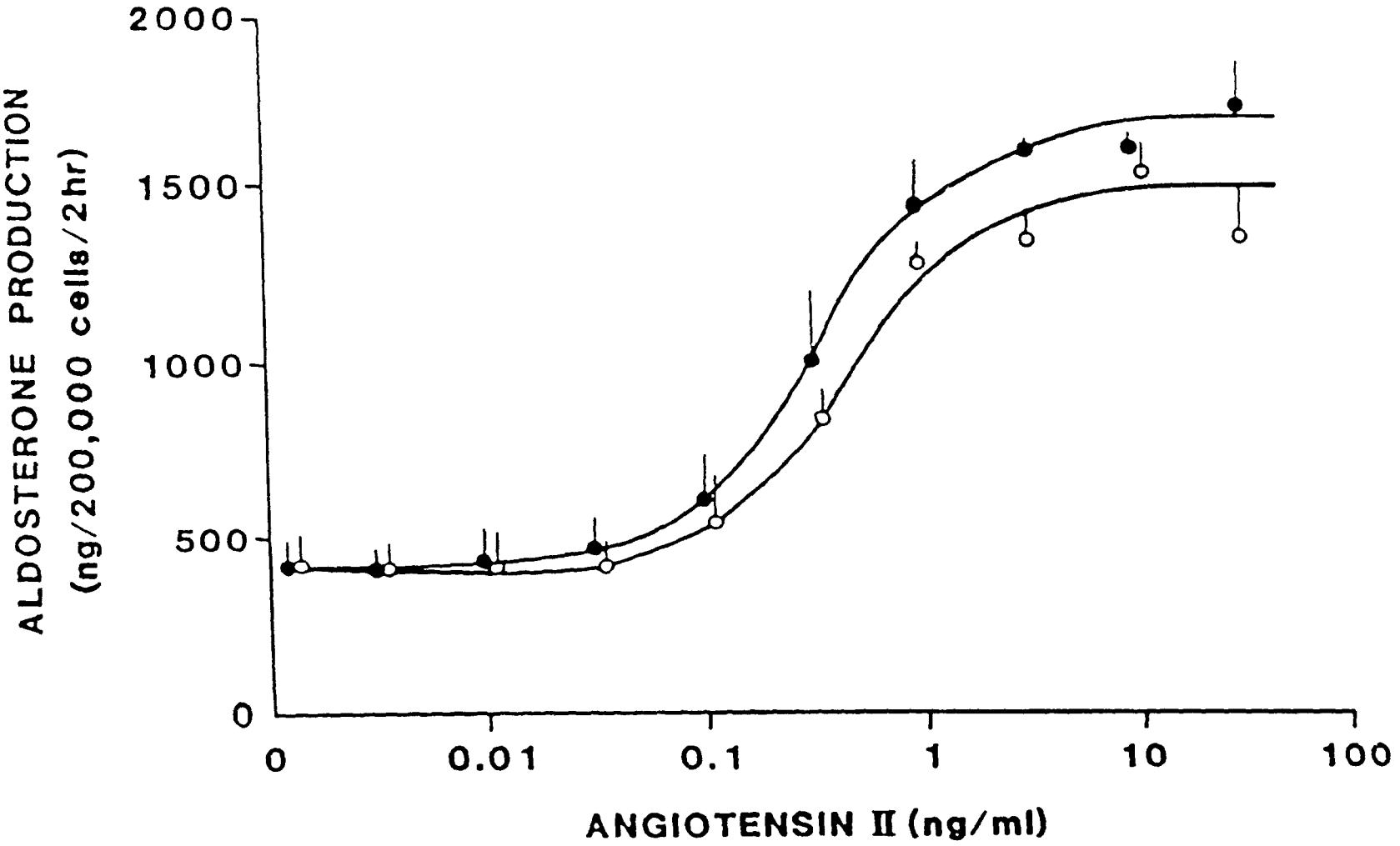

ALDOSTERONE PRODUCTION
(ng/200,000 cells/2hr)
2000
1500
1000
500
0
0
0.01
0.1
1
10
100
ANGIOTENSIN II (ng/ml)

Fig. 4-3 The effect of CSI on the Angiotensin II stimulated-aldosterone production in dispersed zona glumarulosa cell bioassay. Aldosterone log-dose-response to angiotensin II in the absence (o) and presence (●) of 1 μg/ml CSI using rat adrenal zona glomerulosa cells prepared as described in the methods section. The results are the mean of four separate experiments, each measured in duplicate. The vertical lines represent standard deviations.

4.1.2. The Site of Action of CSI on Membrane Signal Transduction

To elucidate the mechanism of action of CS, we used agents which mimic the action of ACTH on adrenal cell steroidogenesis (i.e. dbcAMP, forskolin, cholera toxin). Their possible sites of action are summarized diagrammatically in Fig 4-4.

The effects of increasing concentrations of dbcAMP on corticosterone production in dispersed rat adrenocortical cells in the presence (●) and in the absence (O) of CSI are shown in Fig. 4-5. The effective dosage range of dbcAMP was from 2 to 64 ug/ml. The maximal corticosterone production by the adrenal cells in response to db-cAMP was proximately 300 ng/$2x10^5$ cells/2h, which was of the same order as that obtained by ACTH stimulation. There was no significant difference between the action of dbcAMP in the absence or presence of CSI, even at concentrations of 1 μg/ml of CSI, which completely abolished the steroidogenic response to 150 pg/ml ACTH under the same assay conditions. These results were consistent with the previous observation that CSI reduced the production of cAMP by adrenal cell suspensions in response to ACTH (Fig. 4-1). We can infer from this that CSI acts prior to the production of cAMP in the response of adrenal cells to ACTH.

Forskolin, the adenylate cyclase activator (482), can also stimulate rat adrenal cell steroidogenesis. The difficulty in using this stimulator is its low saturation concentration and

relatively long penetrating time, so the variation of actual concentration of forskolin, especially in a short incubation period, greatly contributed to experiment variation in terms of steroid genesis. The maximal stimulation of steroidogenesis was only 25 ng/2x10^5 cells/2h under our experimental conditions. There was no significant difference between the action of forskolin in the absence or presence of CSI (1 μg/ml) as shown in Fig. 4-6. Thus, the possibility that CSI could act as a adenylate cyclase inhibitor can be excluded by these results.

In most types of cells, G proteins, the link between a hormone receptor and the adenylate cyclase, can be divided into two categories: inhibitory and stimulatory G proteins. The stimulatory G protein (G_s) can be activated by cholera toxin and the inhibitory G protein (G_i) can be activated by pertussis toxin (for a review see ref. 483). Cholera toxin in our rat adrenal cell bioassay system stimulated corticosterone production. The maximal response of steroidogenesis to cholera toxin was 150 ng/2x10^5/2h. There was no significant difference between the presence or absence of CSI (at 1 μg/ml) as shown in Fig. 4-8. Pertussis toxin was found to be able to suppress the inhibitory effect of vasopressin on ACTH-induced cAMP production by Y-1 cells (484). In our rat adrenal cell bioassay system, pertussis toxin had no stimulatory effects or inhibitory effects on the basal or on ACTH-stimulated corticosterone production.

Pertussis toxin also had no effect on the inhibitory action of CSI as shown in Fig. 4-9.

These results taken together suggested that CSI acts at a site(s) prior to the action of the stimulatory G proteins and this site is most likely at the ACTH receptor itself.

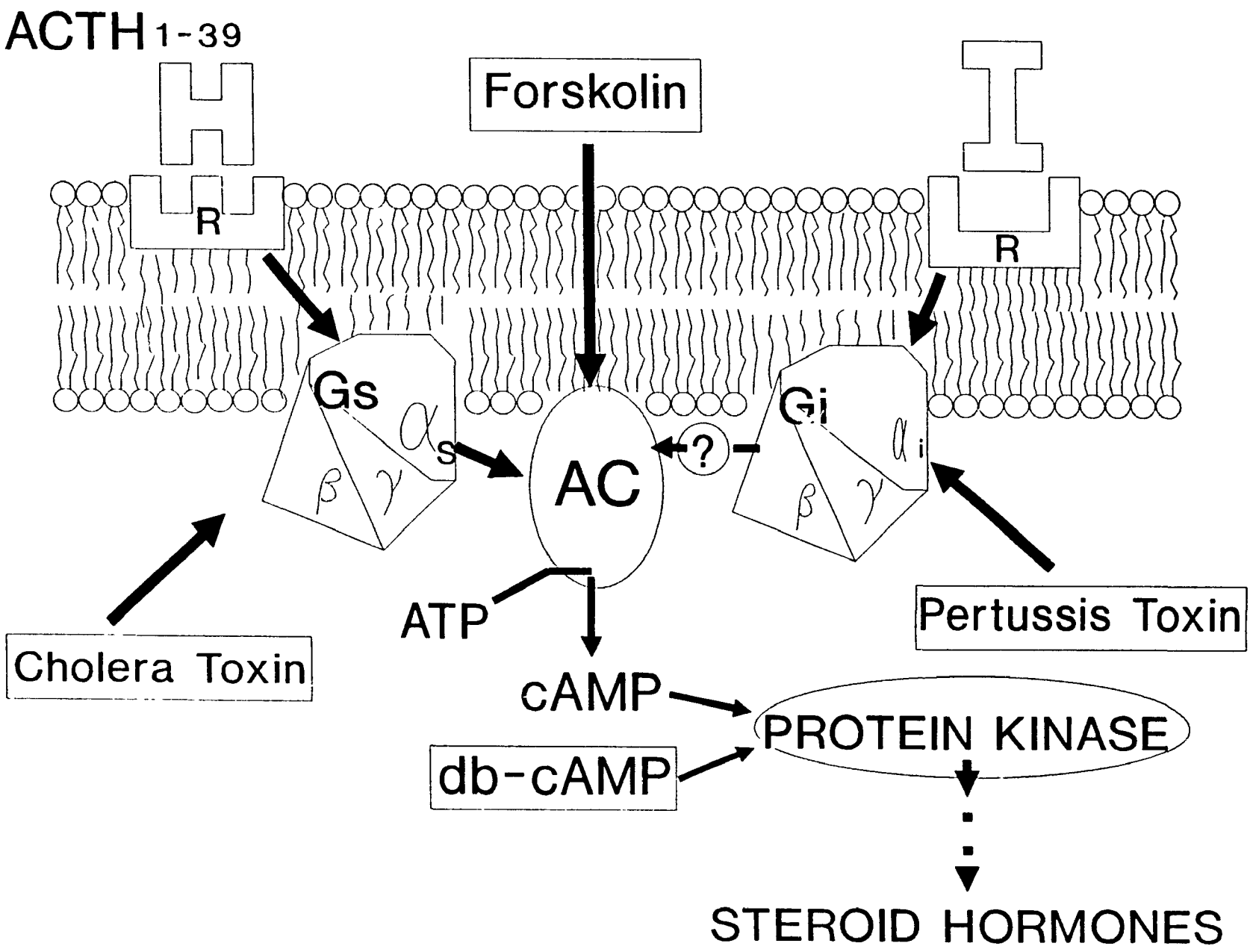

ACTH 1-39
Forskolin
R
R
Gs
Gi
AC
?
ATP
cAMP
Cholera Toxin
Pertussis Toxin
PROTEIN KINASE
db-cAMP
STEROID HORMONES

Fig. 4-4 The adrenal cell membrane. For a detail discussion of the signalling transduction system see section 5.6.

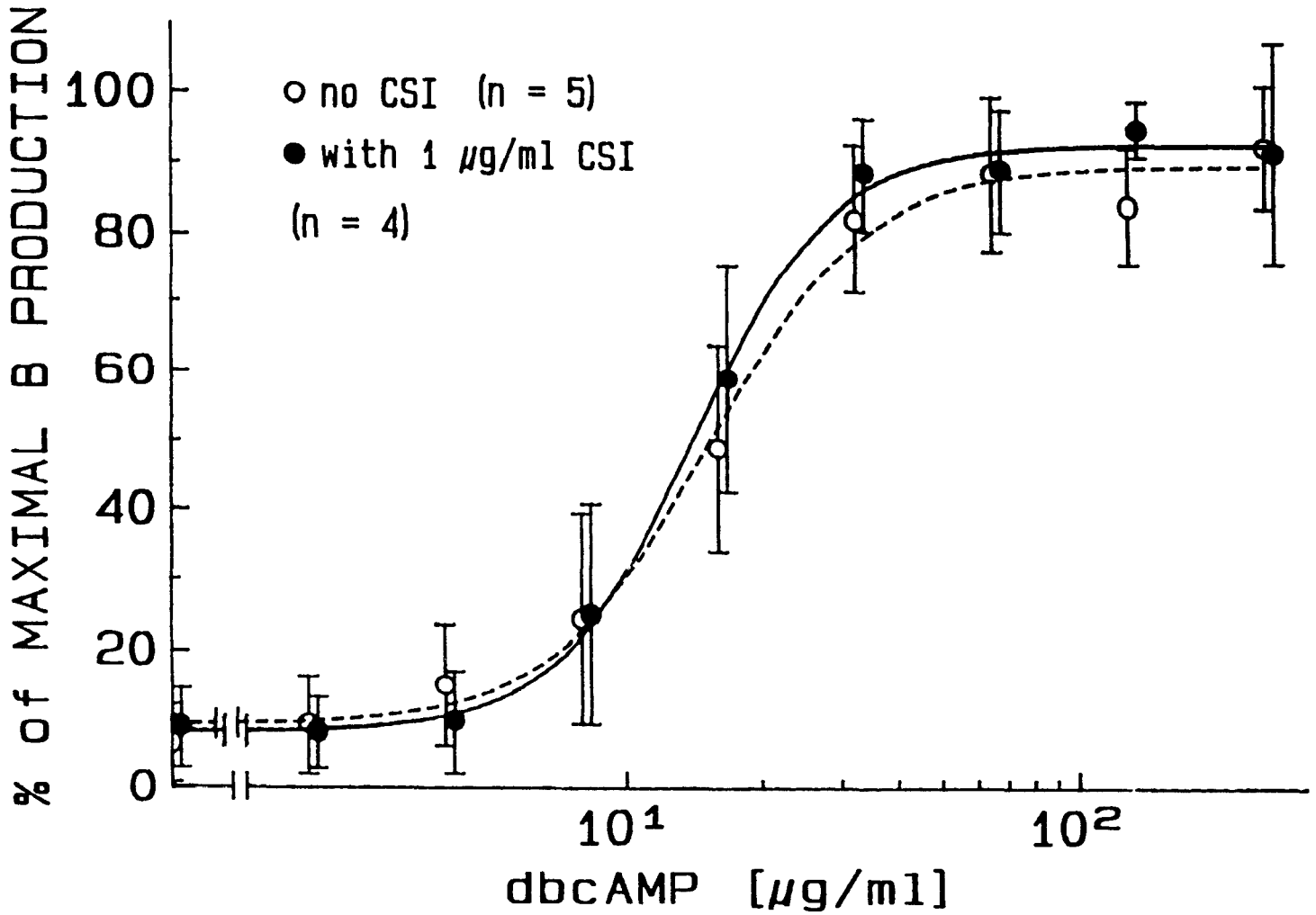

% of MAXIMAL B PRODUCTION
100
80
60
40
20
0
o no CSI (n = 5)
● with 1 μg/ml CSI
(n = 4)
10¹
10²
dbcAMP [μg/ml]

Fig. 4-5 The effect of CSI on dbcAMP stimulated-corticosterone production. Log-dose response of corticosterone production by isolated adrenal cells in response to dbcAMP in absence (o) and presence (●) of 1 μg/ml CSI in isolated rat adrenal cells. The values are the mean of four or five separate experiments of duplicate incubations. The bars represent standard deviations.

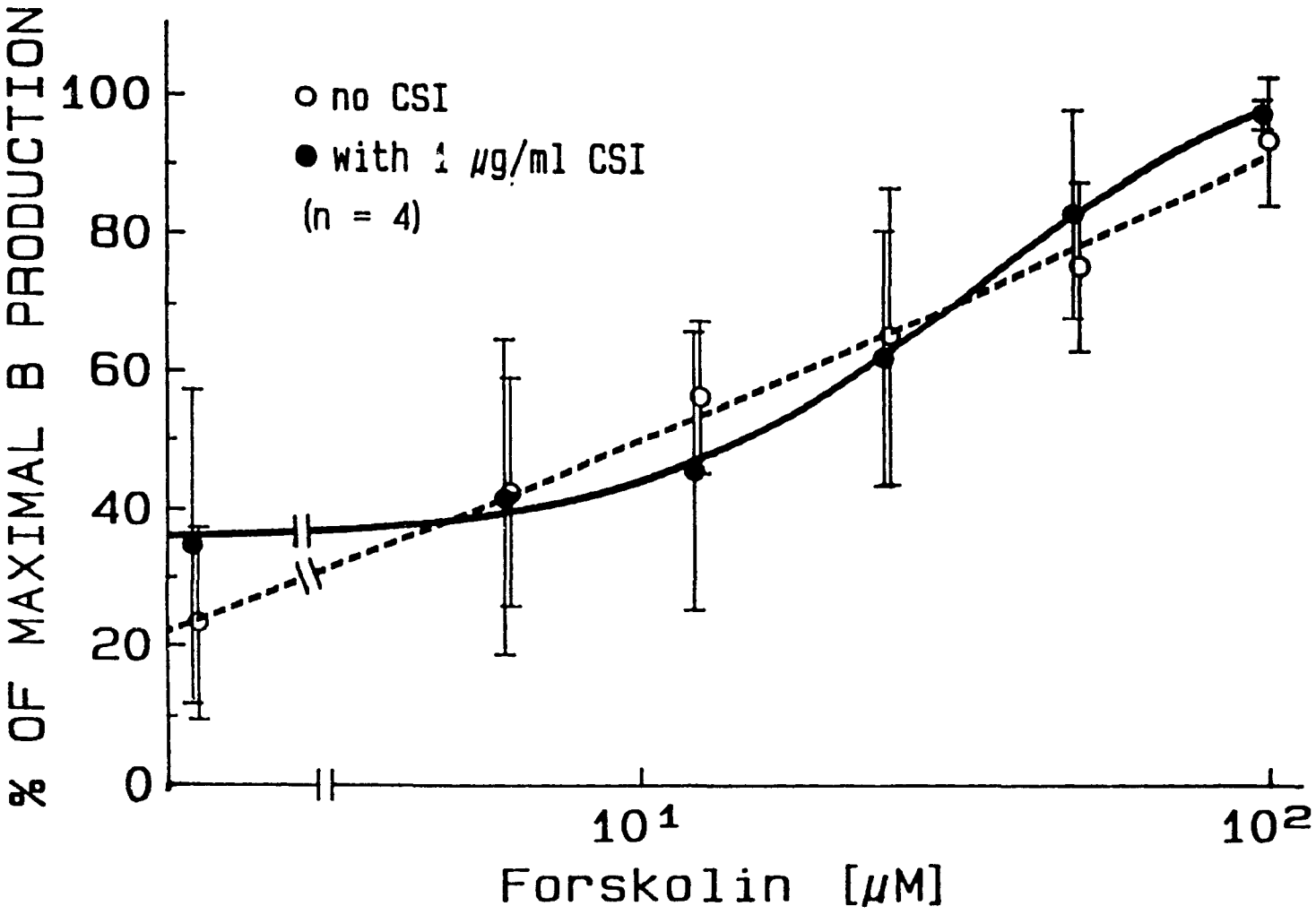

% OF MAXIMAL B PRODUCTION
100
80
60
40
20
0
o no CSI
● with 1 µg/ml CSI
(n = 4)
10¹
10²
Forskolin [µM]

Fig. 4-6 The effect of CSI on forskolin stimulated-corticosterone production. Corticosterone log-dose response to forskolin in absence (o) and presence (●) of 1 μg/ml CSI in isolated rat adrenal cells. The values are the mean of four separate experiments, each measured in duplicate. The bars represent standard deviations.

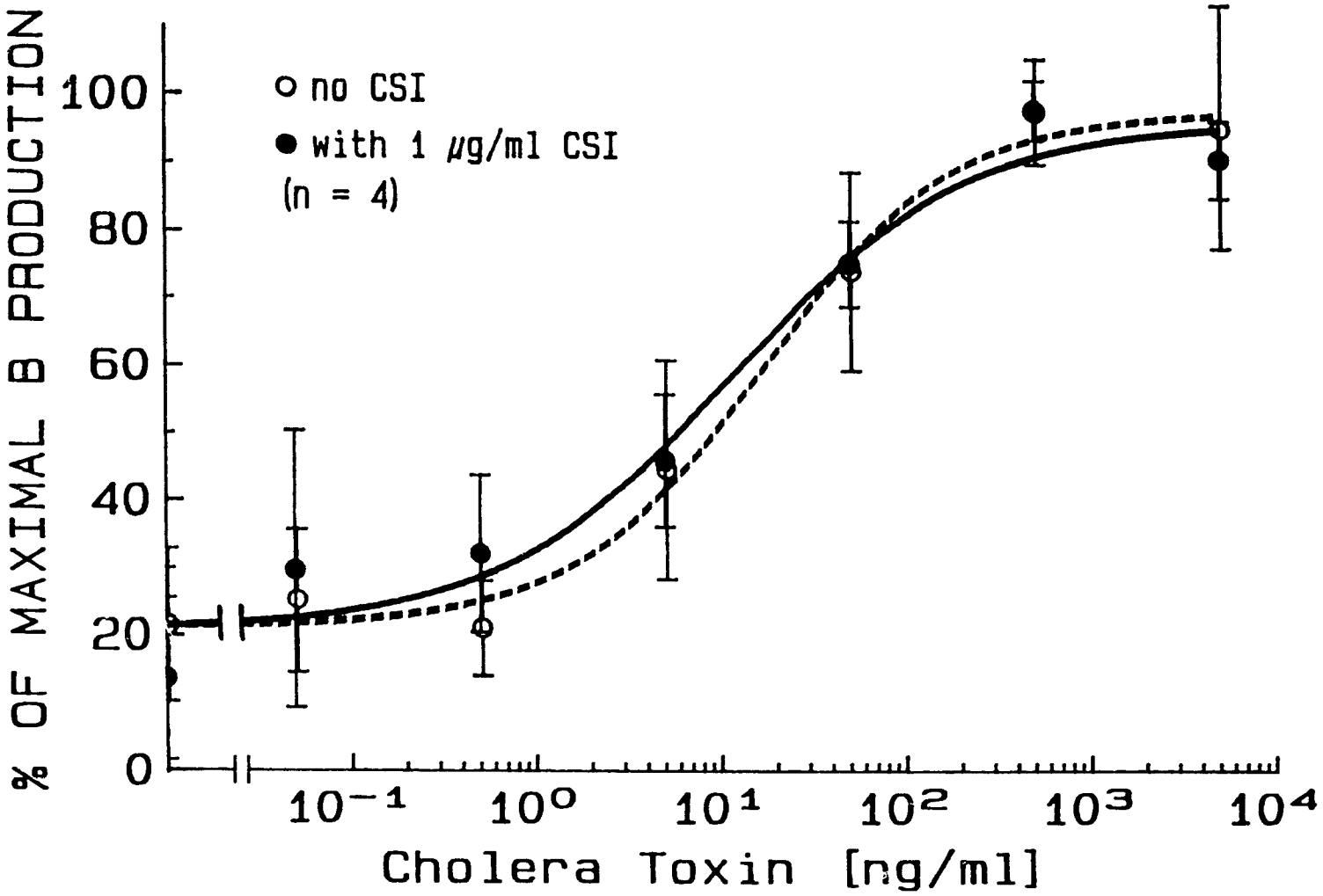

% OF MAXIMAL B PRODUCTION
100
80
60
40
20
0
o no CSI
● with 1 µg/ml CSI
(n = 4)
10⁻¹
10⁰
10¹
10²
10³
10⁴
Cholera Toxin [ng/ml]

Fig. 4-7 The effect of CSI on cholera toxin stimulated-corticosterone production. Corticosterone log-dose response to cholera toxin in absence (o) and presence (●) of 1 μg/ml CSI in isolated rat adrenal cells. The values are the mean of five separate experiments of duplicate incubations. The bars represent standard deviations.

Fig. 4-8 The effect of pertussis toxin on the CSI inhibition. % inhibition of corticosterone production in ACTH-stimulated adrenal cells (150 pg/ml) with increasing concentrations of CSI in the absence (□) and presence (■) of 100 ng/ml pertussis toxin (PT). The values are the mean of two separate experiments, each measured in duplicate.

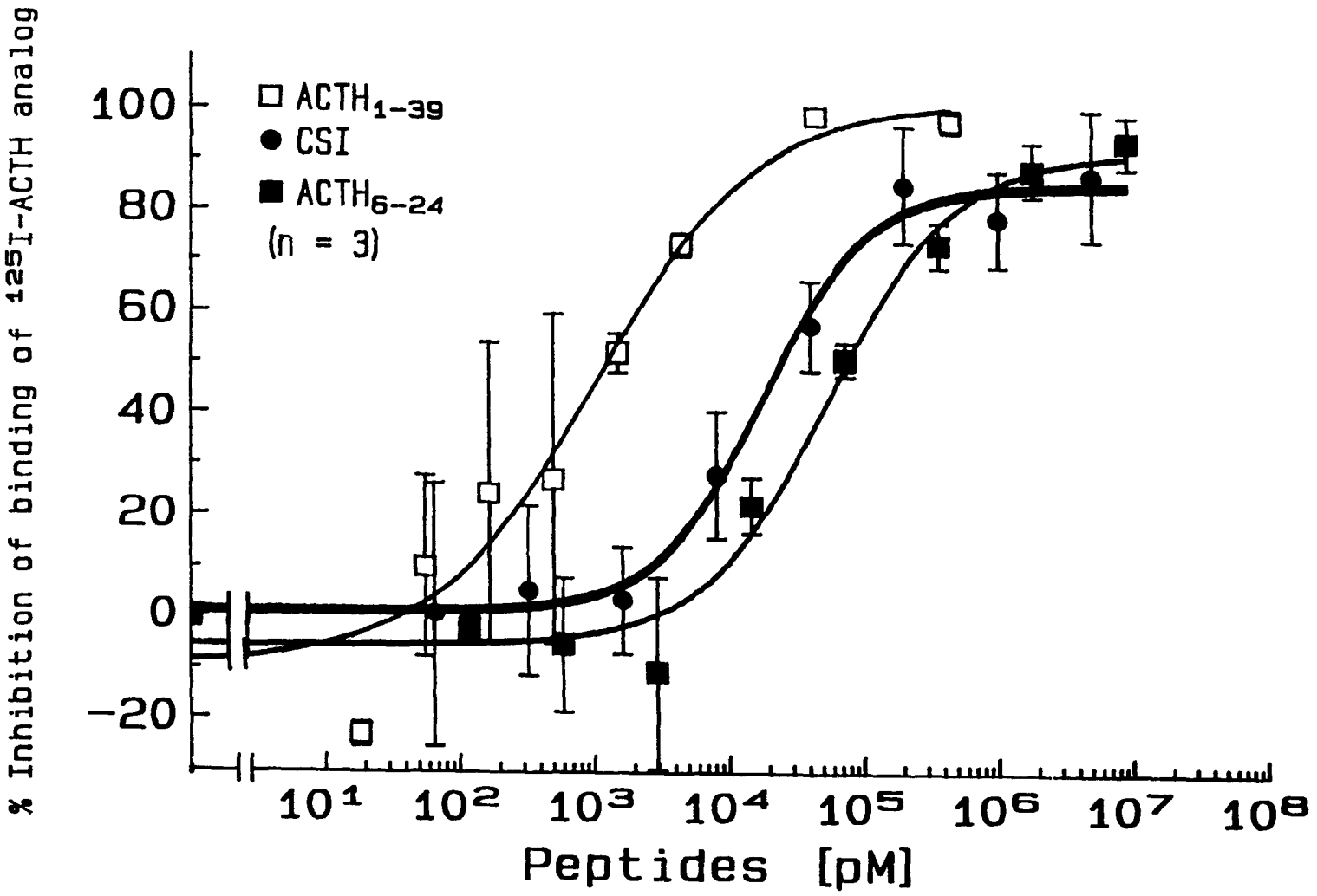

% Inhibition of binding of 125I-ACTH analog
100
80
60
40
20
0
-20
□ ACTH1-39
● CSI
■ ACTH6-24
(n = 3)
10¹
10²
10³
10⁴
10⁵
10⁶
10⁷
10⁸
Peptides [pM]

Fig. 4-9 The ACTH receptor binding assay. The percent inhibition of specific binding of ^{125}I-Phe^{2}, Nle^{4}-ACTH$_{1-38}$ to rat adrenal cell ACTH receptors by ACTH$_{1-39}$, CSI and ACTH$_{6-24}$. The experiment was carried out as described in section 2.6.2..

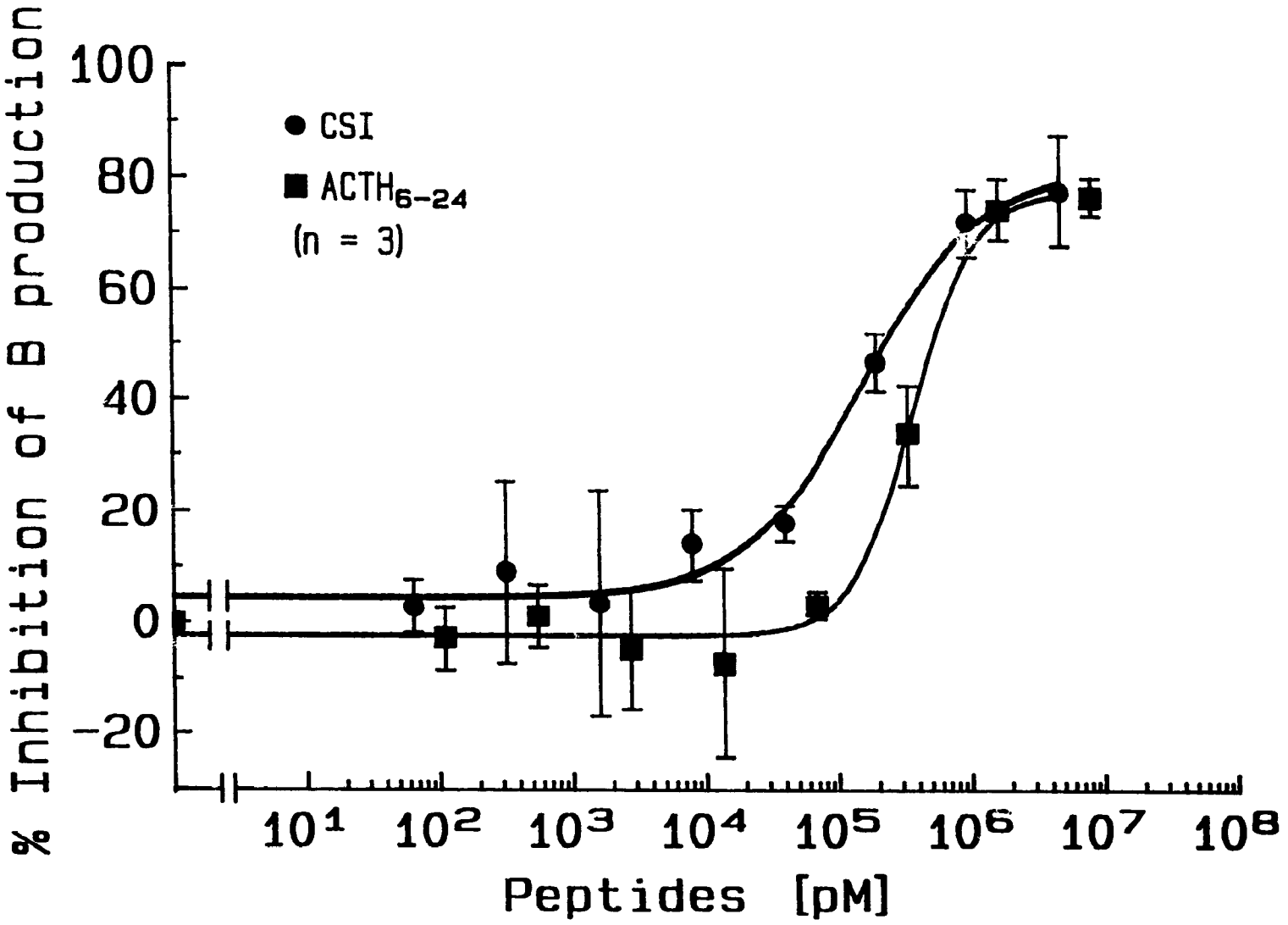

% Inhibition of B production
100
80
60
40
20
0
-20
● CSI
■ ACTH6-24
(n = 3)
10¹ 10² 10³ 10⁴ 10⁵ 10⁶ 10⁷ 10⁸
Peptides [pM]

Fig 4-10 The inhibition of corticosterone production by CSI and $ACTH_{6-24}$. In the same experiments outlined in Fig. 4-9 corticosterone productions were measured in the medium. Values are the mean of three separate experiments ± standard deviations. (Methods are described in detail in 2.6.)

4.1.3. CSI: A Competitive Inhibitor of ACTH

All the evidence presented so far suggested that CSI was a competitive inhibitor of ACTH. The direct evidence that CSI was a competitive inhibitor of ACTH was obtained through ACTH receptor binding studies with the aid of ^{125}I-[Phe^2, Nle^4] ACTH$_{1-39}$, a fully biologically active ACTH analogue (477). The experiments were carried out as discussed in detail in the methods section. Fig. 4-9 shows that CSI was able to displace 175 pM of ^{125}I- [Phe^2, Nle^4] ACTH$_{1-38}$ in the concentration range 10 nM to 250 nM being about 12 times less potent than hACTH$_{1-39}$ and 1.7 times more potent than ACTH$_{6-24}$, a synthetic ACTH antagonist (485). The ability to displace radiolabeled ligand by CSI and ACTH$_{6-24}$ correlates very well with their ability to suppress corticosterone release as shown in Fig. 4-10.

4.1.4. The Site of Action of CSI on the ACTH Receptor

Further evidence of the nature of CS binding to the ACTH receptor was obtained by comparing the inhibition of steroidogenesis by CSI in response to N-terminal fragments of ACTH. It was found that αMSH (N-acetyl-ACTH$_{1-13}$ amide), stimulates adrenal steroidogenesis at a concentration higher than 10^{-7} M, and is not inhibited by 1 μg/ml CSI as is shown in Fig. 4-11. ACTH$_{1-18}$ amide however which is effective at 10^{-12} M, is inhibited by 1 μg/ml CSI, the ED$_{50}$ for steroidogenesis

shifting from about $2X10^{-11}$ to $1x10^{-10}$ M (Fig. 4-12). Now there are only two possibilities left, one is that CSI competitively binds to the ACTH receptor at residues 14 to 18, or that CSI binds to the 14 to 18 sequence of ACTH 14-18 itself. Because the 14 to 18 sequence of ACTH ($-Gly_{14}-Lys_{15}-Lys_{16}-Arg_{17}-Arg_{17}-$) and CSI are both very basic, the latter possibility is unlikely. This view was further supported by the results from repeating the experiment shown in Fig. 3-10B in the presence of 1 ng/ml of $ACTH_{7-39}$ which had 14 to 18 sequence but had no effect on the inhibitory action of CSI against 150 pg/ml of ACTH stimulation. $ACTH_{7-39}$ is an antagonist of ACTH. The potency of anti-ACTH activity of $ACTH_{7-39}$ is close to (slightly weaker than) the potency of CSI therefore 1 ng/ml $ACTH_{7-39}$ will not significantly change the dose-response curve of corticosterone to the stimulation of ACTH.

In conclusion, the experimental evidence suggests that CSI acts by competing with the basic $-Gly_{14}-Lys_{15}-Lys_{16}-Arg_{17}-Arg_{18}-$ sequence of ACTH for its binding site.

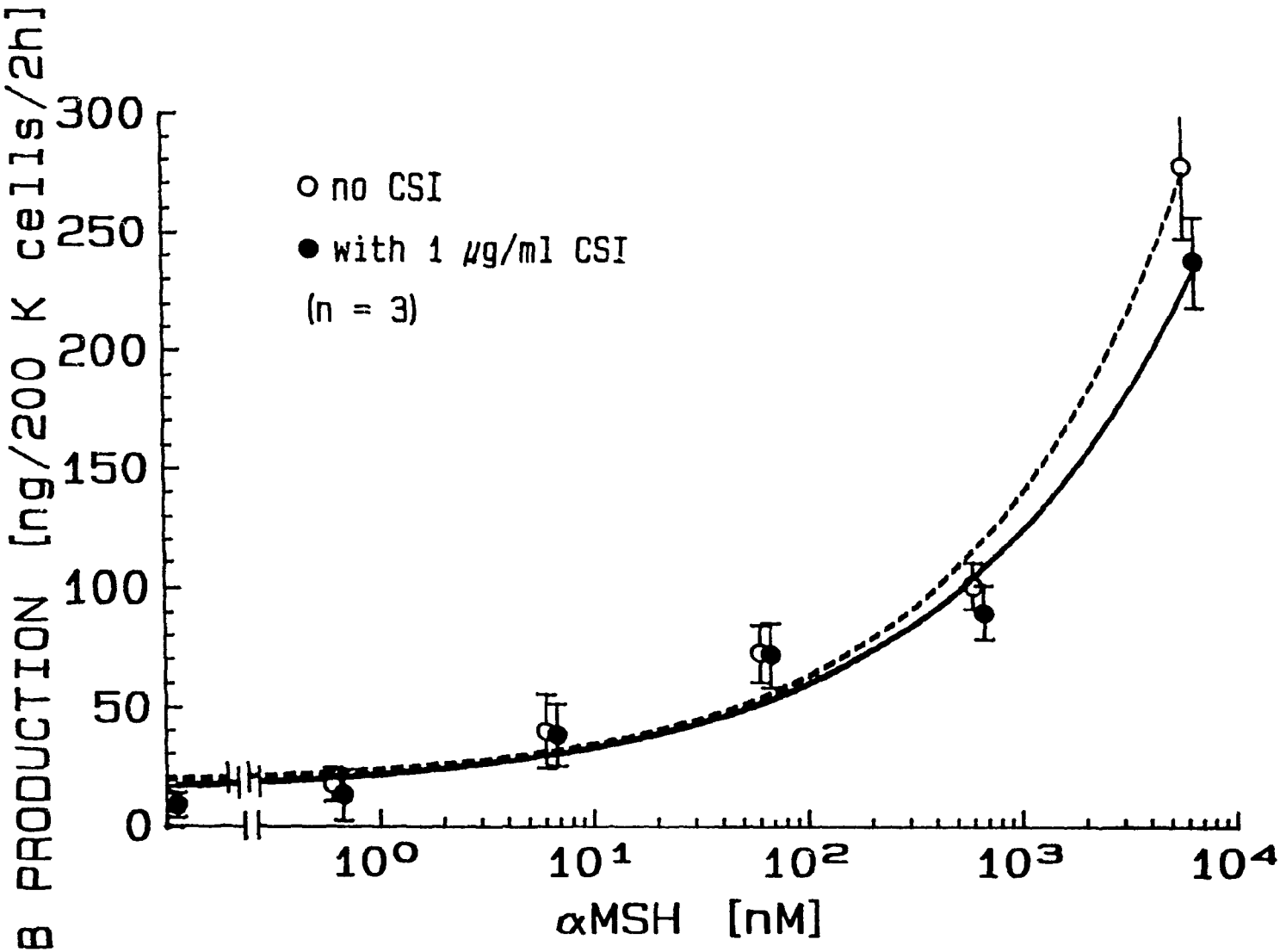

B PRODUCTION [ng/200 K cells/2h]
300
250
200
150
100
50
0
o no CSI
● with 1 µg/ml CSI
(n = 3)
10⁰
10¹
10²
10³
10⁴
αMSH [nM]

Fig. 4-11 The effect of CSI on αMSH stimulated-corticosterone production. Corticosterone log-dose response to αMSH in the absence (o) and presence (●) of 1 μg/ml CSI in isolated rat adrenal cells. The values are the mean of three separate experiments each measured in duplicate. The vertical lines represent standard deviations.

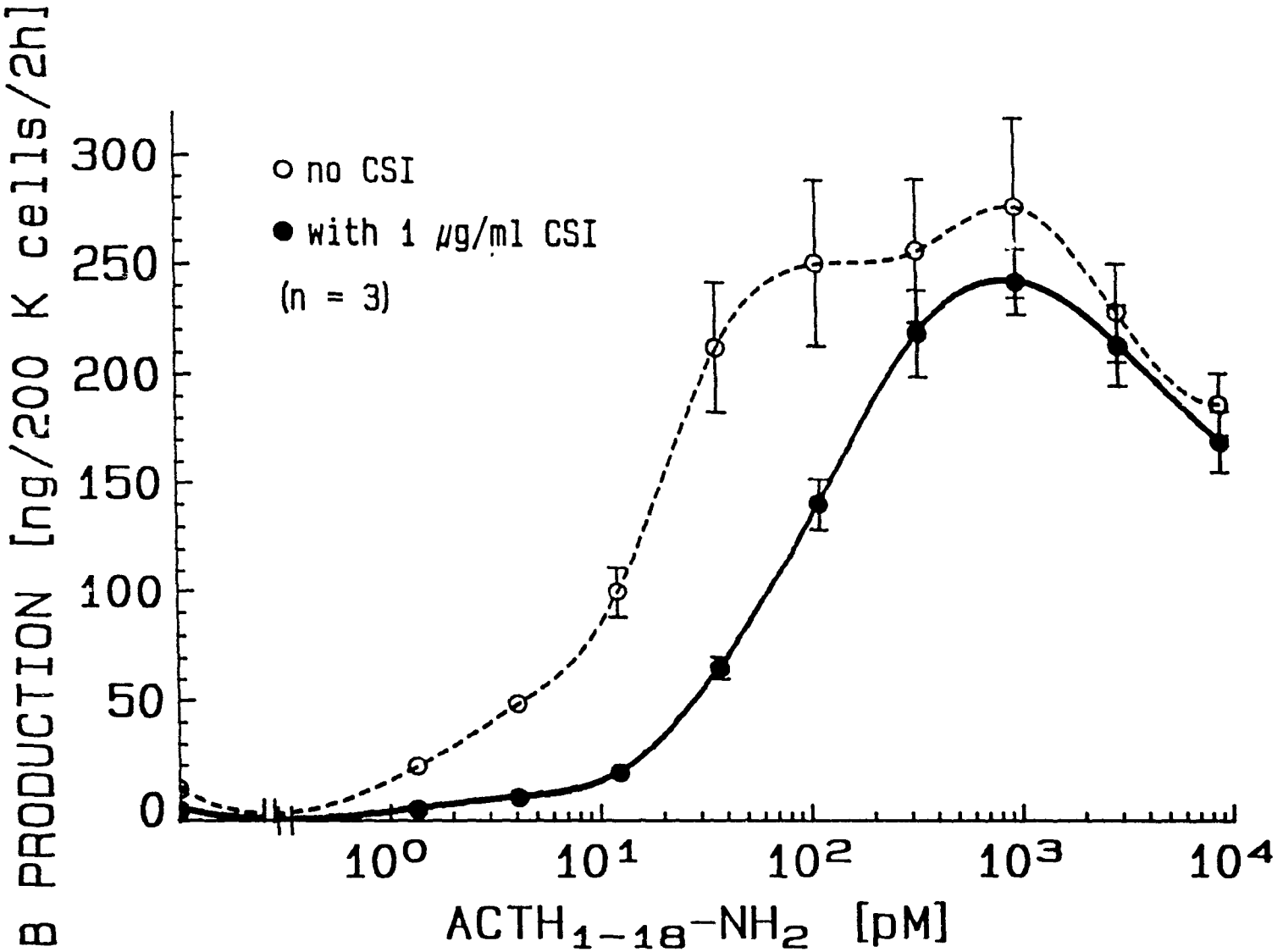

B PRODUCTION [ng/200 K cells/2h]
300
250
200
150
100
50
0
o no CSI
● with 1 µg/ml CSI
(n = 3)
10⁰
10¹
10²
10³
10⁴
ACTH1-18-NH2 [pM]

Fig. 4-12 The inhibitory effect of CSI on the $ACTH_{1-18}$ amide stimulated corticosterone production. Corticosterone log-dose response to $ACTH_{1-18}$ amide in the absence (open circle) and presence (closed circle) of 1 μg/ml CSI in isolated rat adrenal cells. The values are the means of three experiments, each measured in duplicate. The vertical lines represent standard deviations.

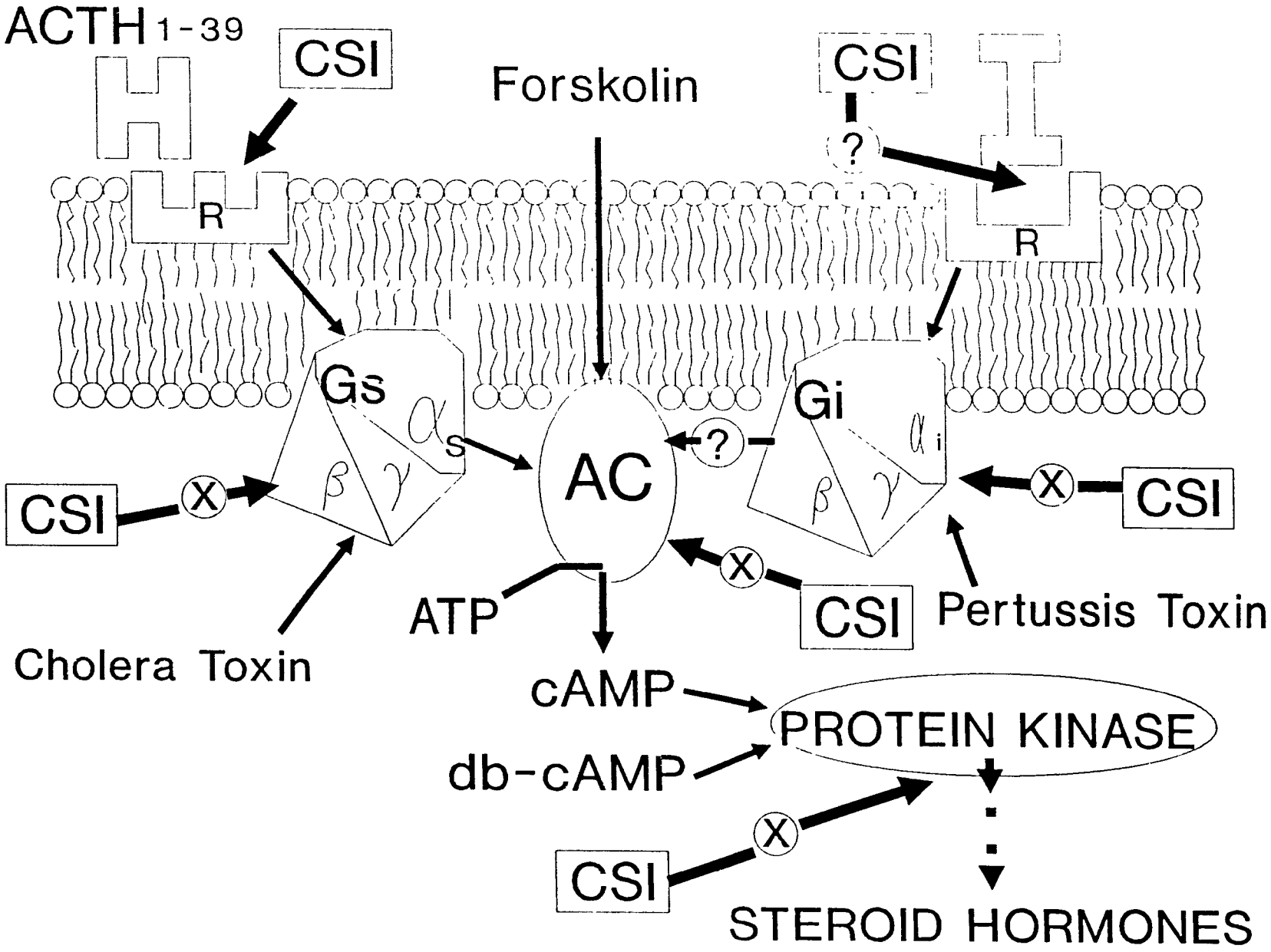

ACTH 1-39
CSI
Forskolin
CSI
?
R
R
Gs
α_s
β
γ
AC
?
Gi
α_i
β
γ
CSI
X
CSI
X
ATP
X
CSI
Pertussis Toxin
Cholera Toxin
cAMP
PROTEIN KINASE
db-cAMP
X
CSI
STEROID HORMONES

Fig. 4-13 The mechanism of action of CSI on the adrenal cell membrane. For a detail discussion of the signalling transduction system see section 5.6.

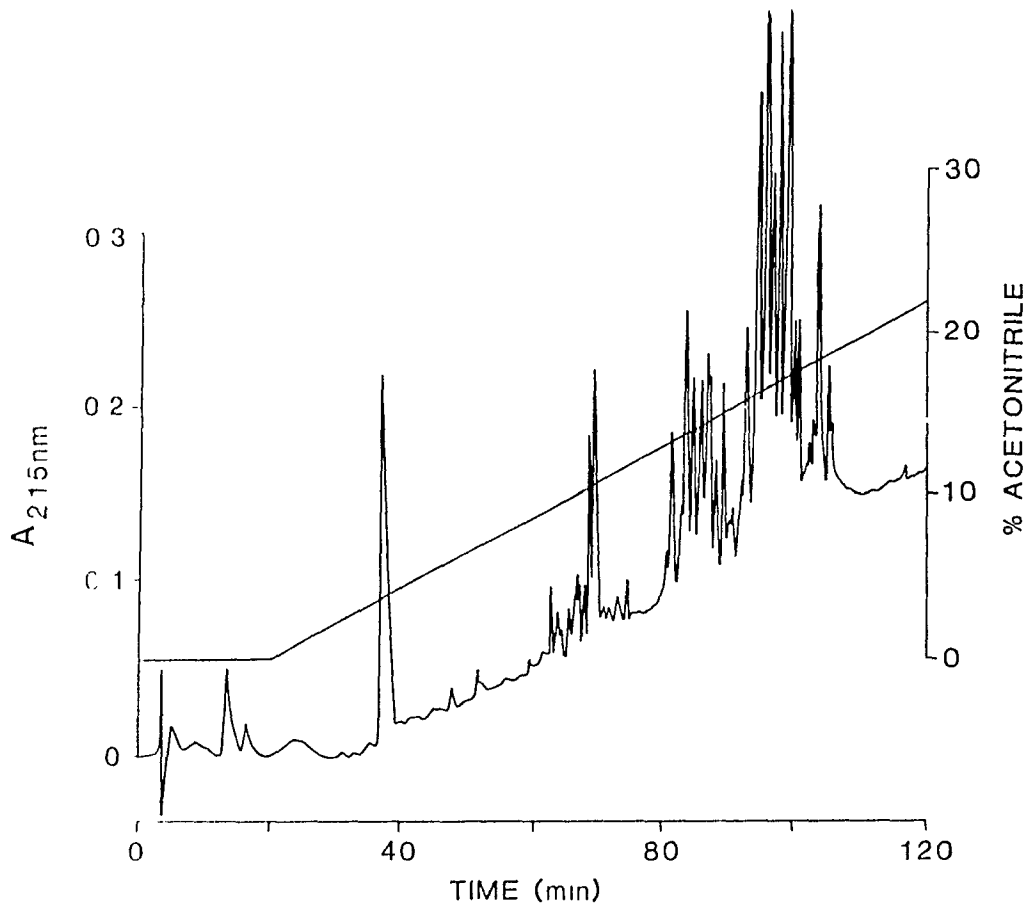
4.2. The Comparisons of the Effects of Natural CSI and Synthetic CSI

Recently CSI has been successfully synthesized by Dr. Varga of American Peptide Company, Inc. (Santa Clara, CA) using solid phase methods in a joint project with our laboratory. They used the BOC/Benzyl strategy and random air oxidation to cyclize the synthetic peptide. The peak material which co-eluted with the natural product has been isolated and purified by HPLC.

The purified synthetic CSI was further characterized by gas phase micro-sequencing after reduction using mercaptoethanol and pyridylethylation. Prior to sequencing one reversed-phase HPLC step was undertaken and the purity of the peptide was confirmed by amino acid analysis. The first 33 cycles of sequencing showed that the synthetic peptide had the same sequence as rabbit CSI. The last cycle of sequencing failed presumably because insufficient sample was loaded. In order to confirm further that the synthetic CSI has the same structure of rabbit CSI, both synthetic CSI and rabbit CSI were digested by trypsin and their HPLC peptide maps were compared (see Fig. 4-14). There were no noticeable differences between two tryptic maps. Not only were the chemical properties of the natural and synthetic CSI very similar, the biological activity of the synthetic peptide was found to be identical to native CSI (see Fig. 4-15).

It is of interest that two side products of synthetic CSI,

named CSIb and CSIc, also had corticostatic activity but were 4 times less potent than synthetic CSI (see Fig. 4-16). The antibody raised against synthetic CSI was able to bind to CSIb and CSIc but to reduced extent with approximately 50% crossreactivity. These results suggest that the correct disulfide bridges in the CSI are not essential but important for its corticostatic activity.


0 3
0 2
0 1
0
A_{215nm}
30
20
10
0
% ACETONITRILE
0
40
80
120
TIME (min)

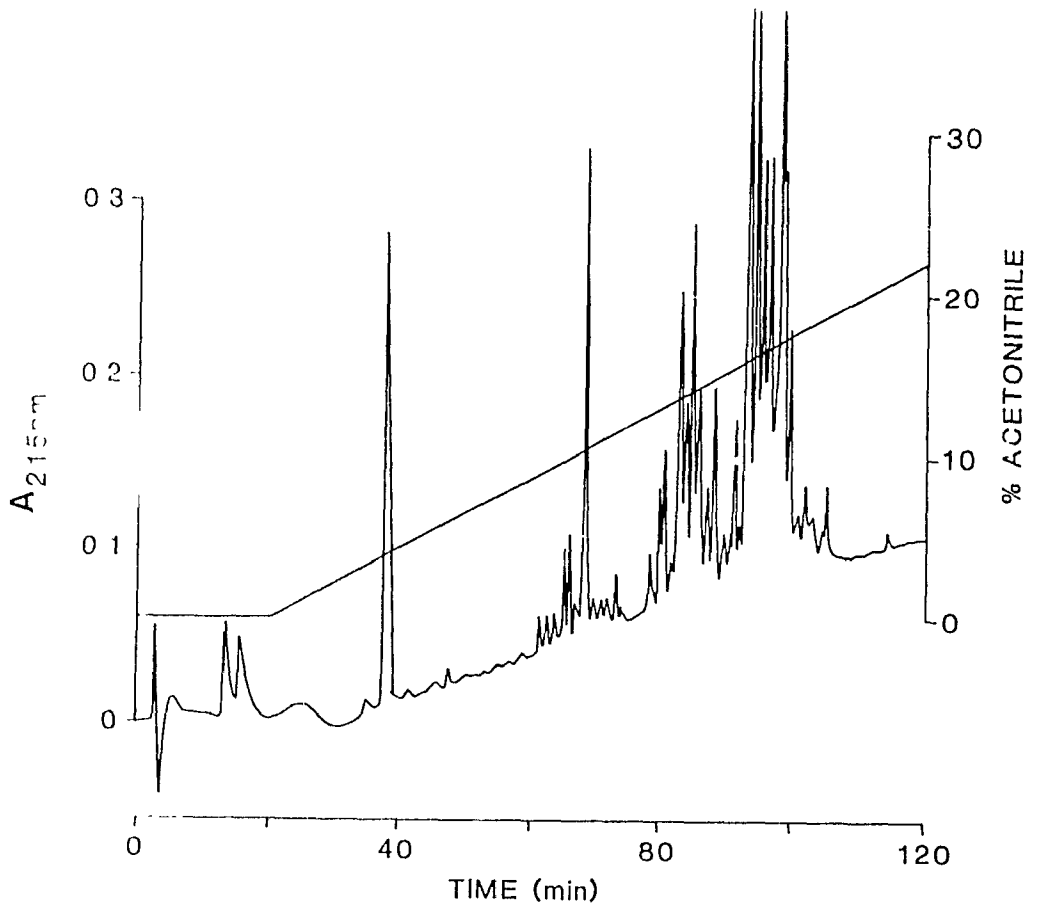

0 3
0 2
0 1
0
30
20
10
0
% ACETONITRILE
0
40
80
120
TIME (min)

Fig. 4-14 Trypsin digestion maps of rabbit CSI and synthetic rabbit CSI. A total of 100 μg of rabbit CSI and synthetic rabbit CSI was digested with trypsin at a concentration of 1:50 (enzyme:peptide/w:w) in 0.5 ml of 0.75% NH_4HCO_3 buffer pH 7.4 at 37°C for 24 hours. At the end of incubation 2 ml of 1% TFA was added to lower the pH to approximately 3 prior to purification by reversed-phase HPLC using 0% acetonitrile for the first 20 minutes, followed by a linear gradient to 22% acetonitrile over 100 minutes. The flow rate was 1.5 ml/min, and 0.1% TFA was used as the counter-ion. The upper panel shows UV absorbance at 215 nm for synthetic CSI. The lower panel shows UV absorbance at 215 nm for natural CSI.

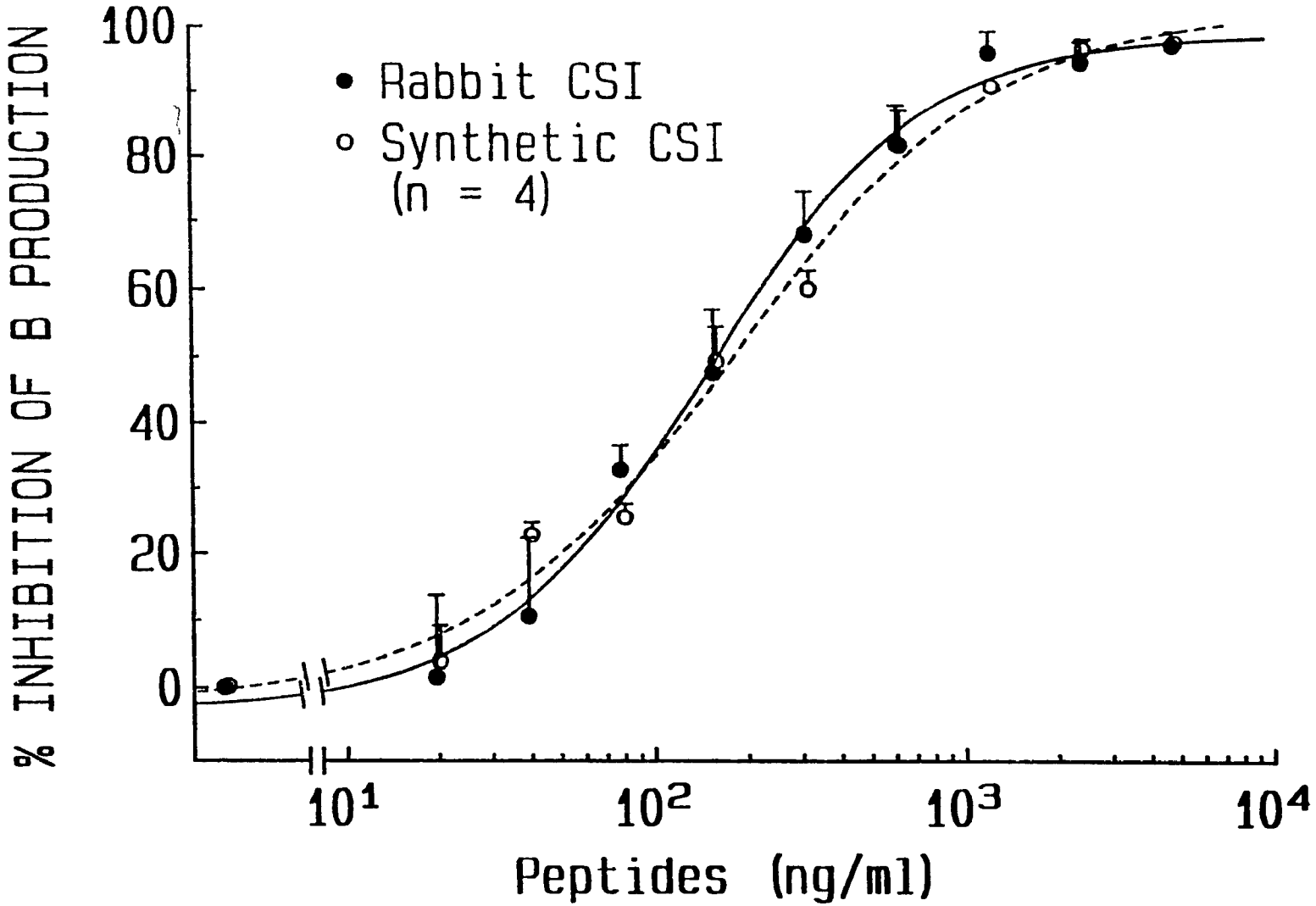

% INHIBITION OF B PRODUCTION
100
80
60
40
20
0
• Rabbit CSI
○ Synthetic CSI
(n = 4)
10^1
10^2
10^3
10^4
Peptides (ng/ml)

Fig. 4-15 Inhibition of ACTH-stimulated corticosterone production by rabbit CSI and synthetic rabbit CSI. ACTH was maintained at 150 pg/ml, and the corticostatins were tested at concentration up to 5000 ng/ml. The values shown are the means (± SD) of four separate and experiments were done in duplicates.

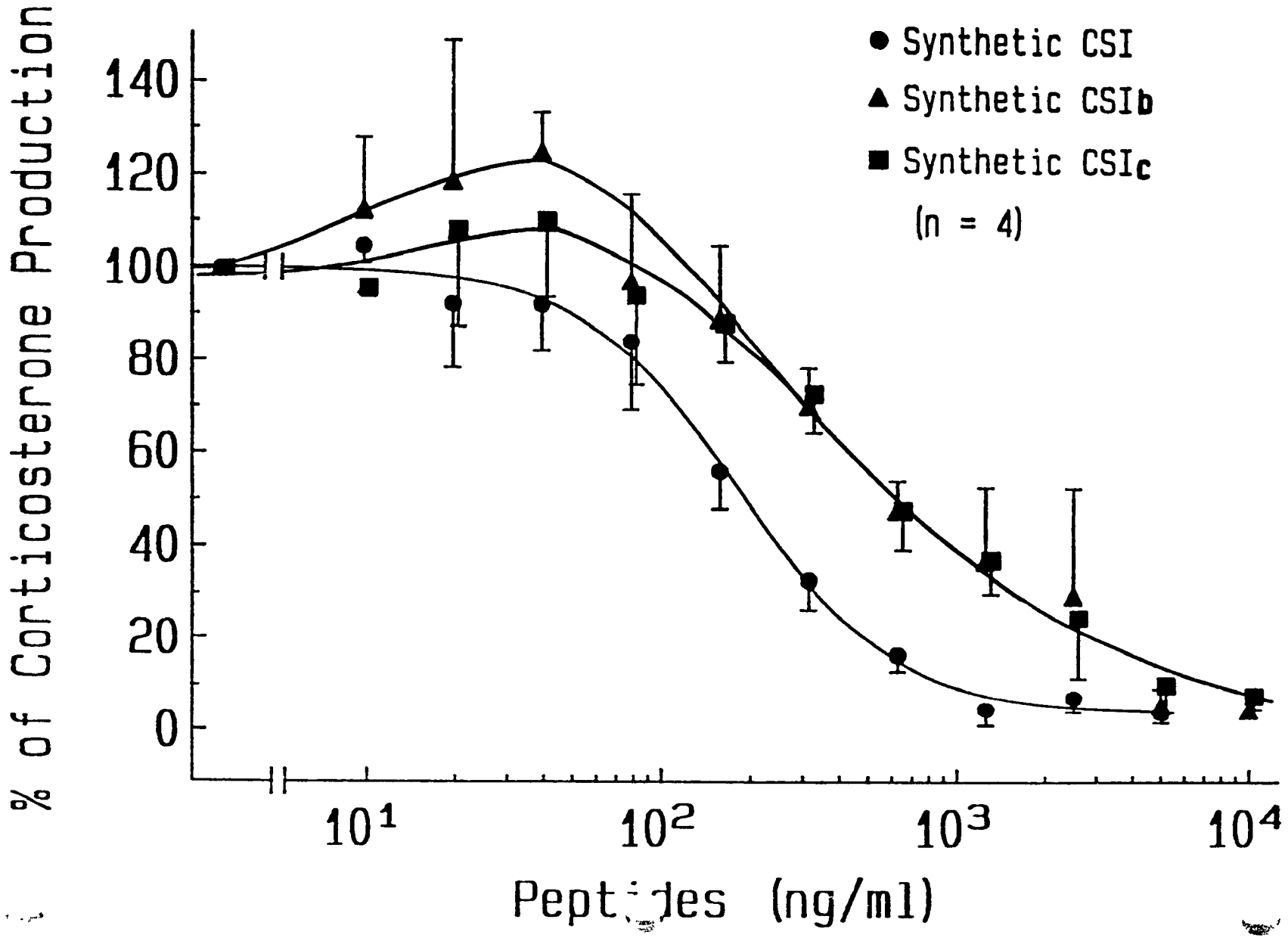

% of Corticosterone Production
140
120
100
80
60
40
20
0
10^1
10^2
10^3
10^4
Peptides (ng/ml)
Synthetic CSI
Synthetic CSIb
Synthetic CSIc
(n = 4)

Fig. 4-16 Inhibition of ACTH-stimulated corticosterone production by synthetic rabbit CSI and synthetic CSIb and synthetic CSIc. ACTH was maintained at 150 pg/ml. The values shown are the means (± SD) of three separate and the experiments were done in duplicates.

CHAPTER 5 DISCUSSION

5.1. The Evidence for the Presence of Anti-ACTH Substances in Rabbit Fetal Lung Extracts.

Initially we set out to determine whether the human fetal lung synthesizes ACTH which had previously been detected in that tissue by radioimmunoassay. We started with 20 pairs of human fetal lungs and indeed could demonstrate that ACTH was present in the fractions eluted from the first HPLC (Fig. 3-1) but that the amount was insufficient for further analysis. We also detected the presence of large concentration of αMSH which we did not further characterize because the desacetly-αMSH had previously been isolated from the human fetal pituitary (486). The postulate we formed at that time dealt with the fact that glucocorticoids had specific receptors in the fetal lung (487) and if ACTH were made in fetal lung it could stimulate the fetal adrenal and thus the lung could control its own synthesis of surfactant which is vital for lung function.

Because of the small amounts of ACTH present in human fetal lung we set out to determine if biologically active ACTH was present and set up the Sayer's adrenal bioassay system (469) to test these fractions. In addition we turned to the fetal rabbit lung to continue this work because of the difficulty in

obtaining human fetal lung tissue. Although we were unable to confirm this hypothesis we noticed ACTH inhibitory fractions in our lung extracts.

The first indication that anti-ACTH peptides might be present in the lung extracts came from the analysis by bioassay of corticosterone production from adrenal cells after the addition of Sep-Pak fractions containing ACTH activity (Fig. 3-2). It was noted that there was an unexpected fall in bioactivity in fraction eluted with 40-80% acetonitrile. One of the possible explanations for this repeated observation was that there was an inhibitor present in those fraction.

To test this possibility, an aliquot of 325 μl from the 40-80% acetonitrile fraction (equal to 1 gram of fetal lung tissue) was co-incubated with an increasing amount of ACTH in the rat adrenal cell bioassay and the results shown in Fig. 3-3 supported this assumption. Later trypsinization (Fig. 3-4) and heat resistance (data not shown here) studies showed that this factor(s) had lost its inhibitory activity after 3.5 hours trypsin digestion but was still active after 30 min incubation in a 100°C hot water bath.

In order to study further this inhibitory substance(s) we turned to a large scale isolation from rabbit fetal lung. Before doing so we had to improve our bioassay and determine how we could isolate these peptides by HPLC.

5.2. The Rat Adrenal Cell Bioassay

Radioimmunoassays have provided a fast, highly sensitive and selective post-column detection procedure for the chromatographic analysis of most known hormones. The problems associated with immunochemical techniques when dealing with unknown peptides are that it can only be used to determine known or some structurally closely related peptides, and in most case it cannot provide any information on the unknown's physiological activity. When studying a new factor or investigating a new physiological activity, the method of choice is the bioassay. In a bioassay the activity of a factor is studied on living tissue or cells. Physiological responses, such as secretion of another hormone or cell growth are monitored and physiologically useful data on secretion and mode of action can be obtained .

Most of the important discoveries in endocrine sciences have followed the establishment of a good sensitive, reliable bioassay.

To set up a new bioassay system and then to use it to pick up a new factor(s) is not easy and very time consuming. The bioassay method we have successfully used and emphasize here is ED_{50} bioassay method which has a wide applicability. The difference between ED_{50} bioassay method and traditional bioassays is that in the traditional bioassay the cell is at a basal level and any factor which can increase the basal secretion or reduce

basal secretion can be monitored. In the traditional bioassay one cannot detect factors of two important categories, potentiator and antagonist. While in the ED_{50} bioassay method basal secretion has been elevated to 50% of maximum by a stimulator, therefore it can detect antagonists and potentiators as well. The disadvantage of this method is high background, and low sensitivity to agonists.

The bioassay for corticostatic peptides is essentially the same as for the ACTH assay. The bioassay for ACTH was carried out first *in vivo* by measuring adrenal weight or histological changes (488), ascorbic acid or cholesterol depletion (489), or corticosterone output (490). The sensitivity was highest by measuring corticosterone output (25 pg) and lowest by measuring adrenal weight. *In vitro* bioassays for ACTH were first reported by Saffran and Schally (491) using adrenal quarters, then by Kloppenborg *et. al* (492) and Swallow & Sayers (493) using isolated adrenal cells. Relatively high concentrations of ACTH were needed to stimulate steroidogenesis in rat adrenal quarters. This was due to tissue heterogeneity and uneven exposure of peptides to cells. The possibility of other factors from medullary cells responsible for this lack of sensitivity in using adrenal quarters bioassays cannot be excluded.

In the dispersed rat adrenal cell bioassay, both collagenase (492, 494) and trypsin (495, 469) can be used to lyse the collagen fibres and ground substance between the outer cellular

layers. The quality of commercially available collagenases has been improved dramatically, therefore the advantage of using the purer trypsin was lost. In addition, it may not be necessary to inhibit the dispersal enzyme with trypsin inhibitor as is the case for trypsin dispersal. Trypsin inhibitor has been shown to inhibit steroidogenesis. Medullary cells are destroyed during the isolation procedure in the Sayer's method.

Sedimentation was suggested by Bennett *et. al* (471) to remove the cell debris thus increasing the apparent potency of ACTH and pre-incubation was proposed by Goverde *et. al* (473) as a means of further increasing the sensitivity of the bioassay.

In the present bioassay system, addition of trypsin inhibitor has been omitted and always keeping cells in the pre-oxygenated buffers at 37°C has been emphasized. These small modifications did not change the observed potency of ACTH, but improved the viability of cells, the reproducibility of the assay, and increased the corticosterone production therefore increasing the sensitivity of the assay.

In the ED_{50} bioassay method, 150 pg/ml ACTH was added to each tube except where an ACTH dose-response curve was constructed. It has been found that the control tubes with 150 pg/ml ACTH should be placed at least every 10 tubes when the total exceed 100 tubes. This is in order to correct for the time difference when adding the reagents in both bioassay and

radioimmunoassay procedures.

As with any cell dispersal or culture method, certain precautions have to be taken. Adrenal glands should be removed immediately after decapitation of rats. The enzymatic activity of collagenase changes from one batch to another, or even during the time of storage, so the amount of enzyme should be adjusted to meet the minimum requirement. This can be achieved by addition of mechanical dispersal at 30 or 45 min of the 1 hour incubation time especially in when using a new batch of collagenase. A shorter or longer than 1 hour of incubation time required for enzymatic dispersal of quartered adrenal glands always indicates the necessity to decrease or increase the amount of collagen used in the experiments. The cells should be treated as gently, and as fast as possible during the entire cell preparation. The temperature in the bioassay has to be controlled at 37°C or a little below. Temperatures higher than 38°C should be avoided at all costs.

The lowest amount of ACTH that could be detected in our assay system was 1 to 3 pg/ml (data not shown here), and ED_{50} was at approximately 150 pg/ml, and reached a plateau at about 740 pg/ml (Fig. 3-5). The maximal corticosterone response to ACTH stimulation could change from one assay to another. This is due to the variations in the conditions of the animal such as the degree of stress before decapitation, and the method of handling the cells, the temperature of incubation, the gas phase

condition, and even the counting of cells and the radioimmunoassay of corticosterone. The value of the maximal response to ACTH stimulation in our bioassay system was around 300 to 700 ng corticosterone/2 x 10^5 cells / 2h and most of results obtained have been normalized as % of maximal corticosterone production.

5.3 Isolation and Identification of Corticostatic Peptides.

5.3.1. Peptide Extraction and Purification

During the course of isolation most peptides are susceptible to proteolytic enzyme degradation. The risk of purifying a peptide that has been generated artificially during the extraction process is very high. The acidic extraction medium at 4°C (homogenization medium) we used, first described by Bennett *et al* (496) is the most efficient way of inhibiting peptidase activity during the first stages of isolation. Furthermore, most of the large proteins are insoluble in this medium and may be removed by centrifugation. The peptides in the supernatant are simultaneously concentrated, desalted and deproteinized by adsorption onto ODS-silica cartridges. Small peptides are able to penetrate the pores of ODS-silica, whereas protein are usually excluded and salts are too polar to be retained. Peptides bound to the ODS-silica cartridge can be recovered by elution with non-polar solvent, 80% acetonitrile

or roughly fractionated by a series of solvent in the order of increasing hydrophobicity. This methodology represents a general approach for the extraction and isolation of peptides.

The eluate from the ODS-silica cartridge usually in 80% acetonitrile containing 0.1% TFA can be stored safely at below -20°C for a long time. At this low pH, in a non-polar solvent and in the cold, most of the peptidases are not active. The eluate cannot be injected onto reversed-phase HPLC columns directly unless the acetonitrile concentration is below 2%. This can be achieved by dilution with aqueous buffer or by evaporation of acetonitrile under vacuum.

The reversed-phase HPLC has been proven to be the best method for the chromatographic separation of peptides (for a review see ref. 497). This is primarily due to the its versatility and its ability to resolve peptides exhibiting very minor differences in structure. There are other advantages of using reversed-phase such as the efficiency, speed and the high reproducibility. The reversed-phase HPLC system used in the present studies employed solvent systems which are completely volatile. Thus following drying, the direct use of radioimmunosassay, bioassay and amino acid analysis on column eluates could be undertaken. The hydrophobic interactions of peptides with ODS-silica packing material are enhanced by using a pH below the pKa of carboxylic groups and the ion-pairing agents (TFA or HFBA) to minimize the polarity of free amino

groups. Thus the relative elution position of peptides depends to a large extent on the number of positive charges and hydrophobic residues. The counter-ion HFBA, is stronger hydrophobic ion-pairing reagent than TFA and maximizes the retention of basic peptides. Both TFA and HFBA systems have high solvating power, and are completly volatile, while TFA is more UV transparent than HFBA. This is the reason why TFA is used more often than HFBA especially during the final step of purification.

5.3.2. Recovery of Peptides in the Initial Protocol

The recovery of peptides when using reversed-phase HPLC methods is variable and depends on the individual peptide, the amount of peptide and the purity of the peptide. The main source of losses are non-specific adsorption of peptide to glass or plastic surfaces.

The discovery of the existence of corticostatic peptides in the fetal rabbit lung extracts was made essentially by chance. As I mentioned earlier HFBA was more UV absorbing at 210 than TFA, therefore TFA is used more often than HFBA, especially with ODS-silica cartridge extraction or fractionation. There was no particular reason to use HFBA buffer in the first instance (Fig. 3-2), since we did not know the peptides would turn out to be so basic in nature. The only reason for using HFBA buffer was its availability (my double distilled water for making new TFA

buffer was not ready yet at that time). Fortunately corticostatic peptides can only be separated from the main peak of ACTH by C_{18} Sep-Pak in the HFBA system, otherwise it will be eluted mainly in the fraction of 0-30% acetonitrile and some of them will be in the fraction of 30-35% acetonitrile in the TFA system, therefore we might not have found the ACTH or corticostatins so readily.

Because of the extremely basic nature of these peptides, they do not behave in the manner expected for most peptides. The retention time of these peptides was not constant in the RP-HPLC system. The retention time shifted from a low percentage of acetonitrile (15% in Fig. 3-5) to a higher percentage of acetonitrile when the peptide became purer (Fig. 3-8). This was due primarily to the protein-protein interaction which can only be noticed when local concentrations of proteins are high and the peptide being purified is highly charged.

The efficiency of purification of corticostatic peptides by reversed-phase HPLC largely depends on the way the sample is handled and the quantity of the material present. In general, corticostatic peptides are very sticky because they are so basic and easily adsorb to glass or plastic surfaces. This property made purification very difficult at first. We failed to realize this, but learned to do rapid purifications and to avoid the complete evaporation of solvents from samples which is very important for their recovery. The evidence of the adsorption of

the corticostatins to the plastic surfaces of the test tubes used was realized by finding that there was very high corticostatic activity in the "empty tubes", which had been washed with the aqueous buffer at least three times, in the rat adrenal cell bioassay.

The recoveries of corticostatic peptides was also improved by using polypropylene tubes and by adding carrier protein such as BSA. The maximum recoveries were achieved while purifying in the 10 to 200 μg range of peptide.

The other characteristic of CSI is related to its high content of arginines (9 out of 34 amino acids). During reversed-phase HPLC the concentration of acetonitrile at which the pure CSI elutes in two counter-ion systems (i.e. TFA and HFBA) are very different. CSI was eluted at 35 to 42% of acetonitrile in the HFBA system, while it was eluted relatively early at 15 to 22% of acetonitrile in the TFA system. The range of difference of acetonitrile concentration indicated here was caused by the age of the column. The older the column being used, the lower the elution concentration of acetonitrile needed to elute CSI. This relatively big difference in these two counter-ion system did cause some problems in the first two trials when we missed scanning the very beginning part of the HPLC run in the rat adrenal cell bioassay because the elution time was much earlier in the TFA system than we had predicted from its elution time in the HFBA system. We soon took advantage of this property.

CHAP. 5. Discussion

We used a very shallow gradient running from 12% to 25% acetonitrile for 2 hours from 60 to 180 min in the first TFA purification as shown in Fig. 3-6. The most abundant inhibitory materials with retention time of 72-96 min was further purified in HFBA system with a steep jump in gradient for the first 30 min to elute most of the peptides followed by a relatively shallow gradient from 32 to 56% acetonitrile for the next 60 min (Fig. 3-7). The corticostatic peptides eluted at a concentration of 38% acetonitrile beyond most of the proteins thereby effecting a large degree of purification. The purpose of using a relatively steep gradient at the beginning of this HPLC purification step was to reduce the sample size later on in the rat adrenal cell bioassay. The aliquot sizes taken for the rat adrenal cell bioassay should be as much as one could afford, that is, there should be enough material left for later bioassay and sequence studies. Here we took 100 μl in duplicates from the fist HPLC purification corresponding to 6 g of fetal lung tissue and 100 μl in duplicates again from the second HPLC purification because we knew, from the results of Fig. 3-3 and Fig. 3-4, that extracts equal to 1 gram of tissue would be sufficient to suppress totally the corticosterone release stimulated by 100 pg/ml ACTH. We also took into account the recovery during HPLC and peak spreading into several fractions. The chromatogram of the third purification step shown in Fig. 3-8 indicated the necessity of carrying out further

purification. We used gel permeation HPLC purification and successfully obtaining a uniform peak as shown in Fig. 3-9. The amino acid analysis of this material indicated that the peptide was homogeneous (Table 3-1). This purified material was sent to Dr. Fred Esch's laboratory at the Salk Institute, San Diego, Calif. for a gas-phase sequence analysis and the following sequence was obtained: Gly-Ile-Cys-Ala-Cys-Arg-Arg-Arg-Phe-Cys-Pro-Asn-Ser-Glu-Arg-Phe-Ser-Gly-Tyr-Cys-Arg-Val-Asn-Gly-Ala-Arg-Tyr-Val-Arg-Cys-Cys-Ser-Arg-Arg. This peptides was named corticostatin (CSI) because of its ability to inhibit the action of ACTH. In order to obtain a large amount of purified CSI for further investigation we modified the purification protocol.

5.3.3. Separation into Basic, Acidic, and Neutral Fraction.

The second protocol that was developed employed CM (carboxymethyl)-silica cartridges as a step between ODS-silica cartridge extraction and reversed-phase HPLC. The use of ion-exchange Sep-Pak cartridges in the batch fractionation of tissue extracts was first introduced by Bennett (466). The CM-silica cartridge is a weak cation-exchanger. The capacity to bind peptide was well correlated with the theoretical charge calculated for a variety of peptides at a given pH. The binding ability of the cartridge for peptides was largely dependent on the total ion strength of the mobile phase including the peptides themselves. The stronger the ion strength, the weaker

the binding. The capability to bind could change slightly from one batch of Sep-Paks to another so the ion strength or pH needs to be adjusted to achieve the best results. In order to decrease other types of interactions which can cause poor recovery, 20% acetonitrile was added to all buffer systems (466). Theoretically peptides bound on the CM-silica cartridges can be recovered by elution with low pH buffer such as 20% acetonitrile in 0.1% TFA, but in the present studies the use of low pH buffer results in very poor recovery. This is due to low dissociation rate for corticostatic peptides such as CSI with 9 arginines being released from the resin. With the use of a 50 mM Tris buffer containing 1 M NaCl and 20% acetonitrile a better recovery was realized. Eluates were then desalted by ODS-silica cartridges. Direct injection of eluates with high concentrations of NaCl onto reversed-phase HPLC column would shorten column life, and should be avoided.

Corticostatins were fractionated into a basic pool such that they could be purified to homogeneity in a single step (in some cases in two steps) by reversed-phase HPLC. The results as shown in Fig. 3-15 are typical examples illustrates the facilitation of the purification after using the CM-silica cartridge as a step between ODS-silica cartridge extraction and reversed-phase HPLC. This could also be seen when we compared the HPLC profiles of fetal rabbit lung extracts without using CM cation exchange (see Fig. 3-6) and using CM cation exchange (see Fig. 3-12a).

There were four major peaks corresponding to the four corticostatic peptides in Fig. 3-15. Although they appeared to be asymmetric, the next reversed-phase HPLC (see Fig. 3-16) demonstrated the first CSI peak only had some contamination at the beginning and at the end of the peak and it had already reached 90% purity. The purity of other three peaks were much higher. They had slight contamination at the begining and end of each peak (data not shown here). The asymmetric shape of these peaks was caused by their high polar properties especially when using the HFBA system. These four peptide were all purified to a homogenous form after the second reversed-phase HPLC in the TFA system. Following reduction and alkylation of these peptides their sequences were determined by the gas-phase microsequencing.

5.4. Tissues that Contain CSI

The retention time of CSI in reversed-phase HPLC purification depends not only on the concentration of acetonitrile but also on protein-protein interaction especially in dealing with cationic (CSI) and anionic peptide interactions. This property makes it very difficult and time consuming to screen tissues with reversed-phase HPLC, followed by the rat adrenal cell bioassay methods because the protein composition is markedly in different tissues. A fast purification step using the pre-column

treatment of samples was desperately needed at that time in order to obtain relatively constant retention time. The use of CM cation exchange made it possible to scan the tissues that contain CSI, because acidic and neutral peptides had been excluded by this method before HPLC purification. As we discussed previously we could even visualize the peak fractions in the chromatogram of the first HPLC purification. This greatly facilitated our studies at that time since an antiserum against CSI was not available yet. The sensitivity of the quantitation by HPLC was limited by the sensitivity of the UV detector. The lowest amount of peptides which could be visualized was approximately 1 to 4 μg in our reversed-phase HPLC system.

Using this method we scanned fetal rabbit lungs, adult rabbit lungs, rabbit peritoneal neutrophils (see Fig. 3-12 a, b, and c), adult rabbit heart, kidney, and liver (see Fig. 3-13 a, b, and c). We were surprised to find that adult rabbit lungs contain the largest amount of CSI like substances because we speculated at that time that CSI was an important regulator of fetal adrenal grands but not in the adult life. Neutrophils contained the highest concentration of CSI. CSI was undetectable in heart, kidney and liver extracts using this methods.

The distribution of CSI in the rabbit was studied later on by radioimmunoassay using a polyclonal antibody raised against CSI as described in the methods section. The results are shown in the Table 5-1. Of interest was that the CSI content had been

found in the central nervous system and they were unevenly distributed. This phenomenon is currently under the investigation in our laboratory.

Table 5-1 CSI levels in the rabbit tissues

Tissues	CSI	(ng/g tissue)
Spleen	21	
Plasma	40	(ng/ml)
Liver	86	
Kidney	108	
Heart	158	
Brain	165	
Adrenal	619	
Lung	1810	
Bone Marrow	202000	(ng/two femur bone)
Pituitary	9590	
Cerebrum	27	
Corpus	43	
Cerebellum	97	
Thalamus	164	
Pons, medulla Oblongata	482	
Hypothalami	682	

5.5 Corticostatic Peptides in the Immune System

5.5.1. Corticostatic Peptides from Neutrophils

The rationale for scanning the CSI content in rabbit neutrophils resulted from a computer search of the Protein Identification Resource of the National Biomedical Research Foundation (Georgetown University, Washington, DC) which revealed that a few months earlier, Selsted *et al* (478) had

found the same peptide in rabbit peritoneal neutrophils. We purified the first four peaks shown in Fig. 3-12c (from retention time 31 to 47 min) by using the exactly same gradients and counter-ions described in the legends to Fig. 3-7, and Fig. 3-8 when we purified the CSI from rabbit fetal lungs extracts. The gas-phase sequence analysis of these purified peptides confirmed that they had the same structures as the ones we purified from adult rabbit lungs. Since lung contains a large amount of neutrophils, the peptides we purified from rabbit fetal and adult lung might come from the neutrophils in the these tissues. We are now trying to identify the cell types which contain CSI cells in the lung tissue using immunocytochemistry techniques.

5.5.2. The Possible Role of CSI in the Interaction of the HPA Axis and the Immune System

The fact that the corticostatins are immune system derived factors makes this project very interesting. As was pointed out in the introduction, the HPA axis and the immune system have bi-directional communication. It has been well established that glucocorticoids exert a negative control over the immune system; and that macrophage derived-IL-1 or IL-6 and possibly other cytokines act as potent CRF releasing factors in the hypothalamus and pituitary. This immune and endocrine bi-directional regulation can explain nicely the old observation

that glucocorticoids cause monocytopenia, and lymphocytopenia (498, 499) (see Fig. 5-1 a and b). However it became difficult to explain another effect of glucocorticoids, that is, that glucocorticoids stimulate neutrophil egress from bone marrow-stores and causes neutrophilic leukocytosis (500) (see Fig. 5-2). The real physiological significance of the increased number of neutrophils in the blood circulation upon the stimulation of glucocorticoids is still unknown. The discovery of the Anti-ACTH activity of the corticostatins in the neutrophils may help explain these findings.

A possible relationship between the immune and endocrine system during the inflammation process has been postulated as follows. As mentioned in the introduction, there are a variety of stimuli that can activate the HPA axis. Very often trauma or infection will cause an increase in glucocorticoid secretion from the adrenal glands. This is not a process mediated by IL-1 or IL-6 since the macrophages or monocytes have not been fully activated during this initial time period. The increased glucocorticoid levels at this first stage is very important. It directs the body into an emergency status (for a review see ref. 7), increasing the number of neutrophils in the blood circulation which are now ready for the later inflammation process. This is the most critical time for the body to survive. In the second stage, the increased glucocorticoids play a negative feed-back role on the hypothalamus. Concurrently it is

possible that and the corticostatic peptides secreted from the neutrophils or escaping from the destroyed neutrophils exert their corticostatic activity to counter act the ACTH action on the adrenals and to ensure a lowered glucocorticoid secretion. A lower glucocorticoid circulatory level is critical for the normal immune response to take place. Third, the leukocyte, particularly neutrophil polymorphs, and to a lesser extent macrophages, migrate out of the capillaries and into the surrounding tissue and an acute inflammation response begins. In the fourth stage, the activated macrophages secrete IL-1 which triggers the immune response process into taking place. In the last stage, the increased levels of IL-1 activate the hypothalamic CRF cells resulting in the higher levels of glucocorticoids in the blood circulation which in turn suppress the IL-1 synthesis and secretion and slow down the immune response process in instances where an over active immune system will do harm to the body.

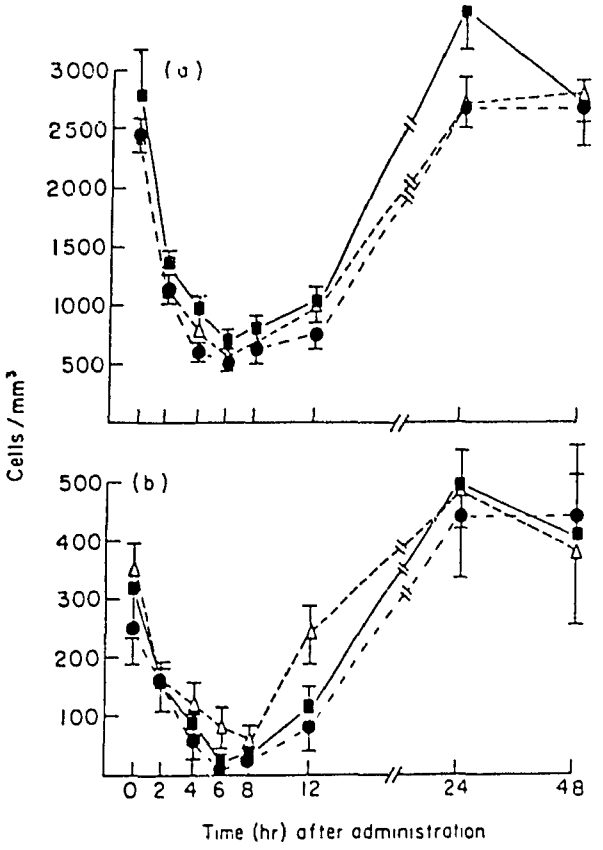


Fig. 5-1. The effect of corticosteroid administration on absolute circulating (a) lymphocyte and (b) monocyte counts. Subjects received a single dose of either hydrocortisone (■, 320 mg, i.v.) prednisone (Δ, 80 mg, p.o.) or dexamethasone (o, 12 mg p.o.). There were eight subjects in each treatment group. Each data point represents the mean (± s.e.m.) counts at various time intervals following corticosteroid administration (copied from ref. 499)

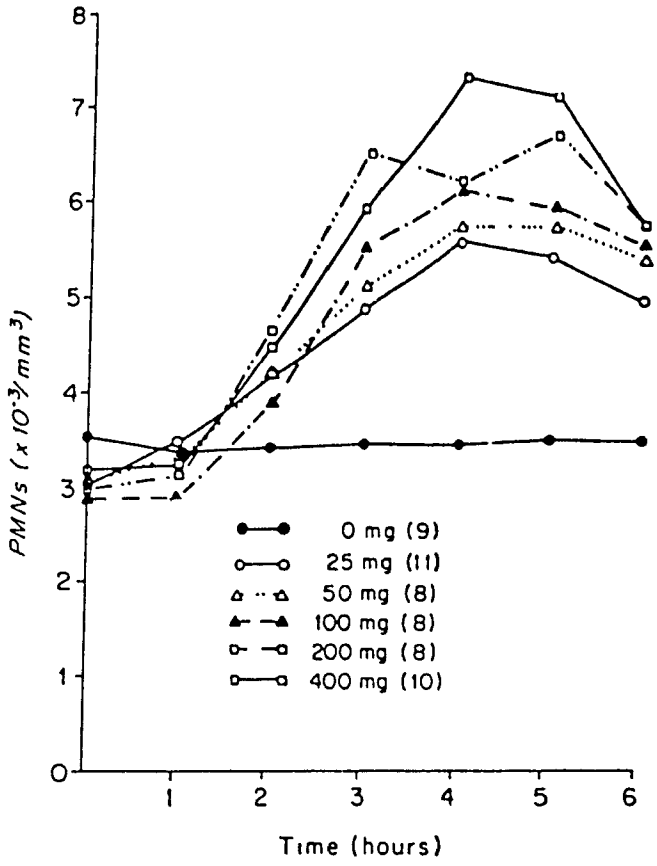


Fig. 5-2. The effect of various doses of intravenous hydrocortisone administration on absolute circulating PMN counts. The point shown are mean values for groups of the sizes indicated in the parentheses (copied from ref. 500).

In some instances in the first step, the increased glucocorticoid secretion in the circulation does not appear, however the concentration of neutrophils in the blood still appear to rise faster than other leukocytes and this is in part because other factors such as bacterial endotoxin (501) will also cause an increase of neutrophils in the blood. Its anti-ACTH, corticostatic peptides found in neutrophils may suppress the glucocorticoids secretion and assure that the normal immune response will take place.

Large amounts of CSI has been found in the blood circulation of surgical infected rabbits. In some infected rabbits, CSI levels were as high as 300 to 500 ng/ml (data is not shown here). This is not surprising if we consider the fact that neutrophils represent about 60-70% of the total normal blood leucocyte content and they are produced in the bone marrow at a rate of eighty million per minute with a short half life of 2 to 3 days.

The negative control of the immune system over HPA axis has been known for quite sometime. The variations in adrenocortical responsiveness has been reported in surgical patients (502) and in patients with severe bacterial infections (503). Corticostatins may in part be responsible for these phenomenona Several other inhibitory factors recently reported including murine macrophage factors (454, 455), TGFß (458, 459) may also contribute to this negative control. However, these negative

mediators of the HPA axis are derived mainly from monocyte and macrophages and their effects may be negated by positive mediators such as IL-1. The detailed mechanisms of how these regulators work together will be the subject of further investigations.

5.6. The Mechanism of Action of CSI

The mechanism of the anti-ACTH action of corticostatins has been studied extensively. The knowledge of its antimicrobial activity reported by Selsted *et al* five months before we obtained the CSI sequence raised the question as to whether the corticostatic activity was due to its cytotoxic activity. We used the Trypan Blue exclusion method to compare the viability of the cells in the absence of CSI and in the presence of an increasing dose of CSI after two hours incubation. We did not find any significant difference in the presence or the absence of CSI. These experimental results were shown in table 4-1. We also compared the activities of the corticostatic agents and found the following rank order of potency, CSI>CSII>CSIII≥CSIV (see Fig. 3-17). Their order of antimicrobial activity was found to be CSIII≥CSIV>CSI≥CSII according to the data of Levitz *et al.* (479). These activities are not related to each other. The idea that the anti-ACTH activity of CSI was not due to its cytotoxic activity was further supported by the facts that CSI only

suppressed ACTH-induced corticosterone, aldosterone and cAMP release (see Fig. 4-1) but was unable to suppress the action of other adrenal stimulators such as angiotensin II (see Fig. 4-3), db-cAMP (see Fig. 4-5), forskolin (see Fig. 4-6) and cholera toxin (see Fig. 4-7).

The other important thing we could establish was the specificity of CSI action. Since CSI could suppress ACTH-stimulated aldosterone production as well as corticosterone production, we wondered whether CSI could inhibit angiotensin II stimulated aldosterone. To our surprise CSI did not affect aldosterone formation stimulated by angiotensin II in rat zona glomerulosa cells as shown in Fig. 4-3 but it did in inhibit ACTH stimulated aldosterone production. This indicated that CSI only acted on ACTH stimulated processes. We then turned to the study of the mechanism on its inhibitory action. Our approach was based on the current knowledge of the trans-membrane signalling system in the adrenal cell.

Cyclic-AMP has been demonstrated to be an intracellular mediator of steroidogenesis (for a review see ref. 504). This concept was based on the following observations. First, the characteristics of the dynamic responses of steroidogensis and cyclic AMP to ACTH stimulation, such as the time to reach maximum output rates, the initial stimulatory rates as well as the subsequent decay rates, were very similar (504). Second, exogenously added cyclic AMP, or its derivatives could mimic the

action of ACTH in terms of steroidogenesis (for reviews see ref 505). Third, Rae et al. (506) demonstrated that the adenylate cyclase and protein-kinase-deficient mutant strains of Y-1 mouse tumor adrenocortical cells totally lost response to ACTH stimulation. Very recently, in elegant studies, Wong *et al.* (507) demonstrated that protein kinase defective (Kin 8) Y-1 cells concomitantly recovered steroidogenic and morphologic responsiveness to ACTH- and 8-bromo-cAMP, after being transfected with an expression vector encoding the catalytic subunit of cAMP-dependent protein kinase. The interesting question we first tried to answer was whether CSI exerted its anti-ACTH activity through a cAMP mediated mechanism, and if so was it prior to cAMP or after cAMP. The experiments shown in Fig. 4-1 strongly suggested that CSI exert its corticostatic action through cAMP mediated mechanism since CSI could suppress ACTH-stimulated cAMP accumulation as well as corticosterone production. We confirmed this using a cAMP derivative db-cAMP to stimulate corticosterone production in the rat adrenal cell bioassay. In this experiment, a total of 1 μg/ml CSI was co-incubated with an increasing dose of db-cAMP (see Fig. 4-5), in the same design we used in the experiments shown in Fig. 4-1. There were no significant difference in the amount of corticosterone released in the presence or in the absence of the CSI. Therefore the site of action of CSI must be prior to the cAMP step in the membrane transduction signal.

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The adenylate cyclase which catalyzes the cAMP formation was the next possible site at which CSI might act. This was tested by checking the effect of CSI on forskolin, an adenylate cyclase stimulator, which stimulates corticosterone production. Although the results shown in Fig. 4-6 had a very big standard deviations, which was caused by the difficulty in preparing accurate forskolin concentrations because of the low solubility of this reagent in aqueous buffer, it still can be seen that there was no significant difference between the corticosterone production in the presence and in the absence of 1 μg/ml CSI. These results suggested that CSI did not act directly on adenylate cyclase.

It is now well established that there is another protein called G protein in the membrane signal transduction pathway between the hormone receptor and adenylate cyclase (for a recent review see ref. 508). G-proteins are membrane-associated heterotrimeric proteins composed of α-, ß-, and γ-subunits (483). It is now generally accepted that G-protein undergoes an activation cycle when interacting with receptors and effector enzymes such as adenylate cyclase. The α-subunit of G-proteins contains the guanine nucleotide-binding site which has an intrinsic GTPase activity that slowly hydrolyzes bound GTP for GDP. In the non-activated state, the guanine nucleotide-binding site is occupied by GDP. In the presence of an appropriate hormone such as ACTH, hormone-receptor complexes activates G-

proteins by catalyzing the exchange of bound GDP for GTP. This leads to the dissociation of the ßγ-subunit complex from α-GTP complex. The α-GTP complex in turn regulates the activity of adenylate cyclase. The bound GTP will be hydrolyzed by the α-subunit and the α-GDP complex then reassociates with ßγ-subunit reforming an inactive heterotrimer. Cholera toxin is an ADP-ribosylating enzyme, that uses NAD as a substrate to covalently modify a specific residue (i.e. Arg 201) (509) in the α-subunits of G stimulatory proteins, resulting in the inhibition of α-subunit's GTPase activity. The consequence of this GTPase inactivation is the accumulation of the active form of α_s-GTP complexes which leads to constitutive activation of adenylate cyclase. The corticosterone release response of the adrenal cell to cholera toxin stimulation is much slower, almost a 1 hour delay, than that to the ACTH stimulation (510). Our results were consistent with this as the maximal response of corticosterone production to the cholera toxin stimulation was only half of the maximal response to ACTH stimulation during a 2 hour incubation period. We did not find any effect on the corticosterone response when co-incubating 1 μg/ml of CSI and an increasing dose of cholera toxin with the rat adrenal cells as shown in Fig. 4-4-7.

Pertussis toxin like cholera toxin is also an ADP-ribosylating enzyme using NAD as substrate. It acts to covalently modify cysteine residues, four amino acids from the

C-terminal (511, 512) in the α-subunits of G inhibitory proteins resulting in the uncoupling G-protein activation (513). The role of G_i protein in the adrenal glands is still not clear. Pertussis toxin was found to be able to suppress the inhibitory effect of vasopressin on ACTH-induced cAMP production by Y-1 cells (514) and enhance the potentiating effect of angiotensin II on ACTH- and cholera toxin- induced cAMP production on cultured bovine adrenal cells between 0 and 3 days of culture (515). We tested the possibility that a G_i protein might be involved in the corticostatic function of CSI by comparising the corticostatic activity of CSI in the absence and in the presence of 100 ng/ml of pertussis toxin. We found no significant difference between these two curves as shown in Fig. 4-8. Therefore, the site where CSI might act, must be prior to the G-proteins, somewhere on the ACTH receptor.

5.7. ACTH Receptor Binding Studies.

The clue that CSI might be a competitive inhibitor of ACTH came from an early experiment in which the weight ratio of CSI and ACTH was fixed at 533 (molar ratio was 600) as shown in Fig. 4-2. In this manner, the corticosterone response decreased to about 50-60% of maximal and remained at this level regardless of the continued increase of ACTH as well as CSI. The direct evidence that CSI was a competitive inhibitor of ACTH could only

be obtained from ACTH receptor binding studies. The interaction of ACTH with specific ACTH receptors on the adrenocortical cell membrane itself remained a controversial issue. The recognition of ACTH in adrenocortical steroidogenesis was made as early as 1955 by Li and co-workers who isolated ACTH from the pituitary, and the discovery of the control of adrenal glands by the pituitary was made even earlier in 1927 by Smith.

A number of reports from various laboratories (516-519) have claimed that binding sites of both high affinity (K_d = 0.25 nM) and low affinity (K_d = 10 nM) sites were observed and that there was a correlation between occupancy of the high affinity binding sites and stimulation of steroidogenesis. The difficulty encountered in solving this issue was to prepare an uniform and fully biologically active radio-iodinated ACTH (520). Studies from Dr. Ramachandran laboratory indicated that ^{125}I-Phe^2,Nle^4 $ACTH_{1-38}$ could be used as it had the same biological potency as ACTH in stimulating steroidogenesis (521). Using this labelled analogue Ramachandran and colleagues were able to show that there was only one type of saturable, high affinity (K_d = 1.41 nM) binding site (477).

We chosen Ramachandran's ACTH receptor assay system to study the binding of the CSI molecule after comparing the biological activity of Phe^2,Nle^4 $ACTH_{1-38}$ and ^{125}I-Phe^2,Nle^4 $ACTH_{1-38}$ with natural $ACTH_{1-39}$ and ^{125}I-ACTH. The amount of biologically active ^{125}I-ACTH found after reversed-phase HPLC purification by our

iodination method was extremely small and for unexplained reasons the material became biologically inactive very rapidly. The half-life was estimated to be less than 20 min (data not shown here) so it was impossible for us to check its biological activity prior to ACTH receptor assay and therefore it was not a suitable radiolabeled ligand to be used to study CSI competition. Compared with natural ACTH, ^{125}I-Phe^{2},Nle^{4} $ACTH_{1-38}$ was a much better ligand with full biologically activity and structural stability. However we found it was still necessary to use freshly iodinated material and to purify ^{125}I-Phe^{2},Nle^{4} $ACTH_{1-38}$ each time before doing receptor studies. As shown in Fig. 4-9 CSI, $ACTH_{6-24}$ and $ACTH_{1-39}$ were all able to displace ^{125}I-Phe^{2},Nle^{4} $ACTH_{1-38}$. Interestingly the potency of CSI in inhibiting the binding of the labelled ACTH analogue lies between $ACTH_{1-39}$ and $ACTH_{6-24}$. CSI was able to displace 175 pM of ^{125}I- [Phe^{2}, Nle^{4}] $ACTH_{1-38}$ in the range of 10 nM to 250 nM, with about 12 times less potency than $hACTH_{1-39}$ and 1.7 times more potency than $ACTH_{6-24}$. $ACTH_{6-24}$ is a synthetic ACTH antagonist synthesized in Schwyzer's laboratory (485). The ability to displace radiolabeled ligand by CSI and $ACTH_{6-24}$ correlates very well with their ability to suppress corticosterone synthesis as shown in Fig. 4-10. As we previously discussed in order to maintain 50 to 60% inhibition of the corticosterone response to ACTH-stimulation, we had to keep the CSI concentration at about 533 fold weight ratio (600 fold molar ratio) higher than the ACTH

concentration. We also demonstrated that the potency for $ACTH_{1-39}$ was only 12 times higher than that of CSI displacement. This is due in part to the fact that the maximal corticosterone response to ACTH stimulation only requires about 5% of receptor occupancy. If we suppose that CSI or $ACTH_{6-24}$ have occupied 50% of the total receptor population there are still half of the receptor sites available for ACTH. Theoretical doubling the amount of ACTH will overcome this CSI inhibitory effect.

5.8. ACTH Structure and the Dual Site Calcium Channel Form ACTH Receptor.

The structural requirement for the ACTH molecule to exert its adrenal corticotropic hormone activity has been studied by testing dozens of ACTH analogues, derivatives, and fragments in the adrenal cell preparations (505). There is a lot of controversy with respect to the steroidogenic activity of some of these synthetic fragments and analogues. This controversy is believed to be due in part to the use of adrenal tissues from different species and to the use of different incubation conditions, such as different Ca^{2+} concentrations, which have been proven to be crucial for the activity of some of the analogues such as $ACTH_{6-39}$ which acts as an antagonist at low Ca^{2+} concentration and as an agonist at high Ca^{2+} concentration (522).

The structural studies of the ACTH molecule by Evans *et al.*

(523) Schwyzer *et al.* (524) Bristow *et al.*, Buckley & Ramachandran and other investigators (525) have contributed to the following concepts:

1. $ACTH_{1-39}$ can first be divided into two parts, $ACTH_{1-24}$ and $ACTH_{25-39}$. $ACTH_{1-24}$ possess full biological activity associated with $ACTH_{1-39}$ and even more potent than the native hormone in *vitro* assays using isolated adrenal cells (470, 471, 473). Attachment of the C-terminal $ACTH_{25-39}$ enhances the antigenicity and prolongs the half life of $ACTH_{1-39}$ in the bloodstream.
2. The first four or five amino acids from N terminal side potentiate the activity of ACTH; from five or six to nine amino acid residues of ACTH molecule are essential for its ACTH activity.
3. The amino acid sequence 11 to 18 is important for the binding to adrenal cortex cells and therefore this region has been called the address region. Within this region, the residues 15-18 containing the sequence Lys-Lys-Arg-Arg are particularly important in the binding of the hormone to the adrenal cell ACTH receptor. The ability of ACTH analogues to bind to the ACTH receptor is proportional to the positive charge associated with this region (526, 527, 477).

In order to find out with which ACTH region the CSI molecule competes for ACTH receptor binding, we added a fixed amount of CSI (1 μg/ml) to increasing concentrations of $ACTH_{1-24}$, $ACTH_{1-18}$ amide, and $ACTH_{1-13}$ amide in the rat adrenal cell system. What we

found was the following: CSI was able to inhibit $ACTH_{1-24}$ (data not shown here) and $ACTH_{1-18}$ amide-stimulated corticosterone production (see Fig. 4-12) but there was no inhibition of $ACTH_{1-13}$ amide-stimulated corticosterone production (see Fig. 4-12). These results demonstrated that CSI competes mainly with amino acids 14-18 for binding to the ACTH receptor.

Very recently Li *et al.* (528) reported that $ACTH_{1-10}$ and $ACTH_{11-24}$ were both biologically active with almost equal potency in terms of stimulation of cortisol production in bovine adrenal cell cultures. They also claimed that both $ACTH_{1-10}$ and $ACTH_{11-24}$ could only elicit about 50% of the $ACTH_{1-24}$ response and that $ACTH_{1-10}$ and $ACTH_{11-24}$ were additive in the stimulation of cortisol production. With the aid of forskolin and calcium channel blockers, they suggested that there were two receptors mediating $ACTH_{1-24}$-stimulated steroid secretion: one class of receptor recognized $ACTH_{1-10}$ but not $ACTH_{11-24}$ and was linked to the cAMP messenger pathway, other class of receptor recognized $ACTH_{11-24}$ and was not linked to the cAMP messenger pathway.

We are in favour of the concept that only one kind of ACTH receptor mediates $ACTH_{1-24}$ or $ACTH_{1-39}$ stimulated steroidogenesis and this is linked to the cAMP messenger pathway. This is because: CSI specifically competes with the ACTH 14-18 sequence for its binding site to the ACTH receptor. If there were two receptors mediating $ACTH_{1-24}$ or $ACTH_{1-39}$-dependent steroid secretion, CSI could not suppress ACTH-stimulated cAMP

accumulation and could only partly suppress corticosterone secretion. In contrast, even though CSI was unable to suppress $ACTH_{1-13}$ amide-stimulated corticosterone production (see Fig. 4-11), CSI was able to inhibit all $ACTH_{1-39}$ actions, namely, stimulation of corticosterone production, cAMP accumulation and aldosterone production (4-1) and CSI could totally suppress ACTH-stimulated corticosterone production when the concentration of CSI was high enough (see Fig. 3-10).

Further evidence, supporting dual receptor mechanisms of ACTH come from receptor binding studies. As mentioned earlier, there are a substantial number of reports from several laboratories (516-518), using ^{125}I-ACTH, which demonstrate that there are two kinds of binding site, high affinity (K_d = 0.25 nM) and low affinity (K_d = 10 nM). The dissociation constants reported from these laboratories were reasonably close. Based on the observation of two populations of binding sites, many models have been proposed, such as dual receptors for ACTH as discussed above, dual sites within a single receptor molecule, negative cooperativity, dimeric receptors, or a tertiary receptor with interaction of the receptor with another membrane protein (see review, 480). However, using ^{125}I-Phe^2,Nle^4 $ACTH_{1-38}$ which had the same biological potency as ACTH in stimulating steroidogenesis (477) Ramachandran and colleagues demonstrated that there was only one type of saturable, high affinity (K_d = 1.41 nM) binding sites (477).

CHAP. 5. Discussion

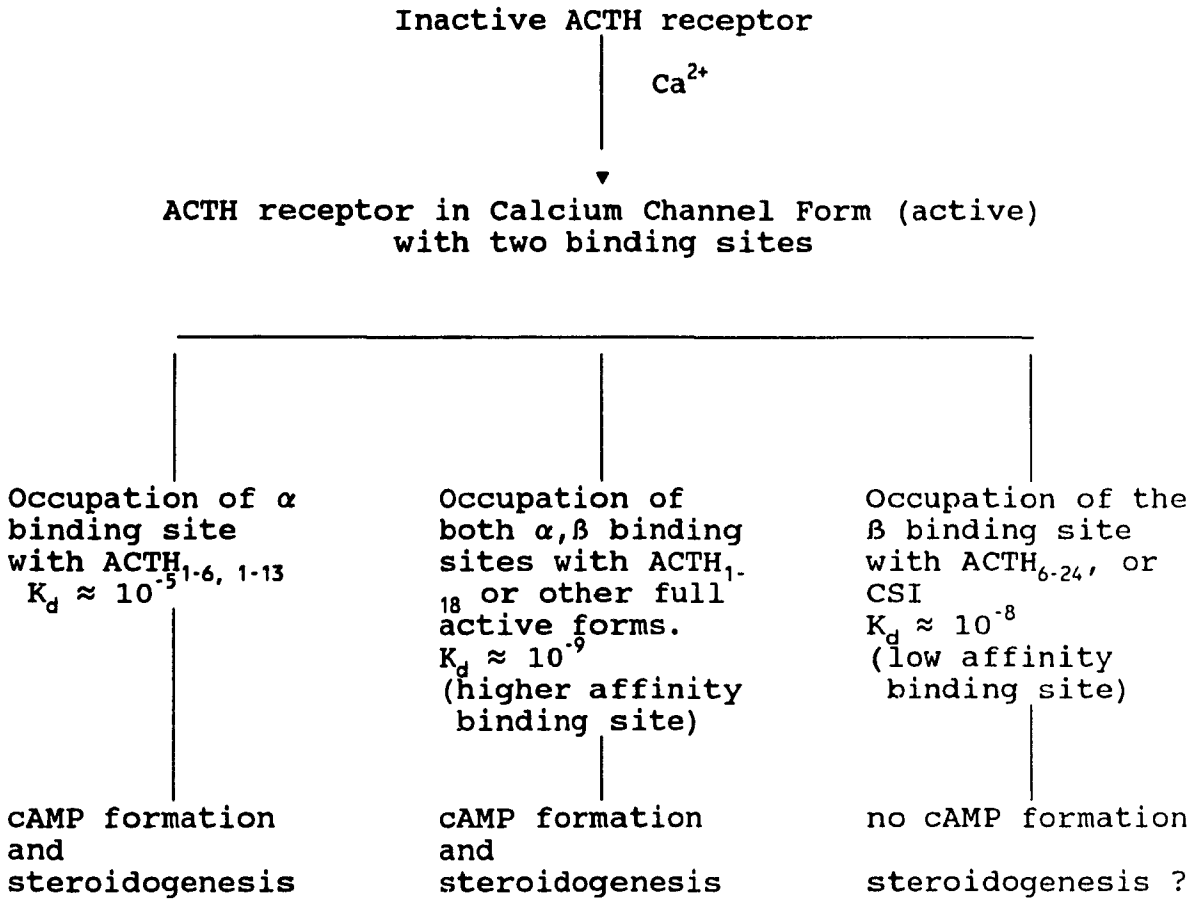
In trial experiments we found that ^{125}I-Phe^{2},Nle^{4} ACTH$_{1-38}$ was superior in many respects to ^{125}I-ACTH$_{1-39}$. First, ^{125}I-Phe^{2},Nle^{4} ACTH$_{1-38}$ was fully biologically active with the same potency as non-iodinated Phe^{2},Nle^{4} ACTH$_{1-38}$ and natural ACTH$_{1-39}$ in our assay system. Second it was much more stable than ^{125}I-ACTH$_{1-39}$, even though we still found it necessary to iodinate it freshly and purify the ^{125}I-Phe^{2},Nle^{4} ACTH$_{1-38}$ tracer immediately before use. In contrast to this, ^{125}I-ACTH$_{1-39}$ was much less potent than ACTH$_{1-39}$ and had a very short half-life for reasons unknown. We postulated that ^{125}I-ACTH$_{1-39}$ used these ACTH receptor binding studies but it was still a mixture of products. The impurity of ^{125}I-ACTH$_{1-39}$ might be caused by poor chromatography which was unable to separate fully biologically active forms, such as ^{125}I-Tyr^{23}-ACTH$_{1-39}$, from partly biologically active forms, such as ^{125}I-Tyr^{2}-ACTH$_{1-39}$. Impurities of ^{125}I-ACTH$_{1-39}$ might form after chromatography and give rise to its short half-life. For instance the methionine residue at position four from N-terminal side may be oxidized by trace amount of ^{125}I^{-} which can possibly detach from the ^{125}I-Tyr^{23}-ACTH$_{1-39}$ or simply be there through contamination. When the mixture is used in ACTH receptor binding studies, ^{125}I-Tyr^{23}-ACTH$_{1-39}$ would bind to the receptor with K_d close to K_d of ^{125}I-Phe^{2},Nle^{4} ACTH$_{1-38}$, while ^{125}I-Tyr^{2}-ACTH$_{1-39}$ or other forms would bind to the ACTH receptor with a K_d close to the K_d of ACTH$_{6-24}$ or CSI.

The other equally important issue in the ACTH receptor

debate is the role of Ca^{2+} in ACTH stimulated-steroidogenesis. It has been known for long time and repeatedly confirmed by many investigators including ourselves (date not shown here), that the extracellular Ca^{2+} is crucial for the ACTH activity. Adding sufficient EGTA or EDTA in the medium could totally abolish the ACTH effect. The concentration of extracellular Ca^{2+} will effect not only the potency of ACTH and ACTH analogs but also the maximal steroid production. It appears to be that the influx of Ca^{2+} from the extracellular fluid is necessary for ACTH biological action. Recently, using ^{125}I-Phe^2,Nle^4 $ACTH_{1-38}$ and a photoreactive derivative of ACTH, Ramachandran *et al.* (529) demonstrated that extracellular Ca^{2+} was absolutely required for the binding and continued occupancy of the receptor by ACTH, but was not needed for steroidogenesis once the hormone was bound to the receptor.

Taken together all information presented so far and from our results, we postulate that dual sites exist within one receptor calcium channel model. In the model, the whole process of the activation of ACTH receptor is divided into three major steps: first step, Ca^{2+} binds to an inactive form of the ACTH receptor, which in fact is more like a calcium binding protein or calcium channel than an ACTH receptor, resulting in conformational changes to the membrane proteins and the formation of two major ACTH binding sites: a site to which ACTH 1-5 (or $ACTH_{1-13}$) binds, which we call the α domain, with a K_d of 10^{-5} M; and a site to

which ACTH 6-24 (or $ACTH_{14-39}$) binds, which we call the ß domain, with a K_d of 10^{-8} M. These two binding sites are structurally separated in the absence of $ACTH_{1-24}$ or $ACTH_{1-39}$ occupancy so that CSI with mass of 4K does not interfere with $ACTH_{1-13}$ amide activity. In a second step , the address region of ACTH molecule, 10 to 18 residues, binds to the ß domain resulting in another type of conformational changes bringing the α domain even more closer to the ß domain. The third step, the N-terminal part of the ACTH molecule binds to the α domain and resulting in the activation of the ACTH receptor. The consequence of the occupancy of the two sites of ACTH receptor, αß domains is the formation with an apparent K_d of 10^{-9} M. The model of the ACTH receptor is schematically shown below:


Inactive ACTH receptor
Ca^{2+}
ACTH receptor in Calcium Channel Form (active) with two binding sites
Occupation of α binding site with $ACTH_{1-6, 1-13}$ $K_d \approx 10^{-5}$
Occupation of both α,ß binding sites with $ACTH_{1-18}$ or other full active forms. $K_d \approx 10^{-9}$ (higher affinity binding site)
Occupation of the ß binding site with $ACTH_{6-24}$, or CSI $K_d \approx 10^{-8}$ (low affinity binding site)
cAMP formation and steroidogenesis
cAMP formation and steroidogenesis
no cAMP formation
steroidogenesis ?

5.9. The Structural Requirement for Anti-ACTH Activity of Corticostatin

In the last two years, corticostatic peptides have been detected in other species such as the human (530), rat (531), guinea pig and bovine (532) Some of the peptides have been fully characterized. Their structures are shown on the below together with the structure of $ACTH_{6-24}$.

Peptides with Corticostatic activity

$ACTH_{6-24}$	HFRWGKPVGKKRRPVKVYP
CSI	GICACRRR FCPNSERFSGYCRVNGARYVRCCSRR
CSII	GRCVCRKQLLCSYRERRIGDCKIRGVRFPFCCPR
CSIII	VVCACRRA LCLPRERRAGFCRIRGRIHPLCCRR
CSIV	VVCACRRA LCLPLERRAGFCRIRGRIHPLCCRR
HP-4	VCSCRLV FCRRTELRVGNCLIGGVSFTYCCTRV
R-4	VTCYCRRT RCGFRERLSGACGYRGRIYRLCCRR
R-5	VTCYCRTT RCGFRERLSGACGYRGRIYRLCCRR
GPCS	RRCICTTR TCRFPYRRLGTCIFQNRVYTFCC

Peptides without Corticostatic activity

HP-1	ACYCRIP ACIAGERRYGTCIYQGRLWAFCC
R-1	ACYCRIG ACVSGERLTGACRLNGRIYRLCC
R-2	VTCSCRTS SCRFGERLSGACRLNGRIYRLCC

R-3 CSCRTS SCRFGERLSGACRLNGRIYRLCC

By comparing the structure-activity relationships of the corticostatins it appears that an arginine residue in the C-terminal end next to the final cysteine is important since this is missing in the inactive analogue HP1 and three rat peptides (531) R-1, R-2, R-3. In preliminary experiments we have removed the last two arginines from CSI. This did not totally diminish its corticostatic activity. This could have been caused by the contamination of the side-products from incomplete digestion. Further experimental studies will be necessary before reaching final conclusions concerning the C-terminal arginines.

By comparing the structures of the two most potent corticostatins rabbit CSI and rat R-4 with $ACTH_{6-24}$, all three peptides have a very similar basic amino acid pattern, namely, basic-X-X-basic-X-X-X-basic-basic. When the basic amino acid in the last 6 position is replaced by other amino acid in the case for CSIII and CSIV, the corticostatic activity falls to 10% of that of CSI. Rat peptides R-1, R-2, R-3 have a basic arginine four residues from the C terminus and they have no corticostatic activity. Therefore it seems that this basic amino acid is not essential for the corticostatic activity, but is a very important potentiator. CSII and CSIII have very similar overall basic amino acid patterns. Interestingly, CSII is missing the last C-terminal basic amino acid but has an extra one at the

second position of the N-terminal site and CSII is almost three time more potent then CSIII. Therefore the amino acid at the N-terminal may be as important as it at C-terminal.

Although the disulphide bridges of these peptide has not yet been characterized, the disulphide linkages for NP-5, an analogue (we predict that it is corticostatic) has been deduced recently (533). The disulfide linkages were C-3-C-31, C-5-C-20, and C-10-C-30. A correct disulphide linkage in the corticostatic peptides will potentiate its corticostatic activity. This can be seen very clearly when we compare the activity of three synthetic rabbit CSIs (see Fig. 4-16). For these three peptides only synthetic CSI has the correct disulphide linkage and it is almost four times more potent than other two. We postulate that correct disulphide bridge brings N-terminal basic amino acids to the C-terminal, such as the tri-basic sequence of residues 6 to 8 in CSI. This feature may account for the higher potency of CSI compared to $ACTH_{6-24}$ and the other analogs. From immunological studies we have obtained further evidence that CSI differs from the less potent corticostatins including synthetic CSIb and CSIc. A polyclonal antibody raised against synthetic CSIa cross reacts not only very weakly with CSII, CSIII, CSIV, but also can distinguish synthetic CSIa (correct form) from the other two side products which have about 40% cross-reactivity.

CONCLUSIONS

1. The anti-ACTH substance(s) extracted from rabbit fetal lungs was thermodynamically stable but susceptible to trypsin digestion suggesting that this substance(s) might be a peptide.
2. Reversed-Phase HPLC followed by rat adrenal cell bioassay revealed that there were at least four anti-ACTH peptides in the acidic extracts of rabbit fetal lungs. The most abundant one was purified to homogeneity and had the following sequence: **Gly-Ile-Cys-Ala-Cys-Arg-Arg-Arg-Phe-Cys-Pro-Asn-Ser-Glu-Arg-Phe-Ser-Gly-Tyr-Cys-Arg-Val-Asn-Gly-Ala-Arg-Tyr-Val-Arg-Cys-Cys-Ser-Arg-Arg.** This peptide was named **corticostatin I** (CSI) because of its ability to inhibit ACTH and it was the first such peptide characterized.
3. Scanning adult rabbit tissue by an improved purification protocol demonstrated that rabbit adult lung and rabbit neutrophils contain higher levels of the peptides.
4. Four major components have been purified from both rabbit adult lung and neutrophils and sequenced. They are called corticostatin I (CSI), II (CSII), III (CSIII), and IV (CSIV) respectively according to their retention time. Among them CSI is the most potent with ED_{50} of 25 nM against a concentration of

0.033 nM ACTH in the rat adrenal cell bioassay.

5. The mechanism of the action of CSI has been studied extensively. CSI shows a high degree of specificity in that it does not inhibit the action of angiotensin II in the adrenal cortex. CSI does not inhibit dbcAMP induced steroidogenesis, however it does inhibit the accumulation of cAMP in response to ACTH in the rat adrenal cell bioassay. CSI does not directly act at adenylate cyclase or G proteins. CSI acts by competing with the basic $-Gly_{14}-Lys_{15}-Lys_{16}-Arg_{17}-Arg_{18}-$ sequence of ACTH for its binding site.

6. Synthetic CSI has an identical anti-ACTH activity of the isolated peptide.

CLAIMS TO ORIGINALITY

This is the first description of a family of peptides which are derived from the non-endocrine systems which specifically inhibits ACTH action on the adrenal cortex. Due to the nature of this research, all the results presented in the chapter III and IV can be considered as original research. **The most significant contributions of this work to original knowledge are listed below:**

1. Discovery of the existence of anti-ACTH substances in the acidic extraction of rabbit fetal lungs.

2. Purification and characterization of the corticostatic (anti-ACTH) peptide from rabbit fetal lungs.

3. Purification and characterization of four corticostatic peptides (CSI, CSII, CSIII, and CSIV).

4. Evidence that CSI is the most active corticostatic peptide of the four.

5. CSI inhibits ACTH but not Agiotensin II-stimulated aldosterone production.

6. CSI is not a direct inhibitor of the cAMP-dependent protein

kinase or adenylate cyclase or G proteins.

7. CSI inhibits ACTH binding by competing with the sequence 14 to 18 of ACTH.

8. Preparation of a highly specific polyclonal antibody for CSI.

9. Evidence that the negative control of HPA axis by the immune system.

10. Fetal and adult lungs are the richest source of corticostatic peptides.

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