REGIONS OF THE CORTICOTROPIN MOLECULE ESSENTIAL FOR THE STIMULATION OF AEROBIC GLYCOLYSIS IN MOUSE ADRENOCORTICAL CELLS

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A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

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The mechanism of ACTH-induced aerobic glycolysis was investigated first by determining the substrate and nucleotide requirements, and secondly, by identifying essential structural elements through structure-activity studies with various ACTH-related peptides.

Glycolysis was stimulated by dibutyryl cyclic AMP, AMP and GMP whereas dibutyryl cyclic GMP was ineffective. AMP and GMP did not stimulate steroidogenesis, unlike dibutyryl cAMP. Basal glycolysis was stimulated by glucose, glucose 6-phosphate, fructose, fructose 6-phosphate, pyruvate, glycerol, and to the greatest extent by fructose 1,6-diphosphate. Only glucose potentiated ACTH-induced glycolysis in cells whereas ACTH steroidogenic action in intact glands was potentiated by pyruvate and glucose.

Structure-function studies revealed that the essential regions of ACTH for glycolytic activity were the basic sequence LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18} and the N-terminal segments MET^{4}-GLU^{5}-HIS^{6}-PHE^{7}-ARG^{8}-TRP^{9} and LYS^{11}-PRO^{12}-VAL^{13}.

The two minimum active sequences were ACTH (4-10) and ACTH (7-13); ARG^{8} was essential for steroidogenesis but not obligatory for glycolysis; TRP^{9} was mandatory for glycolysis and cAMP production, which could be blocked by replacing TRP^{9} by NPS-TRP, without any effect on maximal steroidogenic activity. Parathyroid hormone, but not vasoactive intestinal peptide, could mimick ACTH effect on glycolysis.

Although a steroid mediated component of ACTH-induced aerobic glycolysis is likely in view of the established direct glycolytic effects of exogenous corticosteroids, the present study indicates that the glycolytic response to ACTH may also be dissociated from steroidogenesis. Mechanism for steroid-independent glycolytic effects of ACTH could include enhancement of glucose transport and allosteric modification of glycolytic enzymes induced directly or indirectly by cAMP and changes in ATP/ADP, AMP ratios. The functional significance of ACTH-induced glycolysis is not known but could involve increases in blood flow, ATP-supply and modification of intercellular gap-junctional transport induced by changes in pH. Such effects could also be exerted in extra-adrenal tissue, including the brain.

RÉSUMÉ

Le mécanisme par lequel l'ACTH induit la glycolyse aérobique fut étudié premièrement par la détermination des exigences en termes de substrats et de nucléotides et, deuxièmement, par l'identification des structures essentielles, suite à des études de structure-activité utilisant des peptides reliés à l'ACTH.

La glycolyse fut stimulée par le di-butyrate d'AMP cyclique, par l'AMP et le GMP alors que le di-butyrate de GMP cyclique s'est avéré inefficace. L'AMP et le GMP n'ont pas stimulé la stéroidogénèse contrairement au dibutyrate d'AMP cyclique. La glycolyse basale fut stimulée par le glucose, le glucose 6-phosphate, le fructose, le fructose 6-phosphate, par le pyruvate, le glycérol et, en grande partie, par le fructose 1,6-diphosphate. Le glucose fut la seule substance parvenant à amplifier la glycolyse induite par l'ACTH dans des cellules en culture alors que l'action stéroidogénique de l'ACTH fut amplifiée à la fois par le pyruvate et par le glucose dans la glande intacte.

Les études de structure-fonction on révélé que les régions de l'ACTH essentielles pour son action sur la glycolyse étaient la séquence de base LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18} et les segments N-terminaux MET^{4}-GLU^{5}-HIS^{6}-PHE^{7}-ARG^{8}-TRP^{9} et LYS^{11}-PRO^{12}-VAL^{13}.
Les deux séquences actives minimales furent ACTH (4-10) et ACTH (7-13); l'ARG^{8} fut essentielle pour la stéroidogénèse mais non pour la glycolyse; le TRP^{9} fut indispensable pour la glycolyse et la production d'AMP cyclique, production qui pouvait être bloquée en remplaçant le TRP^{9} par NPS-TRP, tout en conservant une activité

stéroidogénique maximale. L'hormone parathyroidienne, contrairement au peptide intestinal vasoactif, put imiter l'effet de l'ACTH sur la glycolyse.

Quoiqu'une induction de la glycolyse aérobique par l'ACTH avec les stéroides comme modulateurs soit en accord avec l'effet glycolitique direct reconnue aux corticostéroides exogènes, la présente étude indique que la réponse glycolitique à l'ACTH peut être aussi dissociée de la stéroidogénèse. Un mécanisme par lequel l'ACTH exercerait son effet glycolitique indépendamment des stéroides pourrait impliquer une augmentation du transport du glucose, une modification allostérique des enzymes inductibles directement ou indirectement par l'AMP cyclique ainsi que des changements dans les rapports ATP/ADP, AMP. L'importance fonctionnelle de la glycolyse induite par l'ACTH est inconnue mais pourrait impliquer des augmentations de flux sanguin et d'approvisionnement en ATP ainsi que des modifications du transport inter-cellulaire produit par des changements de pH. De tels effets pourraient être aussi exercés dans des tissus extra-surrénaliens, incluant le cerveau.

To

The memory of

John Kofi Hinson

(1904-1982)

PREFACE

This thesis is divided into 3 sections: Section A reviews current knowledge about ACTH, Section B describes various methods applied in the study and Section C presents the data accumulated from the research work.

In reviewing ACTH in Section A, emphasis has been placed on recent advances, molecular principles and current concepts.

The data in Section C are in two parts, representing two independent projects about the same aerobic-glycolysis phenomenon. However, as the thesis title suggests, the main focus of the thesis has been on the latter study, which investigated the relation of ACTH molecular structure to stimulation of aerobic glycolysis. The first project, has been presented as an adaptation of the recently accepted publication (305).

All the schematic diagrams and data figures presented in this thesis were original sketches professionally redrawn and printed at the Montreal Neurological Institute's Photomicroscopy Laboratory.

ACKNOWLEDGEMENTS

I am very grateful to my Research Director, Professor M.K. Birmingham, for her constant encouragement, guidance, financial assistance, and above all, for the exciting science.

For their donation of various synthetic ACTH peptides, I am greatly indebted to Dr. Klaus Hofmann of Pittsburgh, Dr. J. Ramachandran of California, Dr. M. Nakamura of Tokyo, Drs. Andreatta and Schelbi of CIBA-Geigy, Switzerland, Dr. H. Goverde of the Netherlands and Dr. Peter Schiller of Montreal.

Many thanks to Miss Laura Fiorita for her patience and skill in typing and processing this thesis.

Thanks are also due to Mr. Marc Ekker who provided an excellent French translation of the abstract.

To the entire Hinson family, my sincere gratitude for their love and support. And special thanks to Miss Margaret-Mary Poku for the prayers and excellent proof-reading.

LIST OF SPECIAL ABBREVIATIONS*

ACTH	=	Adrenocorticotropic Hormone, Corticotropin
AiB	=	α-Amino isobutyric acid
BSA	=	Bovine Serum Albumin
cAMP or CAMP	=	Adenosine 3':5' - cyclic monophosphoric acid
cGMP or CGMP	=	Guanosine 3':5' - cyclic monophosphoric acid
CEH	=	Cholesterol Ester Hydrolase
CRF	=	Corticotropin Releasing Factor, Corticoliberin
CTP	=	Cytidine triphosphate
Cyt P-450$_{scc}$	=	Side-chain cleavage cytochrome P-450 enzyme system
Cyt P-450$_{11}$	=	11 β-hydroxylase cytochrome P-450 enzyme system
Cyt P-450$_{21}$	=	21-hydroxylase cytochrome P-450 enzyme system
dbcAMP	=	N^6, $O^{2'}$-dibutyryl adenosine - 3':5'-cyclic monophosphoric acid
dbcGMP	=	N^2,$O^{2'}$-dibutyryl guanosine - 3':5'-cyclic monophosphoric acid
DG	=	Diacyl glycerol
EDTA	=	Ethylene diamine tetracetic acid
FSH	=	Follicle Stimulating Hormone, Follitropin
Gpp(NH)p	=	Guanylylimido diphosphate
ITP	=	Inositol Triphosphate
LH	=	Luteinizing Hormone, Lutropin
LHRH	=	Luteinizing Hormone Releasing Hormone, Luliberin

* Abbreviations are explained also in "Materials and Methods", and in the text when they appear for the first time.

LPH = Lipotropic Hormone, Lipotropin

MSH = Melanocyte Stimulating Hormone, Melanotropin

Nle = Norleucine

NPS = Ortho-nitrophenyl sulphenyl

POMC = Pro-opiomelanocortin

PPI = Polyphosphoinositides

PTH = Parathyroid hormone, Parathormone, Parathyrin

PtdIns = Phosphatidyl inositol

$PtdIns(4,5)P_2$ = Phosphatidyl inositol 4,5 biphosphate

SCP = Sterol carrier proteins

TFE = Trifluoroethanol

VIP = Vasoactive Intestinal Peptide

VSM = Vascular Smooth Muscle

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

TABLES PAGE

I. GENERAL INTRODUCTION

In the event of stress the mammalian brain can communicate with peripheral tissues through the chemical information carried by the ACTH molecule.

Neural signals from higher centers of the brain cause certain neurons in the hypothalamus to secrete corticotropin releasing factor into portal vessels to the pituitary. The corticotrophic cells of the anterior pituitary then respond by secreting corticotropin into the blood. Receptors on various target cells then read out the information carried by corticotropin resulting in a variety of cellular responses: adrenocortical cells respond with increased growth and glucocorticoid secretion, glycolysis is increased and the ascorbate content reduced; fat cells respond with increased lipolysis; amphibian and reptile melanocytes respond with increased melanin dispersion. Responses are also produced in certain CNS neurones involved in attentiveness, learning and memory.

The chemical information to bring about all the above responses reside in the 39-amino acid chain of the corticotropin molecule. This raises the question as to whether a single site of the molecule is responsible for the different effects or whether distinct regions occur that are specific for different kinds of responses.

This study is concerned particularly with the two adrenocortical processes - aerobic glycolysis and steroidogenesis. Adrenocortical cells respond to corticotropin with increased steroidogenesis as well as enhanced aerobic production of lactic acid. The exact mechanism of this enhanced glycolysis remains obscure. Is it somehow related to or

dependent on steroidogenesis or are the two processes independent? At which site on the glycolytic pathway does corticotropin exert its effect? Are the two effects coded for differently in the corticotropin molecule? Are the same adrenocortical receptors involved?

The present study has attempted to resolve some of these questions through structure-activity studies of ACTH fragments and analogs, the main objective being the identification and comparison of amino-acid sequences essential for the two processes, as well as the study of the corticotropin molecule itself and of amino-acid side chains implicated in excitation of hormonal receptors.

(1) to identify the active sequences for lactate and steroid production using ACTH fragments of various chain lengths.

(2) to assess the possible role of key amino acid side chains in the active regions.

(3) to determine the involvement, if any, of the active regions in adenyl cyclase activation.

(4) to ascertain the extent to which steroidogenesis and glycolysis are dissociated at the hormone-receptor level based on information obtained from the above studies.

SECTION A

BACKGROUND

CHAPTER I ACTH: BIOLOGICAL ORIGIN

A. Historical Perspective

1. Definition, Discovery, and Isolation:

Adrenocorticotropic hormone (ACTH, Corticotropin) is a straight-chain polypeptide composed of 39 amino-acid residues produced by the opiocorticotroph cells of the adenohypophysis.

P.E. Smith (1) in 1930 first demonstrated that a pituitary factor affects the adrenal and that atrophy of adrenals by hypophysectomy could be reversed by implantation of the pituitary. This discovery was followed by the isolation by Li et al. (2) in 1943 of a 20,000 dalton protein substance from sheep extract, and also from a pig extract by Sayers' group (3). The peptide nature of the substance was confirmed in 1950 by its inactivation by exposure to trypsin and pepsin (4). In 1953 White (5) reported the first isolation of pig corticotropin and called it corticotropin A. This was followed in 1954 by Bell and co-workers (6) of the isolation of a polypeptide of molecular weight 4,500 which they designated β-corticotropin. Dixon et al. (7) in 1955 obtained from pig pituitaries two preparations, corticotropin A_1 and A_2, whose amino acid compositions were identical to β-corticotropin.

It was later established that the various pig pituitary isolates - corticotropin A, β-corticotropin, and the corticotropins A_1 and A_2 all had the same amino acid sequence but differed in their asparagine content. Corticotropin A_1 was the amide form of corticotropin A_2; β-corticotropin was a mixture of the two forms in which A_2 predominated.

The late fifties saw the isolation of ACTH also from beef (8) and human (9) pituitary extracts.

2. Sequence Determination and Synthesis:

The complete amino acid sequence of β-corticotropin was first worked out in 1954 by Bell and co-workers (6). White and Landmann (10) in 1955 obtained identical amino-acid sequence for corticotropin A, and the sequence for beef (8), sheep (11) and human (9) corticotropins were determined by similar procedures.

In 1966 total synthesis of pig (12) and human (13) corticotropins was achieved. However, in 1974, errors were found in the original sequences proposed by Bell. The structure was re-assessed by Riniker, who then reported the corrected primary sequences. The revised structure of human ACTH was synthesized by Fujino, Bajusz and Li in the seventies.

Synthetic ACTH (1-39) showed full corticotropic activity in vivo and was physicochemically indistinguishable from highly purified, natural ACTH.

3. Biosynthetic Pathway:

Following their discovery in the early 70's as the cell type responsible for the secretion of ACTH (14,15) the corticotrophs were later found also to produce β-lipotropic hormone (β-LPH) and its cleavage products especially β-endorphin (16). The Pelletier group (16) then showed that ACTH and β-LPH are localized in the same secretory granules of the corticotroph cell. Around that period Yalow and Berson demonstrated the existence of high molecular weight forms of ACTH by gel chromatography. Later studies in the mid-seventies on the biosynthesis of ACTH in intact cell (17) and cell-free systems (18) confirmed the

hypothesis that ACTH is biosynthesized as a large precursor form of molecular weight 28-31K which is later transformed into 13K and 4.5K molecules.

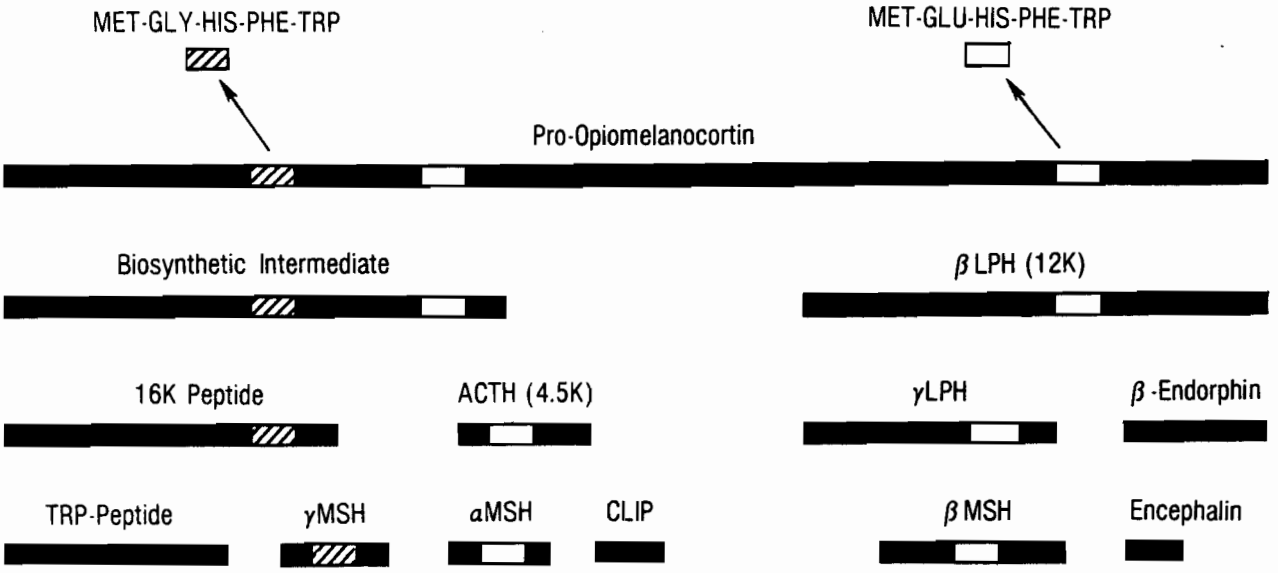
The precursor molecule was demonstrated to contain β-LPH, which itself has β-MSH and β-endorphin sequences in its molecule (19,20). In 1979 the complete structure of this common sequence for ACTH and β-LPH was elucidated by Nakanishi et al. (21) based on the nucleotide sequence of complimentary DNA produced from the mRNA that codes for the precursor. The Chrétien group (22) named the precursor "pro-opiomelanocortin" (POMC) to take into account the three main biological activities of its known secretory components.

Fig. 1 illustrates the biosynthetic pathway of ACTH and related peptides. Processing of POMC begins with the cleavage of the C-terminal β-LPH off the precursor. The remaining intermediate form is split into ACTH and a 16K-fragment (also called big γ-MSH), β-LPH undergoes further cleavage into γ-LPH and β-endorphin. This occurs fully in the intermediate lobe but incompletely in the anterior pituitary.

β-endorphin is processed further to form δ-endorphin or acetylated forms. The production of β-MSH from β- or γ-LPH is still controversial, and furthermore, mouse and rat β-LPH's lack the β-MSH sequence (23). A portion of the 16 K-fragment is named γ-MSH (or little γ-MSH).

Thus, in the anterior lobe the final products are ACTH, β-LPH, and big γ-MSH (16K-fragment), whereas in the intermediate lobe, they are β-endorphin, α-MSH, CLIP, and big and little γ-MSH.

FIG 1



Biosynthetic pathway from POMC to ACTH and related peptides

B. Recent Advances

1. Occurrence of ACTH in extra-pituitary tissues

Molecular forms of immunoreactive ACTH as well as γ-MSH and β-endorphin have been identified in the human brain (24,25,26), the sympathetic adrenal system (27,28,29), the gastrointestinal system (30,31), the human placenta (32), and human tumors (33). There has been some controversy as to the origin of these peptides in those tissues. In the case of the brain, Yalow and Berson (34) have postulated that the pituitary is the sole site of ACTH synthesis and that ACTH may be transported to brain by retrograde flow along the portal vessels. But although definite proof has not yet been obtained accumulating evidence suggests that these tissues produce ACTH and related peptides from the common precursor, POMC. For instance, it has been reported (24,25) that brain ACTH is not affected by hypophysectomy in rats. Liotta further showed that bovine hypothalamic tissue in culture incorporated ^{3}H-labelled amino acids into the POMC precursor of molecular wt 31-K dalton.

The physiological significance of ACTH and related peptides in tissues is largely unknown.

However, Racz et al. (29) have reported that Met- and Leu-enkephalin, well known products of the adrenal medulla, inhibit basal and stimulated corticosteroidogenesis. In the gastro-intestinal tract, it is thought that β-endorphin may be involved in the regulation of gastro-intestinal motility (30,31). Other tissues that have been claimed to contain ACTH or β-endorphin include the kidney, lung, eye and the pineal gland (28,31).

2. Occurrence of ACTH in the CNS:

Krieger and co-workers (35) reported in 1977 the presence of immunoreactive and bioreactive ACTH in the brain of intact and hypophysectomized rats. The highest concentrations were found in the hypothalamus, significant amounts in the limbic system structures, and low concentrations in the midbrain, pons, medulla, striatum, and cortex. These findings were confirmed by subsequent studies (36) which further reported the presence of β-LPH, α-MSH, β-endorphin, and γ-MSH-like peptides in the brains of several mammalian species, including the human. The brain content of these peptides has been reported to be in the range of 1/50 - 1/1000 the content of the whole pituitary gland.

Distribution

Immunocytochemical studies have indicated that POMC-derived peptides occur widely throughout the mammalian brain (36). However, so far the only cell bodies containing the peptides are those of the mediobasal hypothalamus, specifically the arcuate nucleus region (37). Fibers from this region leave the hypothalamus and innervate other brain structures: the thalamus, amygdala, the reticular formation, inferior colliculus and various nuclei within the pons and the brainstem. All such fibers have immunoreactive POMC-derived peptides. The overwhelming evidence to date supports the concept that the extensive fiber systems in brain containing POMC, ACTH, β-lipotropin and the peptides derived from them originate from hypothalamic neurones in the arcuate region and those encircling the ventromedial nucleus. It is believed that such a hypothalamic center distributes those peptides via axons to regulate many other brain regions.

So far except for the opioid peptides (38), no specific receptors for the ACTH-related peptides have been demonstrated in the central nervous system. Nevertheless, specific binding of ACTH to rat brain structures has been detected in the median eminence and arcuate nucleus (39).

Origin

There is indirect evidence that the POMC-related peptides are synthesized in the brain, rather than transported from the pituitary (36). These peptides have been found to persist after hypophysectomy. Direct evidence also exists for the de novo synthesis of POMC-related peptides by hypothalamic neurons. Herbert and associates using a complimentary DNA to mouse pituitary POMC gene, have detected POMC-like mRNA in rat hypothalamus, amygdala, and cortex.

3. Other Extrapituitary ACTH

Immunocytochemical techniques have revealed also the presence of ACTH-related peptides in invertebrates, such as pond snail (neurons) and Drosophila melanogaster (nervous and reproductive systems) (40). ACTH-like peptides have also been detected in a unicellular eukaryote, Tetrahymena pyrimformis (41).

4. Regulation of ACTH secretion

Evidence for the regulation of pituitary ACTH release by a releasing factor from the hypothalamus was first obtained in 1948 by Geoffrey Harris (42), was confirmed in vitro in 1955 by Guillemin and Rosenberg (43) and Saffran and Schally (44). Later in the 70's it became apparent that other substances could act as corticotropin-releasing factors. Thus epinephrine, norepinephrine, vasopressin, oxytocin, and

melanotropin were all reported to stimulate ACTH secretion (45,46). It was not until recently that a principal hypothalamic ACTH releasing factor has been established. Vale and co-workers have reported the isolation, sequence, synthesis, and biological activity of a 41-amino-acid ovine hypothalamic CRF (47). A peptide with CRF activity has also been isolated from porcine hypothalami (48). The ovine CRF has been found to contain amino acid sequences in common with several known peptides: Sauvagine, urotensin I, calmodulin, and angiotensinogen. Seventeen residues of CRF's 41 are common to Sauvagine and Urotensin I. The three peptides exhibit similar potencies to stimulate ACTH and β-endorphin secretion (49,50).

Recent studies indicate that ACTH can also be released by angiotensin II (51), histamine, and acetylcholine (52), synthetic α-melanotropin in interaction with arginine-vasopressin (53), and vasoactive intestinal peptide (VIP) (54,55).

ACTH secretion is also under inhibitory control by glucocorticoids (47,56) and somatostatin (57,58). In the case of glucocorticoids Roberts et al. (59) have shown that the effect is in the nucleus at the level of POMC-gene transcription.

5. Regulation of CRF release

The release of CRF itself is under neurotransmitter control. CRF release from the hypothalamus is stimulated by acetylcholine (60,61) serotonin (60), and the enkephalins (62). On the other hand CRF secretion is inhibited by norepinephrine (52,60), gamma-aminobutyric acid (61, 62) and glycine (63).

Corticosteroids, again, are known to influence CRF release by both fast and slow feedback mechanisms (64,65,66).

CHAPTER 2 THE BIOLOGICAL INFORMATION CARRIED BY ACTH

The ACTH molecule acts on peripheral as well as central receptors. The molecule exhibits the following biological functions (67,68,69):

A. Adrenocortical functions

1. Steroidogenic activity

a. Stimulation of corticosterone and cortisol in vivo and in the isolated zona fasciculata-reticularis cells.

b. Stimulation of aldosterone and other corticosteroids in zona glomerulosa cells.

In mammals the adrenal gland is composed of the capsule, the glomerulosa, fasciculata, and reticularis zones. The glomerulosa cells are located underneath the outer capsule of the adrenal gland, while the fasciculata and reticularis cells comprise the bulk of the adrenal cortex and are arranged in columns between the glomerulosa cells and the inner core of medullary cells. In common laboratory animals the zona fasciculata makes up most of the adrenal cortex (69).

2. Adrenal growth-promoting activity

ACTH increases the weight of the adrenal gland in hypophysectomised animals. The weight increase is paralleled by increases in RNA, DNA, and protein synthesis (70). The following cytological and ultrastructural changes are caused by ACTH administration (69):

- enlargement of zona fasciculata cells and increase in their mitotic rate

- enlargement of cell nuclei and nucleoli

- increase in the number of mitochondria

3. Adrenal blood flow

ACTH can double adrenal blood flow although in unphysiologically large doses (71). However, within the concentration range capable of stimulating adrenocortical secretion, ACTH exerts no effect on adrenal blood flow (71).

4. Adrenal uptake of cholesterol

ACTH stimulates the adrenal uptake of cholesterol from blood especially in large doses (72).

5. Xylose uptake

Adrenal cell permeability to xylose and other sugars is increased by ACTH (73).

6. Glycogen content

At high doses, ACTH has been shown to decrease adrenal glycogen content (74), but at smaller, steroidogenically-effective doses, no decrease in glycogen content is observable (75).

7. Aerobic glycolysis

Bartova and Birmingham (76) demonstrated the <u>in vitro</u> stimulation by ACTH of lactic acid production in rodent adrenal glands. (The phenomenon is fully reviewed in Chapter 4, paragraph (V)).

8. Adrenal ascorbate content

ACTH causes a depletion of the adrenal's rich content of ascorbic acid (77). It has been suggested that the ACTH-induced depletion of ascorbic acid results, at least in part, from inhibition of transport of ascorbate from blood into adrenocortical cells (78). It is also believed

that this inhibitory effect may be mediated by one or more corticosteroids (79).

B. Other peripheral effects

1. Action on fat cells

ACTH stimulates lipolysis in fat cells with release of glycerol and free fatty acids. Thus result increased plasma concentration of tissue, increased hepatic fat content, ketosis and other biochemical changes consistent with increased breakdown of lipids (80). However, adrenal cells are usually 10-100 times more sensitive to ACTH than fat cells (68).

2. Action on melanocytes

ACTH causes melanin dispersion in skins of amphibians and reptiles, similar to the effects observed with the melanotropins (81). Melanin pigmentation of human skin can also be increased by ACTH (82). The first 13-amino-acid residues of ACTH are identical to α-MSH except for N-terminal acetylation and C-terminal amidation in the latter hormone.

3. In vivo effect of ACTH in pancreatic islets

Studies by Sanchez-Franco and associates (83) have demonstrated the presence of immunoreactive ACTH in pancreatic islets, suggesting a possible influence by the hormone on glucagon and insulin release.

Recent in vivo experiments by Knudtzon (85) have shown that ACTH (1-24) and ACTH (1-39) have acute stimulatory effects on plasma levels of glucagon, the effect mediated by stimulation of the -adrenergic receptor. Plasma glucose levels are increased

simultaneously with glucagon. A direct stimulatory effect on insulin release in the pancreas has also been demonstrated in vitro with the mouse pancreas (84) and in vivo in the rabbit (85). This stimulation could be inhibited by stimulation of the α-receptor. The mechanism by which ACTH stimulates the α-adrenergic receptors is still not clear, although recent findings indicate that -receptors are linked with the phosphoinositide-turnover mechanism of intracellular signalling and Ca^{++} mobilization (see Chapter 4). ACTH fragments: (1-4), (4-10), (1-10) and ACTH (11-24), (7-38), (18-39) had no effect on the plasma levels of glucagon, insulin and glucose, in the study by Knudtzon (85). By contrast, Beloff-Chain et al. (86,87) have recently demonstrated the insulin-releasing properties of ACTH (18-39) (CLIP) and ACTH (22-39).

C. ACTH actions in the CNS

1. Biochemical effects in brain cells

ACTH and its fragments stimulate the incorporation of amino acids into newly formed proteins in brain cells (88). In a cell-free system from rat brain tissue, ACTH in low concentrations stimulates protein synthesis while at higher concentrations it is inhibitory. The amino acid sequence 1-24 is responsible for the stimulatory effect whereas the inhibition is attributable to the C-terminal segment (89). ACTH also increases cerebral polyamines through the stimulation of ornithine decarboxylase (90). Botticelli and Wurtman (91) have recently reported the influence of ACTH on serotonin metabolism, on the turnover rates of brain dopamine and norepinephrine and on the regulation of some cholinergic neurons. Dopamine synthesis can be selectively activated by ACTH (1-24) in the frontal cortex (92). ACTH may affect synaptic

transmission through the hormone's stimulation of the synthesis of polyphosphoinositides (93).

Recent findings indicate that ACTH peptides act in the brain via mechanisms similar to those thought to mediate ACTH-induced adrenal steroidogenesis. Thus ACTH-related peptides have been shown to influence neuronal adenylate cyclase activity, phosphorylation and dephosphorylation of specific membrane proteins, activation of acyl cholesterol hydrolase and triacyl glycerol lipase activities, and modulation of the turnover of membrane phosphoinositides (36).

2. ACTH as a neuromodulator

ACTH and N-terminal fragments in certain regions of the brain have been demonstrated to modulate the activities of several central neurotransmitters -- norepinephrine, dopamine, serotonin, acetylcholine, and gamma-aminobutyric acid. It is believed that all the central actions of these peptides may be mediated by modulation of neurotransmission. For example, a dopaminergic system has been implicated in ACTH-induced excessive grooming (94) and this induced behavior can be suppressed with the dopamine antagonist, haloperidol (95).

3. ACTH and central temperature control

ACTH has been linked with thermoregulation ever since Hench and co-workers (96) reported the ability of the hormone to reduce rheumatoid fever and arthritis, when given to patients. Recent research on ACTH (1-24) and α-MSH has further implicated these peptides in the control of normal body temperature (97). These hypothermic and antipyretic effects of ACTH have been shown not to be dependent on adrenal stimulation since the effects are also observed in adrenalectomized rabbits (97).

4. Behavioral effects of ACTH

ACTH and related peptides (i.e., N-terminal fragments and the melanotropins- α-MSH, β-MSH, and γ-MSH), have a short-term effect on the facilitation of learning and memory processes, possibly by temporarily increasing motivation and alertness. These effects are believed to be mediated through the limbic system and midbrain structures (98).

The smallest behaviorally effective ACTH peptide is MET-GLU-HIS-PHE (or ACTH (4-7)). It has no adrenocorticotropic activity and acts directly on central neurons.

Miller (99) found that ACTH (1-24) causes hippocampal or occipital EEG to shift to higher frequencies indicating arousal. Injection of ACTH or fragments elicits exaggerated grooming in rats (100). ACTH fragments are also antagonistic toward opiate binding to the opioid receptor and hence towards analgesia (101). ACTH (1-13) stimulates sexual behavior such as lordosis, in the female rat (102).

5. ACTH and learning

a. Animal studies:

Various experiments (103) have indicated that ACTH and shorter N-terminal fragments facilitate in animals the acquisition and retention of learned behavior such as conditioned avoidance of electric shock, food-motivated behavior in hungry animals, conditioned taste aversion, the kindling effect, approach behavior, and sexually motivated behavior (104). The effects of amnesia-inducing agents are reversed by the peptides.

Recently, Jacquet and Abrams (105) have shown that the ACTH peptides, when injected into the locus ceruleus of rat brainstem can

induce symptoms resembling human Parkinson's disease, and the investigators suggested that these peptides may have a normal role in modulating posture and movement in the brainstem.

b. Human studies:

A number of studies have shown that ACTH peptides influence the visual system, memory and mood in humans. The peptides induce arousal, maintain a high vigilance level, and improve visual motor learning (106). However, according to Veith et al. (107) there are sex differences in the case of visual memory. This was enhanced in males by ACTH whereas in females visual memory was impaired. Instead, the peptides facilitated auditory and verbal memories in females.

Fragments of ACTH have been observed to improve attention and enhance interpersonal and environmental awareness in mentally retarded individuals (108). Van Riezen et al. (109) have used ACTH peptides to help elderly patients in their memory retrieval. ACTH (4-10) also has been found to improve short term memory and attention.

ACTH treatment in humans improved mood and reduced depression and anxiety. This led Gaillard and Narey (110) to propose that the main CNS effect of ACTH and related peptides in humans is to influence processes related to arousal, attention, motivation, mood, and vigilance.

The neurochemical bases of these ACTH actions have been studied by Scharrer (111) who suggested that these peptides may function as a peptidergic neuronal system which may modulate the motor, sensory, and autonomic systems by altering the effect of the classical neurotransmitters on their receptors, by altering their turnover or by modifying membrane proteins leading to a change in membrane

permeability. It is thought that such a system might help to coordinate central and peripheral responses to stimuli that disturb the endocrine system.

CHAPTER 3 STRUCTURAL FEATURES OF ACTH AND THEIR ROLE IN INFORMATION TRANSFER

A. Characteristic features of the primary structure (Fig. 2)

Three distinct regions can be recognised in the primary sequence of the molecule:

(1) A hydrophobic hexapeptide core -

with some complex amino acid side chains. This is located within first 9 residues of the N-terminal region and is highly conserved in all species. The sequence also occurs in α-MSH, β-MSH and to some extent in γ-MSH.

4 5 6 7 8 9

-MET - GLU - HIS - PHE - ARG - TRP -

(2) A basic tetrapeptide sequence -

occurring in the mid-portion of the ACTH molecule; a highly positively charged region that could interact with a complimentary negatively charged region in the receptor.

15 16 17 18

- LYS - LYS - ARG - ARG -

(+) (+) (+) (+)

(3) A relatively acidic C-terminal region -

that makes up one-third of the C-terminal portion. This sequence is the least conserved among species. The sequence below is that of human ACTH:

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

-ASN-GLY-ALA-GLU-ASP-GLU-SER-ALA-GLU-ALA-PHE-PRO-LEU-GLU-PHE-COOH-

(-) (-) (-) (-) (-)

FIG 2

Primary Structure of ACTH

1 2 3 4 5 6 7 8 9 10 11 12 13
NH-SER-TYR-SER-MET-GLU-HIS-PHE-ARG-TRP-GLY-LYS-PRO-VAL-

14 15 16 17 18 19 20 21 22 23 24
-GLY-LYS-LYS-ARG-ARG-PRO-VAL-LYS-VAL-TYR-PRO-

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
-ASN-GLY-ALA-GLU-ASP-GLU-SER-ALA-GLU-ALA-PHE-PRO-LEU-GLU-PHE-COOH-

B. Secondary Structural Features

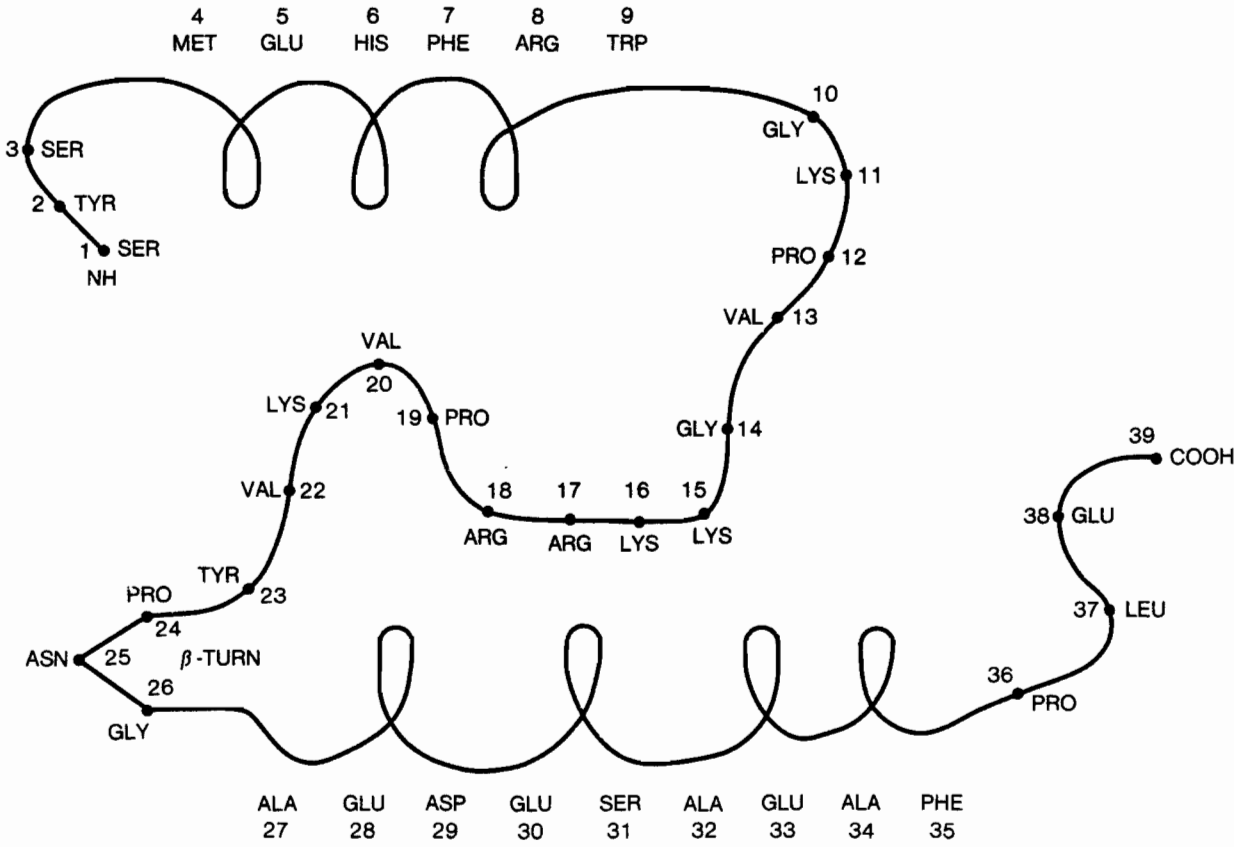
ACTH was considered from earlier spectroscopic studies, to have a completely random structure in aqueous solution. However, in 1975, Fermandjian and co-workers (112) conducted circular dichroism studies with human ACTH and its fragments and found that helix formation can be induced by trifluoroethanol (TFE) which according to Urry et al. (113) mimics the natural environment of membrane proteins. They observed that human ACTH (1-32) underwent a linear random coil-helix transition when the percentage of water was decreased. It had been suggested that the ordered structures induced in TFE could be likened to preferred conformation induced by contact of ACTH with cell surface lipids and receptor proteins.

Using the calculations developed by Chou and Fasman (114), they could predict which part of the molecule had a tendency to form secondary structures. Their calculations (112) showed that the regions 3-9 and 27-35 may adopt an alpha-helix and the residues 23-26 are very favorable for a β-turn formation. Their proposed secondary structure for ACTH is illustrated in Fig. 3. The helix-forming ability of ACTH has since been confirmed by Circular Dichroism (CD) spectroscopic measurements of Greff et al. (112). Their CD-studies of shorter ACTH fragments indicated that the N-terminal part of the molecule was very prone to helix formation. Further, Toma and associates (115) conducted CD and Nuclear Magnetic Resonance (NMR) studies which indicated that the N-terminal part of the ACTH molecule has an organised structure even in water. This has been

observed with Infra-Red (IR) studies (116). Such spectroscopical studies had also confirmed the predicted β-bend formation at sequence 23-26 (115).

FIG 3

Secondary Structure of ACTH



C. Biological significance of the primary and secondary structural features

Earlier structure-function studies of synthetic ACTH peptides by the 4 groups of Hofmann (117), Li (118), Schwyzer (119) and Sayers (120) have established that the N-terminal segment (4-9) of ACTH is responsible for eliciting the hormonal responses, whereas the basic tetrapeptide (15-18) LYS-LYS-ARG-ARG is involved in the binding of the hormone. The acidic C-terminal sequence, on the other hand seems to be important for protecting the hormone during transport in vivo. These properties can be related to certain distinct features of the molecular structure of the hormone.

1. The hexapeptide core:

4	5	6	7	8	9
MET	GLU	HIS	PHE	ARG	TRP

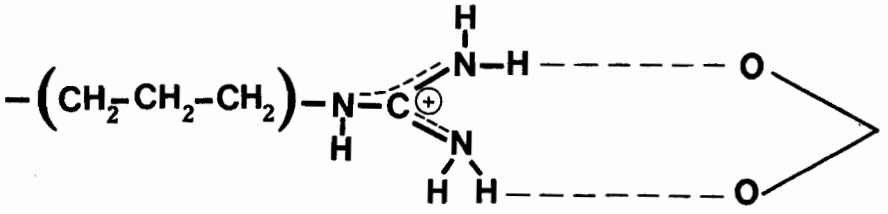
a. Role of amino-acid side chains:

The biologically active N-terminal hexapeptide is rich in residues with complex side chains.

MET:	Hydrophobic interaction
HIS: (imidazole)	Proton donor/acceptor, H-bonding
PHE:	Hydrophobic interaction
ARG: (guanidyl)	Multiple H-bonding (recognition*); cationic interaction
TRP: (indole ring)	Charge donation by pi-orbitals
	H-bonding
	Hydrophobic interaction

*The planar guanidyl group is known to play an important role in the structure, interaction and action of proteins (121). Positively charged,

it interacts primarily with negatively charged carboxylate groups of aspartate and glutamate residues in proteins, as well as with negatively charged phosphate groups. It can also form geometrically specific H-bond interactions with carboxylate or phosphate groups. Recognition is thought to imply geometrically specific interactions involving at least 2 hydrogen bonds. The Figure below shows such possibilities with arginine:



b. Importance of the α-helical structure

It is generally believed that the active core regions of peptide hormones assume an ordered conformation at the receptor (123). The binding of the active core region of ACTH to its receptor has been predicted principally to involve an α-helix association (123). De Wied and co-workers (124) have carried out behavioural studies with ACTH (4-9) and ACTH (4-10) in which the amino-acid residues at the places 4 and 7 or 8 were modified. From their findings they concluded that the ACTH fragments have a helix-type structure in which Met^4 comes close to Arg^8 and Phe^7. This is supported by the earlier circular dichroism studies (112, 115) and recently by the crystal structural determination of ACTH (4-7) (125).

The N-terminal α-helix region of ACTH orients the side chains of TYR^2, MET^4, and PHE^7 in a manner similar to the orientation of

TYR^1, PHE^4 and MET^5 of enkephalin in a β-bend conformation. Thus ACTH is known to have significant affinity for the opiate receptor (126). Toma et al. (127) have also provided evidence that residues which are important for the biological activity of ACTH are located in the α helical segment of the hormone. Substituting the L-amino acids with the D-isomers from position 2 to 8, caused a gradual loss of helical content, the largest loss being observed for ARG^8. These losses correlated with loss in biological activity. They explained such functional changes by the loss of the adequate positioning of the functional side chains.

2. The basic tetrapeptide (LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}):

a. It is now generally accepted that the basic tetrapeptide of ACTH is not essential for activity but acts as a strong binding site which may be involved in the interaction with a negatively charged surface of the specific hormone receptor (128). Hofmann and co-workers (129) showed that although the fragment ACTH (11-20) NH_2 was completely inactive, it was quite capable of binding to the subcellular fractions prepared from beef adrenal cortical tissue to cause the displacement of ACTH which had been specifically bound there. They further observed that the ACTH peptides, in which the basic amino acid residues in positions 15, 16, 17 and 18 were partially missing or blocked were less effective at displacing bound ACTH than ACTH (11-20) NH_2. Thus removal of the basic residues leads to peptides with drastically reduced potencies.

b. Similarity of the basic tetrapeptide to polyamines

The basic core of ACTH could share the general characteristic with polycations in causing membrane perturbations. Wolff and Cook (130)

after studying various polycations concluded that non-specific cationic interactions with the membrane could lead to surface changes that mimic effects produced by hormones. With polylysine they could stimulate steroidogenesis in intact mouse adrenal tumor cells and mouse leydig tumor cells maintained in culture (135). The action of this polycation also led to stimulation of adenylate cyclase in membranes prepared from the tumor cells. The effect of polylysine was biphasic; the stimulation being followed by inhibition at higher concentrations. Keung et al. (131) have also reported biphasic effects with a basic protein, cardiotoxin, in isolated rat adrenal cells and they noticed that the changes paralleled CAMP accumulation. However, smaller polycations, such as tetralysine or spermine, did not stimulate steroidogenesis although spermine was effective on the adenylate cyclase activity of membranes from these cells (132). Notably, polylysine did not compete with ACTH (1-24) for binding, the polycation showing rather a mixed-type inhibition (130) unlike ACTH (11-24) which is a competitive inhibitor (133). Nevertheless, low concentrations of polylysine inhibited ACTH (1-24)-stimulated adenylate cyclase activity (130). Whether the basic core of ACTH causes membrane perturbations upon binding the ACTH receptor remains to be determined.

c. The basic core as part of a calmodulin recognition site:

Weiss et al. (134) first reported in 1980 that ACTH and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase and demonstrated that these peptide hormones compete with the enzyme for calmodulin. (Cyclic nucleotide phosphodiesterase as well as adenylate cyclase, are

both activated by the calcium-calmodulin complex (135)). Recently, Malencik and Anderson (136) have shown that ACTH, β-endorphin, glucagon, and substance P undergo calcium-dependent binding to calmodulin. They observed that these peptide hormones and other calmodulin-binding proteins contain identical amino acid sequences. This homologous recognition region has been determined to consist of a strongly basic tripeptide sequence with at least 2 residues, which are either ARG or LYS, three positions away from a pair of bulky hydrophobic residues (136) (Table 1). The hydrophobic sequences have high β-sheet potential according to the Chou-Fasman calculations. These rules indicate that the basic sequence in ACTH is also associated with a possible β-strand (137).

^{15}LYS-LYS-ARG-ARG-PRO-VAL-LYS-VAL-TYR-PRO^{24}

\+ + + + β-Strand +

It has been suggested that the cluster of basic amino acid side chains interact with complementary anionic sites on calmodulin (137). Malencik and Anderson also found that the recognition sequence is similar to the recognition sequence in the substrates of CAMP-dependent protein kinase (136). It has therefore been proposed that calmodulin and protein/kinase act on common sequences in proteins subject to dual control by calcium and CAMP and that modification of certain calmodulin binding sites is one of the functions of CAMP-dependent protein kinase. However, the physiological relevance of the interaction of ACTH with calmodulin is still unknown, although similarities have been observed between the peptide-binding sites of calmodulin and some of the cell-surface neurohormone receptors (138).

3. The importance of the β-bend region and the acidic C-terminal sequence of ACTH

Various studies have suggested that the C-terminal sequence of ACTH is capable of protecting the molecule from metabolic degradation (139). This protective effect has been explained by the presence of the β-bend, which by folding back may bring the C-terminal portion close to the sensitive basic tetrapeptide LYS-LYS-ARG-ARG of the peptide chain, thereby hindering proteolysis of the molecule by trypsin-like enzymes (140).

D. Interaction of the ACTH molecule with lipid bilayers:

ACTH is known to exert its effect through reversible interactions with specific receptors located in the outer membranes of its target cells (67). The lipid layer of the membrane functions as a matrix for the correct orientation and folding of the receptor proteins (141). Findings from recent experiments by Gremlich and Schwyzer (142,143,144) have indicated that the lipid layer also functions by inducing the correct conformations and orientation of the ACTH molecule favorable for the optimal contact and interaction with the receptors.

Using artifical lipid membranes the Gremlich team studied ACTH-membrane interactions employing biophysical methods (Infra Red attenuated total reflection spectroscopy (142,143), and vesicle-mediated hydrophobic photolabeling (144)). They showed that the hydrophobic N-terminal decapeptide segment enters the membrane as a helical structure with the helix axis perpendicular to the membrane surface, whereas the hydrophilic C-terminal tetradecapeptide segment assumes an extended random conformation on the membrane surface (143). Their observations

TABLE 1. Structural homologies in calmodulin binding peptides

Substance P	-ARG-PRO-LYS-PRO-GLN-GLN-PHE-PHE-GLY
Glucagon (16-26)	-SER-ARG-ARG-ALA-GLN-ASP-PHE-VAL-GLN
ACTH (16-26)	-LYS-ARG-ARG-PRO-VAL-LYS-VAL-TYR-PRO
Beta-Endorphin (29-19)	-LYS-LYS-HIS-ALA-ASN-LYS-ILE-ILE-ALA

correlated well with pharmacological properties of the hormone (145). Their studies demonstrated the importance of the amphiphilic nature of the ACTH molecule. Neither the hydrophobic message segment (1-10) (responsible for triggering the receptor nor the hydrophilic address segment (11-24), is alone capable of being adsorbed to and interacting with the model lipid membranes). It is only their covalent combination that enables distinct membrane interactions accompanied by membrane-induced specific conformation transitions and unique orientations. Thus it was shown that ACTH (1-10) showed preference for a rather aqueous phase and adopted an antiparallel β-pleated sheet structure in hydrophobic surrounding (142). Thus the fragment would only be well recognised by target cells that have their receptors exposed on the hydrophilic surface of the membrane. (ACTH (1-10) is a very weak agonist for the steroidogenic receptors) (142).

It is now postulated that the message segment of ACTH provides the hydrophobic contact by entering the membrane as a perpendicularly oriented helix, whereas the positively charged address segment provides electrostatic contact to the head-group layer of the membrane.

E. Organisation of the ACTH molecule into functional domains

Several structure-function studies have been carried out to determine how the various biological effects of ACTH are coded for by the molecule. The first such study was conducted by White in 1955 (146) who found that ACTH was considerably inactivated when more than half of the N-terminal residues were released by the action of leucine aminopeptidase. Later, Bell et al. (147) observed that ACTH (1-24) possessed the full ACTH and MSH activities. Thus began a series of

structure-function studies conducted in the laboratories of Hofmann (148), Li (149), Schwyzer (139) and Seelig et al. (120), in which both ACTH fragments and analogs were utilized. The findings from these studies indicated that the species-variant segment of the ACTH molecule is ACTH (25-39) and that this is not necessary for biological action but serves to protect against biological degradation in vivo. The (5-10) segment was found to be the steroidogenic active site, while the (11-18) sequence was demonstrated to be involved in binding of the hormone to the receptor and was referred to as the "address" segment.

Schwyzer (150) has proposed on the basis of structural analysis of the ACTH molecule that different regions of the same molecule can adapt to different receptors with different binding constants and produce a variety of different hormonal effects.

In the ACTH molecule, two types of domains can be described:

(1) Message domains -- that excite specific receptors and

(2) Auxilliary domains -- consisting of

i) potentiating sequence -- that enhance potency but lack the ability to excite the receptor by themselves.

ii) addressing sequence -- that provides information for addressing the message to the right receptor.

iii) protecting sequence -- that stabilizes the peptide during transport.

Different ACTH-responsive tissues, cells, or receptors have different structural requirements and recognize different portions of the total ACTH sequence as their own message, potentiator, and address. The organization of information in ACTH (150) is depicted in Fig. 4, and is

described as follows.

Potentiator Sequence I

NH_2-SER^1-TYR^2-SER^3

- enhances the potencies and intrinsic activities of the hormone in cyclic AMP production, steroidogenesis and lipolysis. Schulster and Schwyzer (68) have indicated that the potentiator exerts its effect in two ways:

(i) by providing additional binding force for Message I (see below) through direct interaction with the receptor, and/or
(ii) by promoting α-helix formation and thus stabilizing the α-helical conformation of Message I in contact with the receptor. Rae et al. (151) have recently suggested that the sequence SER-TYR-SER also plays a role in the binding of ACTH to its receptor.

Potentiator Sequence II

NH-SER^1-TYR^2-SER^3-MET^4-GLU^5-HIS^6

- potentiates pigment dispersion in amphibian melanophores and tyrosinase activation in Cloudman melanoma cells (150).

Message Sequence Ia (contains also messages Ib, Ic, Id, Ie)

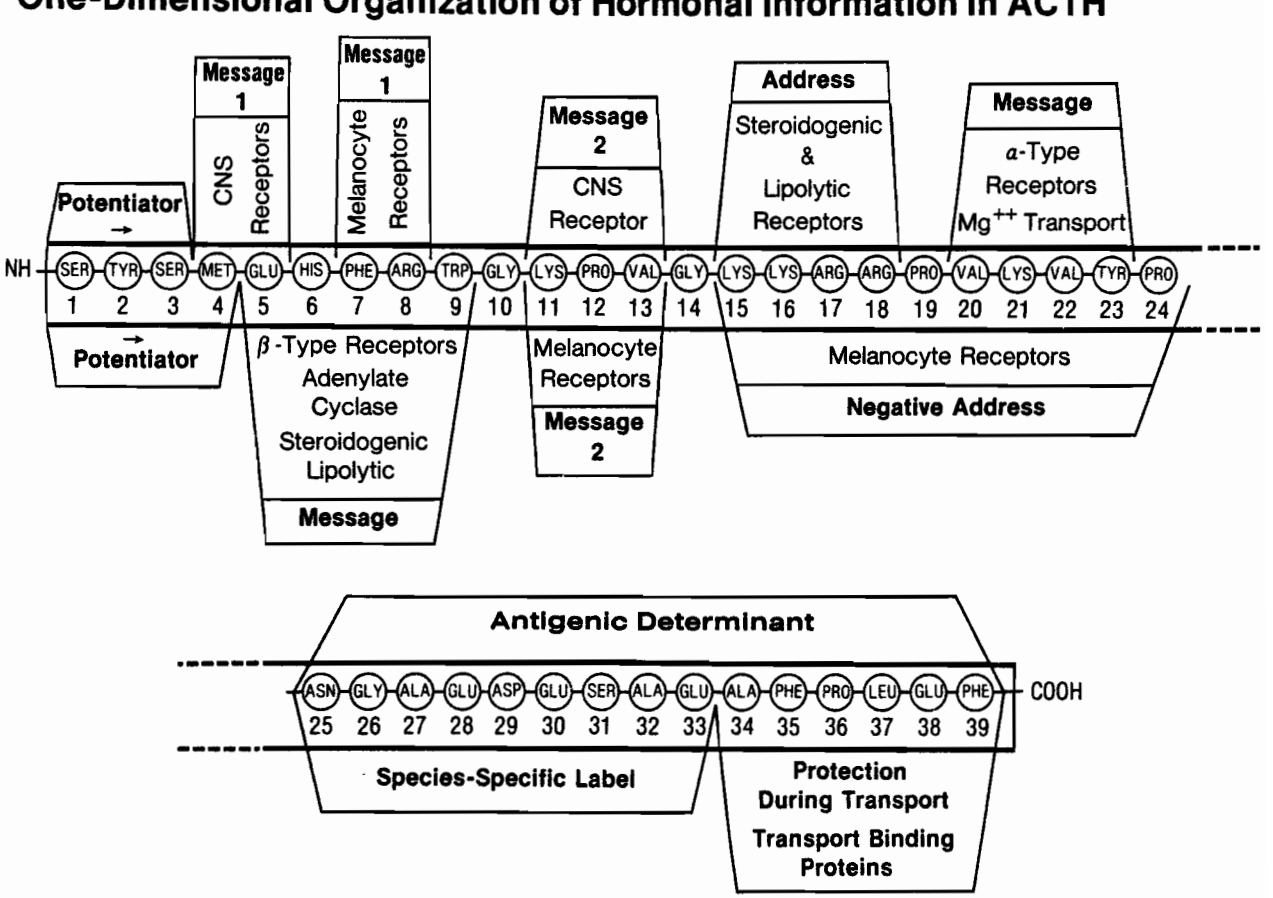
-MET^4-GLU^5-HIS^6-PHE^2-ARG^8-TRP^9-

- triggers β-type receptors, those receptors that, in analogy to β-adrenergic receptors, respond with enhanced cyclic AMP production, thereby stimulating steroidogenesis in rat adrenocortical cells and lipolysis in adipocytes. (In fact the β-adrenergic agonists can also stimulate lipolysis and, in certain species, melanin dispersion (68)).

Message Sequence Ib

-MET^4-GLU^5-HIS^6-

FIG 4


One-Dimensional Organization of Hormonal Information in ACTH
Message 1
CNS Receptors
Message 1
Melanocyte Receptors
Message 2
CNS Receptor
Address
Steroidogenic & Lipolytic Receptors
Message
α-Type Receptors
Mg++ Transport
Potentiator
NH
SER TYR SER MET GLU HIS PHE ARG TRP GLY LYS PRO VAL GLY LYS LYS ARG ARG PRO VAL LYS VAL TYR PRO
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Potentiator
β-Type Receptors
Adenylate Cyclase
Steroidogenic
Lipolytic
Message
Melanocyte Receptors
Message 2
Melanocyte Receptors
Negative Address
Antigenic Determinant
ASN GLY ALA GLU ASP GLU SER ALA GLU ALA PHE PRO LEU GLU PHE
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
COOH
Species-Specific Label
Protection During Transport
Transport Binding Proteins

- influences central nervous system receptors involved in attentiveness, motivation, learning and memory processes (152).

Message Sequence Ic

$-PHE^{7}-ARG^{8}-TRP^{9}-$

- second CNS message for behavioral effects (159).

Message Sequence Id

$-GLU^{5}-HIS^{6}-PHE^{7}-ARG^{8}-TRP^{9}-$

- stimulates cyclic AMP-independent steroidogenesis in rat adrenocortical cells. This is postulated to occur through some other mechanism such as calcium re-distribution, cyclic GMP production or utilization of background amounts of cyclic AMP (153).

Message Sequence Ie

$-PHE^{7}-ARG^{8}-$

- activates amphibian melanophore melanin-dispersing receptors and stimulates tyrosinase activity in Cloudman melanoma cells (154).

Message Sequence II

$LYS^{11}-PRO^{12}-VAL^{13}-$

i) activates amphibian melanophore melanin-dispersing receptors and stimulates tyrosinase activity in Cloudman melanoma cells.

ii) third CNS message for behavioral effects especially avoidance behavior and excessive grooming in rats (152).

iii) CNS message for thermoregulation and fever reduction (97).

Message Sequence III

$-VAL^{20}-LYS^{21}-VAL^{22}-TYR^{23}-PRO^{24}$

- triggers the receptors of the Mg^{++} pumping system on rat adipocytes. These are the alpha-type receptors, and Mg^{++} transport in adipocytes can

be blocked by alpha-blockers such as phentolamine (155).

Address Sequence

$$-LYS^{11}-PRO^{12}-VAL^{13}-GLY^{14}-LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}-$$

- adds receptor-specific affinity for adrenocortical and rat fat cells.

Negative Address Sequence

$$-LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}-$$

- reduces the melanophore-specific activity of ACTH (1-13) (α-MSH).

Protecting and Antigenic Sequence

$$\text{(human ACTH)} - ASN^{25}-GLY^{26}-ALA^{27}GLU^{28}-ASP^{29}-GLU^{30}-$$

$$-SER^{31}-ALA^{32}-GLU^{33}-ALA^{34}-PHE^{35}-PRO^{36}-LEU^{37}-GLU^{38}-PHE^{39}-$$

COOH

-provides (i) information for transport and stability in the blood

(ii) species-specific label; induction of antibody formation in other species.

Although this C-terminal ACTH (25-39) seems to have no intrinsic activity, recent findings by Yamashimo (156) suggest that certain hormonal activities may be sensitive to alterations in the C-terminal portion of the ACTH molecule. This was the case with the lipolytic activities of ostrich and turkey ACTH in isolated rat fat cells. Although the N-terminal sequences (ACTH 1-19) were identical to the human sequence there were wide differences in lipolytic potencies:

Relative Lipolytic Potencies	Species	15	16	17	18	19	20	21	22	23	24
100	Human	-LYS	-LYS	-ARG	-ARG	-PRO	-VAL	-LYS	-VAL	-TYR	-PRO-
2	Ostrich	-ARG									
13	Turkey	-ARG	-ARG	-LYS						-ILE	-

Potency	Species	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
100	Human	-ASN-	GLY-	ALA-	GLU-	ALA-	GLU-	SER-	ALA-	GLU-	ALA-	PHE-	PRO-	LEU-	GLU-	PHE-COOH
2	Ostrich			-VAL-	GLN-	GLU-		-THR-	SER-	GLU-	GLY					
13	Turkey			-SER-	VAL-	ASP-		-GLU-	GLN-	ALA-	SER-	TYR-	VAL-			

In addition the C-terminal sequences ACTH (18-39) and ACTH (22-39) have recently been associated with insulin release following studies by Beloff-Chain and coworkers (86,87).

CHAPTER 4 MECHANISMS OF ADRENOCORTICAL RESPONSE TO ACTH

A. The Adrenocortical ACTH Receptor

It has been demonstrated (151) that ACTH receptors are located on the outer plasma membrane surface and that ACTH acts without entering the cell. ACTH chemically coupled to insoluble and inert polymers (such as cellulose, agarose and polyacrylamide) was effective in stimulating adrenal steroidogenesis in cell suspensions. The size of the complexes apparently ruled out entry into the cell and it could further be demonstrated that this bioactivity was not due to cleavage of the active peptides from the complex.

Until very recently, the investigation of ACTH receptors by direct binding methods have been technically very difficult and inconclusive, first due to the strong tendency of the hormone to bind to inert materials and non-receptor components of the target tissue, and secondly, owing to the low biological potencies of the radiolabeled ACTH preparations used. The loss of biological potency resulted from the introduction of the bulky iodine atom in position 2 of the ACTH molecule and the oxidation of the methionine residue in position 4 (157). Lefkowitz et al. (158) in 1970 first investigated the binding of ^{125}I-ACTH to extracts of mouse adrenal tumor cells and found two types of binding sites: 60 sites of very high affinity and 360,000 sites of much lower affinity. Studies by McIlhinney and Schulster (159) in 1975 of the binding of ^{125}I-ACTH preparations to isolated rat adrenocortical cells also suggested the presence of high and low affinity receptors. In 1978 Yanagibashi et al. (160) reported similar findings: 7350 high

affinity sites per cell and 57,400 low affinity receptors. These studies employed ^{125}I-ACTH preparations with rather low biological potencies (50% or less).

Recently, Buckley and Ramachandran (161) have carried out new binding studies using an ACTH analog in which the tyrosine in position 2 is replaced by phenylalanine and the methionine in position 4 by norleucine. The radioligand was prepared by iodinating this analog at tyrosine in position 23 using the chloramine-T method. This labeled analog, with high specific radioactivity was shown to be equipotent with ACTH in stimulating steroidogenesis in adrenocortical cells. Their binding studies revealed only one single class of ACTH receptors. (4,000 high affinity sites per cell; Kd = 1.41 x 10^{-9}M). ACTH binding occurred at physiological concentrations capable of eliciting steroidogenesis and no evidence for a second class of receptors was found.

B. Intracellular mediators of ACTH action

1. Cyclic AMP

In the classical scheme of the mechanism of ACTH action, the formation of an active hormone-receptor complex leads to stimulation of adenylate cyclase, elevation of CAMP levels and increase in steroidogenesis. This is the "Second Messenger Concept". Several investigators now question the obligatory mediator role of CAMP in ACTH-stimulated steroidogenesis, for the following reasons:

a. No change in CAMP production has been observed with low but steroidogenically effective concentrations of ACTH (162,163,164).

b. Some ACTH analogs are able to stimulate steroidogenesis maximally without a significant increase in CAMP levels (165,166).

Studies have shown that cholera toxin is capable of stimulating elevation of CAMP levels and steroidogenesis in adrenocortical cells. However, in one of such studies, no increase in steroidogenesis was observable until 60 min after the addition of cholera toxin, whereas cyclic AMP production had been initiated 20 min earlier (167).

c. Certain ACTH fragments can stimulate membrane adenylate cyclase without causing steroidogenesis in whole cells (168).

d. An increase of tissue CAMP in perfused cat adrenal glands was found to be insufficient to elicit steroid release (169).

e. Adrenocortical effectors such as angiotensin (170) or acetylcholine (171) can induce steroidogenesis without the mediation by CAMP.

Several hypotheses have been developped to explain the above discrepancies:

One suggests that minute changes in CAMP which are not detectable by the available methods, or translocation of the nucleotide within a small intracellular pool might be sufficient to stimulate steroidogenesis (172).

Another postulates that cyclic GMP (173) or the cAMP/cGMP ratio (174) might be the mediators of the steroidogenic effect of ACTH.

A hypothesis has recently been postulated by Bristow et al. (175) from their inhibition studies with ACTH (6-24). It required 1000 times less ACTH (6-24) to inhibit ACTH (5-24)-induced steroidogenesis than to inhibit ACTH (1-39)-induced steroid production. Also, the concentration of ACTH (1-39) required to elicit an increase in cyclic AMP accumulation

was just about 10 times that required to stimulate steroidogenesis, whereas with ACTH (5-24) cyclic AMP accumulation occurred only at peptide concentrations about 1000 times higher than that required to stimulate steroidogenesis. Maximal rate of steroidogenesis could be reached at concentrations of ACTH (5-24) which elicited virtually no cyclic AMP production. From these findings, the Bristow group proposed that ACTH (1-39) can act via either of two receptors and that binding to one (receptor A) elicits steroidogenesis through the mediation of cyclic AMP, and that binding to the other (receptor B) also elicits steroidogenesis without the mediation of CAMP but through some other mechanism such as calcium re-distribution, cyclic GMP production, or utilisation of background amounts of CAMP (153). This is the "Dual Receptor Concept", which proposes that cyclic AMP is a sufficient but unnecessary condition for producing the ACTH effects; and that these effects could be caused via other pathways or second messengers. This hypothesis is the basis of a newer concept, the "Compartment Guidance Concept" recently proposed by Schulster and Schwyzer (68). They postulated that cyclic AMP (either basal or stimulated concentrations) is necessary but not sufficient to produce the effect and that other actions of ACTH, on the same or other receptors, are required to guide cyclic AMP into the correct compartment for eliciting steroidogenesis. This concept further suggests that this other pathway cannot produce the ACTH effects independently of cyclic AMP and that it functions by inhibiting the action of ACTH on the adenylate cyclase pathway, and it operates only in the presence of a sufficient external supply of Ca^{++} (169). According to this concept, adenylate cyclase activation by ACTH would be a kind of

emergency effect, being called into play at low basal concentrations of CAMP. Schwyzer (153) has used this concept to explain the action of ACTH (5-24). He postulated that ACTH (5-24) contains only the message (GLU-HIS-PHE-ARG-TRP) for activating the non-CAMP dependent receptor B, and that this message can also stimulate the CAMP-coupled receptor A only at very high concentrations. He suggested that receptor A needs more and perhaps different information being supplied by the methionine in position 4.

The foregoing concepts are based on the existence of two classes of ACTH receptors. However, the recent conclusive evidence by Buckley and Ramachandran (161) for the existence of only a single class of ACTH receptor has brought about a novel hypothesis, the "Spare Receptor Concept", that can also be used to explain the apparent dissociation between steroidogenesis and CAMP formation. The hypothesis states that adrenocortical ACTH receptors are present in excess of the amount necessary for maximal stimulation of steroidogenesis and that ACTH acts through a cyclic AMP-dependent process, but only a small fraction of the total CAMP formed is required for the effect. Evidence exists for the presence of spare receptors in adrenal cells. Studies on the binding of ^{125}I-ACTH to intact adrenal cells revealed that only about 12% of the cellular binding sites were filled when steroidogenesis was maximal. It also appears that the large amounts of CAMP produced at high concentrations of ACTH ($>10^{-9}$M) are due to the occupation of these spare receptor sites not directly involved in steroidogenesis. A more accurate description of the concept has now come from mathematical

considerations which suggest that "spare receptors provide the necessary total number of receptors such that the maximal response can be attained at very low hormone occupancy" (68).

The concept is also strongly supported by studies by Hornsby and Gill (176), conducted on bovine adrenocortical cells in culture, and by investigations with mutant doses of Y1 mouse adrenocortical tumor cells (177).

SUMMARY OF HYPOTHESES (Ref.68)

		Hormone	Effect 1	Effect 2
1) Classical Second-Messenger Concept / Spare Receptor Concept	CAMP necessary and sufficient	ACTH →	CAMP →	Steroidogenesis lipolysis etc.
2) Dual Receptor Concept	CAMP sufficient but unnecessary	ACTH →	CAMP → / OTHERS →	Steroidogenesis Lipolysis Etc.
3) Compartment Guidance Concept	CAMP necessary but not sufficient	ACTH →	{CAMP, OTHERS}	Steroidogenesis Lipolysis Etc.

2. Intracellular cyclic AMP pools

The existence of intracellular compartments of cyclic AMP has also been considered in order to explain the observation that ACTH can elicit measurable changes in steroidogenesis without detectable changes in CAMP levels. It has been proposed (178) that low doses of ACTH can elevate

levels of cyclic AMP, but in restricted pools that were too small to be measured. Evidence for such ACTH-responsive CAMP pools has come from the studies by Schimmer and Zimmerman (179). Using a mouse adrenocortical tumor cell-line, they found that CAMP accumulation by ACTH occurred mainly in the extracellular fraction though the nucleotide was synthesized intracellularly. With very low concentrations of ACTH (5 and 10 μU/ml) they could detect an approximately 2-fold increase in extracellular CAMP levels. However, this increase represented only 9% of the total cellular cyclic AMP and thus could easily have gone undetected. They postulated that the extracellular fraction of cyclic AMP reflected the activity of an intracellular pool and thus could be considered a sensitive index of cyclic AMP production. However Podesta et al. (180) and Sala and colleagues (181) have indicated that the measurement of intracellular bound cyclic AMP gives a more accurate measurement of active nucleotide concentrations. They measured the fraction of CAMP bound to the regulatory subunit of protein kinase during ACTH-induced steroidogenesis in isolated rat adrenal cells. They found that in the presence of low concentrations of isobutyl methyl xanthine (IBMX) a very small concentration of ACTH (10^{-12}M) caused a significant rise in extracellular and receptor-bound CAMP concomitant with an increase in free CAMP-receptor sites. These changes correlated well with steroidogenesis. Receptor-bound CAMP rose to twice the basal values while corticosterone increased to its maximum at 10^{-9}M ACTH. An increase to 10^{-7}M ACTH concentration caused a 6-fold elevation of receptor-bound CAMP with no further increase in steroidogenesis.

Petreymann et al., however, have argued that the use of the phosphodiesterase inhibitor may have amplified the effect of ACTH on CAMP accumulation without a comparable effect on steroidogenesis (182). Tait et al. (183) have further suggested that one cannot overlook the effect of using shorter time intervals and pre-incubation of the cells. For it has been reported that total cyclic AMP levels rise rapidly in response to effective doses of ACTH and reach a peak 5-10 min after initiation of stimulation and then fall towards baseline levels.

Nevertheless the work by the Podesta and Sala teams is supported by the correlation of protein kinase activation to steroidogenesis at all ACTH concentrations (10^{-11}M to 10^{-6}M) although the concentration of ACTH required for half maximal stimulation was lower for protein kinase activation than for steroidogenesis (184). Further evidence for the obligatory role of CAMP-protein kinase in steroidogenesis has come from studies with human adrenocortical tumors. Saez et al. (185) reported that ACTH failed to stimulate either protein kinase activity or steroidogenesis in a tumor in which the adenylate cyclase was unresponsive to the hormone although the tumor did respond to prostaglandin E, and to dibutyryl cyclic AMP. In another situation in which adenylate cyclase and protein kinase activities were unresponsive to prostaglandin, the tumor cells responded to ACTH with increases in both protein kinase activity and steroidogenesis. In contrast Moyle et al. (186) found that NPS-ACTH which inhibited the effect of ACTH on cyclic AMP accumulation, caused an increase in steroidogenesis without affecting protein kinase activity, except at a very high dose of NPS-ACTH in which case the analog stimulated protein kinase activity.

3. Cyclic GMP

A number of investigators have demonstrated that exogenously added cyclic GMP could stimulate steroidogenesis (187) and cyclic GMP-dependent protein kinase (188) in isolated adrenal cells. Furthermore cyclic GMP appeared to influence steroidogenesis at the same site as ACTH or cyclic AMP the conversion of cholesterol to pregnenolone (189) and cycloheximide was shown to inhibit cyclic-GMP stimulated steroidogenesis (189). However, in most of these studies cyclic GMP was much less effective than cyclic AMP, particularly when the derivatives were used. Thus 8-bromo-cGMP was 100-fold less potent than 8-bromo-CAMP on steroidogenesis in isolated rat adrenal cells and dibutyryl cyclic GMP was without effect (190).

Recently Hum et al. (191) have reported generic differences in the actions of cyclic GMP in rodent adrenal glands. In intact mouse adrenals in vitro cyclic GMP caused about 50% inhibition of steroidogenesis whereas in the intact rat adrenals, steroid production was stimulated by the nucleotide nearly as effectively as with cyclic AMP.

ACTH has been shown to increase the intracellular levels of cyclic GMP 1.5 to 3-fold over unstimulated levels in rat and bovine adrenal cells, but only at low concentrations (2.5 to 10 μU/ml) of the hormone that marginally stimulated steroidogenesis. As the concentrations of ACTH was raised above 10 μU/ml steroidogenesis continued to rise while the level of cyclic GMP fell, and according to one report I read, even returned below basal levels (190,192,193). This transient rise of cGMP was attributed to the activation of a cyclic GMP phosphodiesterase. Perchellet and Sharma (194) observed that ACTH (5 μU/ml) transiently

increased the activity of cyclic GMP phosphodiesterase and that this effect by ACTH was mediated by cyclic GMP which was found to be 100 times more potent in this inhibitor than cyclic AMP. They also noticed that the stimulation of cyclic GMP accumulation by ACTH in intact rat adrenal cells required the presence of Ca^{++} (195). Sharma and associates (192) suggested that cyclic GMP mediated steroidogenic activity of ACTH only at low concentrations of the hormone whereas at higher concentrations cyclic AMP served as the intracellular mediator.

However, other studies have not provided much support for this possible role of cyclic GMP in ACTH stimulated steroidogenesis. Hayashi et al. (190) found that the transient increase in cyclic GMP levels were confined exclusively to the extracellular fraction and no change was detected in intracellular levels of cyclic GMP or in the amount of cyclic GMP specifically bound to protein kinase. Furthermore ACTH had no direct effect on guanylate cyclase activity in soluble or particulate fractions from rat or bovine adrenals (193). It was shown also that cyclic GMP levels could be increased with sodium nitroprusside or ascorbic acid without any stimulation of adrenal steroidogenesis (196). In contrast, cyclic GMP seems to play an important role in crocodilian adrenal steroidogenesis. Honn and Chavin showed that porcine ACTH markedly inhibited cyclic AMP accumulation in crocodile adrenal slices, while raising cyclic GMP levels (197).

Rubin et al. (174) following their studies with bovine adrenal cells suggested that the regulation of steroidogenesis may be related to the relative amounts of cyclic AMP and cyclic GMP rather than the absolute concentration.

It has recently been suggested that the observed effect of ACTH on cyclic GMP levels may be mediated by glucocorticoids produced following ACTH action. When Guillemant and Guillemant (198) administered dexamethasone to intact or hypophysectomized rats, cyclic GMP levels were raised in the adrenal cortex.

4. Calcium ions

a. Adenylate cyclase activation:

Birmingham et al. (199) first demonstrated that Ca^{++} was required in the steroidogenic action of ACTH. They further showed that in mouse adrenals the requirement for calcium in ACTH action was confined to events preceding cyclic AMP formation (200).

ACTH itself has been reported to stimulate Ca^{++} influx (201) and the net accumulation of calcium inside the cells (202).

Grahame-Smith et al. (203) first reported the direct stimulation of adenylate cyclase by ACTH in rat adrenal homogenates. The enzyme is made up of a catalytic subunit and a nucleotide regulatory protein ("N" subunit) which binds guanine nucleotides (204). Binding of GTP to the regulatory protein transiently activates the catalytic activity of adenylate cyclase. The action of a GTPase causes hydrolysis of the bound GTP with subsequent inactivation of the enzyme. The GTP analogue -- guanylyl imidodiphosphate, Gpp (NH)p, being a poor substrate for the GTPase, induces an almost irreversible activation of adenylate cyclase (204). The enzyme is also activated by Mg^{++} and ACTH, and GTP appear synergistically to reduce the requirement of adrenal adenylate cyclase for Mg^{++} (205).

A recent report by Katz et al. (206) indicates that micromolar concentrations of free Ca^{++} were required for the stimulation of rat adrenal adenylate cyclase, and that other divalent cations (Mn^{++}, Co^{++} and Ba^{++}) could substitute for Ca^{++}. They also showed that high concentrations of free Ca^{++} (above 10 μM) inhibited basal and ACTH-stimulated enzyme activity. His work agrees with the original postulate by Birmingham and Bartova (200) that calcium promotes ACTH-induced CAMP formation. They demonstrated that dibutyryl CAMP could stimulate steroidogenesis and glycolysis in a calcium-free medium. It has been suggested (206) that the divalent cations act at the nucleotide regulatory site to enhance coupling of ACTH receptors to the catalytic subunit of adenylate cyclase. It is believed that Ca^{++} promotes the reaction of guanine nucleotides with their binding site. However, it has been shown that Ca^{++} is not required for the actual binding of ACTH to adrenocortical receptors (200, 172). This role of calcium on adrenal adenylate cyclase activation is specific for ACTH since EDTA and EGTA do not impair the activation induced by prostaglandin PGE (207). Recent studies by Mahaffee and Ontjes (208) suggest that the cation appears to act at two different sites in opposite ways. In one way it acts as a direct inhibitor of the enzyme by competing with Mn^{++}, at the site thought to be located on the catalytic subunit. Higher concentrations of free Ca^{++} (above 10 μM) have been found to inhibit basal and ACTH-stimulated adenyl cyclase activity (206). (But this work on isolated enzyme preparation differs from the results obtained using isolated adrenal cells in which exogenously added calcium (up to 7mM) increased ACTH-stimulated CAMP production (209). This discrepancy has

been explained with the suggestion that in intact cells adenylate cyclase resides in a compartment of low Ca^{++} concentration and is unaffected by extracellular Ca^{++} changes).

In another way Ca^{++} acts on the second site as a potent stimulator of the enzyme. This stimulation is postulated to occur at a step involving guanyl nucleotide interaction with the N-component, which reacts with the catalytic subunit to form the holoenzyme that transforms ATP to CAMP.

Other studies (208,210) have indicated that Ca^{++} also facilitates the coupling of the ACTH-receptor complex to the regulatory component (N) of adenylate cyclase. According to Haksar et al. (210) this effect might be through the stimulation by Ca^{++} of a contractile membrane protein that brings the ACTH receptor and adenylate cyclase in contact.

A number of reports have indicated that Ca^{++} is not only involved in the ACTH-stimulated CAMP formation but the cation is also required for other steps in the steroidogenic pathway. The steroidogenic effect of dibutyryl CAMP in rat adrenals is diminished by using calcium-free media or medium containing EGTA (209). It should be noted that La^{+++} which blocks the influx of Ca^{++} does not block the steroidogenic effect by dibutyryl CAMP (210). Thus it is believed that intracellular stores of Ca^{++} are available for calcium's involvement in the steroidogenic pathway. Intracellular calcium has also been found necessary for adrenal RNA (211) and DNA (212) synthesis.

b. Calcium-Calmodulin

Calcium binds to calmodulin inducing a conformational change in the protein (toward a more helical structure) that enables calmodulin to interact with a variety of enzymes: adenylate cyclase, cyclic nucleotide phosphodiesterase, guanylate cyclase, Ca^{++}-dependent protein kinase and phospholipase A_2, to name a few (213).

Hall et al. (214) recently reported some evidence that the Ca^{++}-calmodulin acts as a mediator in the steroidogenic response to ACTH or dibutyryl CAMP by mouse adrenal tumor cells.

Through inhibition studies with the calmodulin antagonist, trifluoperazine, they showed that the Ca^{++}-calmodulin system may facilitate the transport of free cholesterol to the inner mitochondrial membrane. They suggested that calmodulin-Ca^{++} may influence the function of adrenal actin similar to the role of troponin C in skeletal muscle. They had previously demonstrated that intracellular transport of cholesterol to mitochondria in mouse adrenal tumor cells involves the action of actin and microfilaments. In addition, studies by Sekimoto et al (215) with the calmodulin inhibitors, chlorpromazine, trifluoperazine and W-7, have indicated that the Ca^{++}-calmodulin system might activate the process of cholesterol ester hydrolysis in the steroidogenic pathway in bovine adrenocortical cells.

5. The Polyphosphoinositide System

The inositol phospholipids (phosphatidyl inositol (PtdIns), and polyphosphoinositides (PPI)) have recently been found to play a key role in receptor activation and signal transmission for a variety of neurotransmitters, growth factors, and hormones such as ACTH. The

interaction of these agents with their receptors leads to the turnover of membrane inositol phospholipids with an accompanying increase in intracellular calcium ion. An increase in cytoplasmic calcium ion concentration is now recognized as the most important intracellular signal in many hormonal effects. The main inositol phospholipid influenced by the ligand-receptor interaction is phosphatidyl inositol 4,5 biphosphate (PtdIns (4,5) P_2). Its hydrolysis releases diacylglycerol and Inositide triphosphate. These two are now considered to be the second messengers of hormone action acting to mobilize intracellular levels of calcium, which is currently regarded as the tertiary messenger.

The polyphosphoinositides -- PtdIns (4,5) P_2 and PtdIns 4 P (phosphatidyl inositol 4-phosphate) exist in trace amounts mainly in the plasma membrane and in other membranes that bear a close functional relationship with the plasma membrane (216). (The inositol phospholipids make up only 10% of total cellular phospholipids (217)). The polyphosphoinositides are derived from phosphatidyl inositol (PtdIns). PtdIns is synthesized from phosphatidic acid, inositol, and cytidine triphosphate (CTP). Phosphatidic acid itself is formed in the endoplasmic reticulum from available diacylglycerol in the presence of ATP and diacylglycerol kinase. Diacylglycerol could also be synthesized de novo via successive addition of fatty acyl groups to alpha-glycerophosphate.

In the plasma membrane specific kinases catalyse the conversion of PtdIns to PtdIns 4 P and then finally PtdIns (4,5) P_2 (216). These polyphosphoinositides also interact strongly with proteins, and because of their very negative headgroup which contains 3 or 5 negative charges,

these phospholipids could carry the negative potential of the plasma membrane (217). Thus changes in the amounts of membrane phosphoinositides may affect the conformation of membrane proteins and change the amount of Ca^{++} and Mg^{++} bound to the membrane (218).

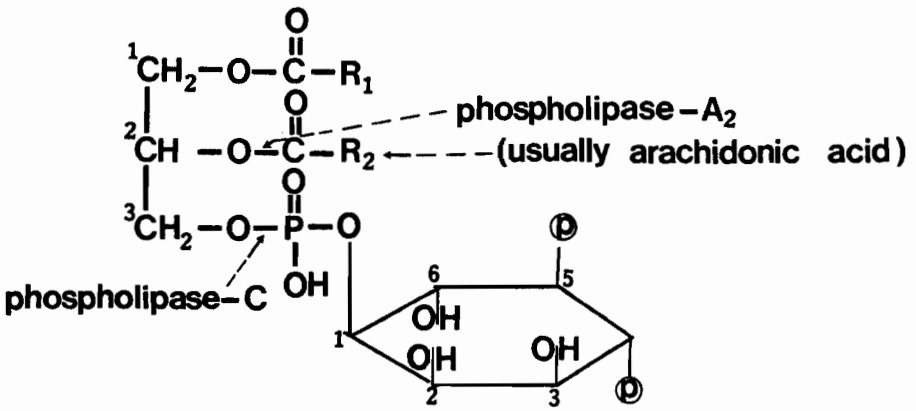
Phosphoinositide turnover can occur by 3 main mechanisms:

(1) Phospholipase-A_2 -mediated deacylation

(2) Phospholipase-C-mediated hydrolysis or

(3) De novo synthesis of phosphatidic acid for PtdIns formation.

Ptd Ins (4,5) – Biphosphate



All three mechanisms have been observed in some actions of ACTH. The first mechanism is Ca^{++}-dependent and may precede or accompany (but not follow) cAMP generation. The second mechanism has been observed in the actions of steroidogenic agents (e.g. Angiotensin II in adrenal glomerulosa cells) utilize Ca^{++} instead of cAMP as their main second messenger. Third pathway is typical of all steroidogenic agents, and is considered to be implicated in the stimulation of the steroidogenic pathway (219).

a. Phospholipase-A_2-mediated deacylation

This occurs at the position 2 of the glycerol backbone which often carries arachidonic acid. Calcium is required for phospholipase A_2 activity, and hence this reaction takes place after prior elevation of cytosol Ca^{++} concentration. The released arachidonic acid may subsequently be utilized by the cyclooxygenase and lipoxygenase enzyme systems for the synthesis of prostaglandins and derivatives.

This mechanism occurs in an early action of ACTH on plasma membrane. ACTH induces a calcium-dependent increase in phospholipase A_2 activity and production and release of prostaglandins of the E and F series (220). These prostaglandins are capable of influencing adenylate cyclase and/or may act as calcium ionophores to regulate intracellular Ca^{++} distribution (217,235). Both ACTH and NPS-ACTH have been shown to stimulate prostaglandin release and steroidogenesis without measureable effect on cAMP levels. Calcium deprivation blocked the prostaglandin and steroid release by the two peptides but did not affect steroidogenesis induced by monobutyryl cyclic AMP (235), similar to the earlier findings by Birmingham and Bartova (200).

Rubin and Laychock (231) suggested that ACTH may have separate actions on adenylate cyclase and phospholipase A_2. They postulated that in situations in which steroidogenesis is stimulated without an effect on cyclic AMP, such as stimulation by the analog NPS-ACTH, a mechanism involving prostaglandins might be operating via the peptides' action on phospholipase A_2. However, the role of prostaglandin in the

stimulation of steroidogenesis does not seem to be an obligatory one. Indomethacin blocked stimulation of prostaglandin release by ACTH but had no effect on steroidogenesis.

b. Phospholipase-C-mediated hydrolysis

This is an important pathway especially in situations in which physiological stimuli do not elevate cyclic AMP levels in cells. The current view is that there are two major classes of receptors for controlling cellular functions and proliferation. One class triggers cAMP production (e.g. β-adrenergic receptors), while the other induces inositol phospholipid turnover, Ca^{++}-mobilization, and frequently, arachidonic acid release and cyclic GMP production (22). This second class of receptors, in which activation causes an increase in intracellular Ca^{++} levels, include the muscarinic cholinergic receptor and the α_1-adrenergic receptor (223).

Stimulation of the calcium-mobilizing receptors causes phospholipase-C-mediated hydrolysis of PtdIns (4,5)P_2 yielding diacylglycerol (DG) and inositol triphosphate (ITP) both of which act as second messengers. The phenomenon is Ca^{++}-gating or release (224). (Actually, phospholipase C requires calcium ions for its activity but according to a new proposal, apparently the levels in the unstimulated cell are already adequate for the enzymatic requirement (223)).

A new type of protein kinase has recently been discovered (236) that has an absolute requirement for diacylglycerol and physiological concentrations of Ca^{++}. The enzyme, Protein Kinase C, exists in an inactive form but in the presence of Ca^{++} associates with membranes to exhibit full activity. Just recently, a calcium-activated,

phospholipid-dependent protein kinase C has been characterized in bovine adrenocortical cells (225). This enzyme was found to be capable of generating a smaller calcium phospholipid-independent form known as M-kinase. M-kinase has been found to be formed in bovine adrenal cortex by the action of a calcium-activated endogenous protease, present in that tissue (225). These studies also demonstrated that the activity of adrenocortical C-kinase may be under the control of ACTH.

The other "second messenger", inositol triphosphate mobilizes intracellular Ca^{++} stores to increase calcium ion concentrations which can further modulate cellular reactions (222).

Resynthesis of phosphoinositide requires regeneration of inositol from inositol phosphate, and in the brain this step is blocked by Lithium, thereby diminishing the responses of neurons to appropriate neurotransmitters (223). Also in the brain ACTH has been shown to exert its effect on synaptic transmission by regulating phosphoinositol metabolism in neuronal membranes. Jolles et al.(226) demonstrated that ACTH action led to the inhibition of a protein kinase responsible for phosphorylating a protein known as B-50. In its non-phosphorylated form, B-50 stimulates the conversion of PtdIns 4 P to PtdIns $(4,5)P_2$ by PtdIns 4 P kinase.

c. De novo synthesis of phosphatidic acid

This is the major effect of most steroidogenic agents (ACTH, cAMP, LH, Angiotensin II, K^+, and serotonin) and other agents such as parathyroid hormone in the kidney and insulin in fat cells (219).

Farese and colleagues have conducted extensive studies on the ACTH effect on de novo synthesis of inositol phospholipids and their

involvement in the stimulation of steroidogenesis in the adrenal cortex (217). They demonstrated that ACTH and CAMP effected about 1.5 to 2-fold increases in the adrenal contents of phosphatidic acid, PtdIns, PtdIns 4 P, PtdIns (4,5)P_2, phosphatidyl glycerol and diacylglycerol. There was also 25% increase in phosphatidyl choline and phosphatidyl ethanolamine levels. Concommitant with the above increases in phospholipids, there was a 3 to 4-fold increase in corticosterone production. Similar effects were produced by CAMP. These effects by ACTH and CAMP required Ca^{++} and were blocked by cycloheximide. Addition of PtdIns 4 P to adrenal mitochondria or dispersed adrenal cells caused increased production of pregnenolone and corticosterone, and these effects were not blocked by cycloheximide.

ACTH-induced increases in phosphatidyl inositol levels have also been observed in cat adrenal cells (227).

ACTH is considered to act through cAMP to increase phosphatidic acid synthesis by three possible mechanisms (228):

(a) increased availability of glycerol-PO_4 by stimulation of glucose uptake, glycogenolysis and glycolysis

(b) increased fatty acid and diglyceride availability through lipolysis and

(c) activation of glycerol-PO_4 acyl transferase.

(Inositol is ultimately derived from glucose 6-PO_4 and concentrated within cells).

C. Role of the inositol phospholipids in steroidogenesis

Exogenous addition of polyphosphoinositides to adrenal mitochondria and to whole cells was found to stimulate mitochondrial

cholesterol side chain cleavage and overall adrenal corticosteroidogenesis and these effects were not inhibited by cycloheximide. Farese (219) proposed that inositol phospholipids may directly stimulate the activity of cytochrome $P450_{scc}$ complex or enhance the movement of cholesterol to the enzyme complex in the inner mitochondrial membrane. In addition, he suggested that increases in phosphatidic acid may influence intracellular Ca^{++} mobilization. The polyphosphoinositides are strongly anionic and have been shown to avidly bind Ca^{++}. Such binding can influence membrane properties as well as directly affect cytochrome $P450_{scc}$ and steroidogenesis. The increase in phosphatidic acid levels may also facilitate transmembrane Ca^{++} ionophore (228). The polyphosphorylated phospholipid --cardiolipin -- is known to promote the formation of a complex between lipoproteins or liposomes and cholesterol ester. It is thus thought that the inositol phospholipids may promote similar complex formation and facilitate transport of cholesterol from the outer mitochondrial membrane to the inner side on which is located the cytochrome P450 complex. In spite of the foregoing advances the exact mechanism of stimulatory action by these phospholipids on the steroidogenic pathway is still unclear.

In summary, the following sequence of events had been suggested:

		site of	De novo	steroido
ACTH → cAMP	⟶	cycloheximide inhibition	polyphospho-	⟶ genesis
			inositide	
			synthesis	

D. Transfer of ACTH stimulus from cyclic AMP to cholesterol side-chain cleavage

It is now generally accepted that the rate-limiting step in the steroidogenic pathway is the binding of cholesterol to cytochrome $P450_{scc}$ (side-chain cleavage P450) leading to side chain cleavage and the primary site of the acute action of ACTH is this binding step. The hormone increases the association of the substrate and the enzyme in the inner mitochondrial membrane. How this is effected by ACTH is explained by the current model (229) described below:

(a) increased availability of free cholesterol

(b) increased translocation of cholesterol to mitochondria

(c) enhanced translocation of mitochondrial cholesterol from the outer mitochondrial membrane to the inner membrane for increased binding of cholesterol to cytochrome $P450_{scc}$.

1. Availability of free cholesterol - activation of cholesterol hydroxylase

(a) cAMP-dependent protein kinase: Upon the binding of cAMP to the regulatory subunit of protein kinase, the catalytic subunit is freed from the regulatory moiety, resulting in the activated form of the enzyme. The activated kinase then catalyses the phosphorylation of cholesterol ester hydrolase (CEH), converting the enzyme from an inactive to an active form. The active CEH catalyses the hydrolysis of stored cholesterol esters in the lipid droplets thereby making available more free cholesterol to the mitochondria.

(b) γ_3-MSH: γ_3-MSH is a 27-amino acid sequence containing the sequence of ACTH 4,6-9, in the N-terminal fragment of bovine POMC

(i.e. 16K-fragment). (γ_3-MSH contains -MET-GLY-HIS-PHE-ARG-TRP-ASP-).

Pedersen and Brownie (230) have recently provided evidence that γ_3-MSH stimulates cholesterol ester hydrolase activity and acts synergistically with ACTH to stimulate corticosterone and aldosterone synthesis in the rat adrenal, by a mechanism independent of cAMP. Farese et al. (231) have also shown that this action by γ_3-MSH does not involve any effect on inositol phospholipid turnover.

(c) Ca^{++}-Calmodulin: Cholesterol ester hydrolysis is stimulated by calcium-calmodulin as demonstrated in bovine adrenal cells by Sekimoto et al. In this study they employed the calmodulin inhibitors chlorpromazine, trifluoperazine, and W-7 to inhibit cholesterol ester hydrolysis (215).

2. Translocation of cholesterol to mitochondria

(a) Mediation by cytoskeletal elements: The cytoskeletal system consists of microtubules composed of tubulin, and microfilaments composed of actin as well as intermediate filaments made up of vimentin, desmin, and other proteins.

The constituent proteins of this system have the ability to assemble into filaments and this process can be inhibited by the drugs cytochalasin B or colchicine.

The system controls various biological processes such as cell shape, motility, growth and secretion.

In the adrenocortical cell, the network of microfilaments and microtubules facilitates the transfer of cholesterol from lipid droplets to the outer mitochondrial membrane. Thus, steroidogenesis in rat adrenal cells is inhibited by blockers of microtubule and microfilament

formation (232). For instance, when Hall et al. incubated mouse adrenal tumor cells with anti-actin, the antibody inhibited ACTH- or cAMP-induced increase in the cholesterol content of inner mitochondrial membranes isolated from cells incubated with amino-glutethimide and ACTH. The investigators also showed that ACTH, dibutyryl cAMP, and Ca^{++}-calmodulin stimulate the transport of cholesterol to the inner mitochondrial membrane, and trifluoperazine (inhibition of calmodulin) blocks the stimulation produced by all three agents. Once cholesterol had reached the inner membrane, the 3 agents and trifluoperazine had no further effect on cholesterol (side-chain cleavage, etc.). The investigators proposed that increased Ca^{++}-calmodulin could affect adrenal actin in the fashion of troponin C action in skeletal muscle. Ca^{++}-calmodulin presumably influences the assembly and organization of the microtubule-microfilament system via phosphorylation of the component proteins by Ca^{++}-calmodulin-dependent protein kinase (214).

(b) Mediation by sterol carrier proteins (SCP)

These proteins recently described in the adrenal have been found to be similar to the rat liver sterol carrier protein SCP_2, which can stimulate the utilization of cholesterol for pregnenolone formation by adrenal mitochondria (233). The sterol carrier proteins have been found in other steroidogenic tissues such as ovary and testes (234,235) and they might be involved in the transport of cholesterol from lipid droplets to the mitochondria. However, their involvement in ACTH-induced cholesterol utilization remains to be determined.

3. Translocation of cholesterol from the outer to the inner mitochondrial membrane, and binding to cytochrome $P450_{scc}$

Mitochondria have an outer and an inner membrane, and the cytochrome $P450_{scc}$ electron transport complex is located within the inner membrane. To reach the complex, cholesterol must be transported from extramitochondrial stores to the mitochondria, and then to the inner mitochondrial membrane.

Recent advances indicate two possible mechanisms:

(a) Mediation by a labile protein factor

Cholesterol transfer from the outer to the inner mitochondrial membrane is stimulated by ACTH and requires a labile protein factor (236). New evidence (237) has just recently been provided for the existence of this factor: Pedersen and Brownie have characterized a small peptide of molecular weight 2200 with a blocked N-terminal. This peptide caused 4.5-fold stimulation of pregnenolone formation by adrenal mitochondria from rats treated with ACTH and cycloheximide. The investigators proposed that an inactive precursor of this labile peptide is converted to the active form by limited proteolysis, and ACTH influences this through cAMP-mediated phosphorylation. The labile peptide is thought to play the role of cholesterol transporter, moving cholesterol from the outer membrane to sites within the inner mitochondrial membrane. Evidence for this has recently been provided by Ohno et al. (238) from their studies with rat adrenal cortex mitochondrial preparations. By measuring changes in cholesterol concentration in the outer and inner mitochondrial membranes in response to ACTH, they discovered that cycloheximide pretreatment caused cholesterol accumulation in the outer and not in the inner membrane, whereas aminoglutethimide caused cholesterol accumulation in the inner

but not the outer membrane. By its small polypeptide size, the labile peptide factor would be readily permeable across the outer mitochondrial membrane. Kimura (239) has recently postulated that the labile protein acts on the mitochondrial membrane by changing membrane polarity or by activating the enzyme system responsible for phosphotidate synthesis.

(b) Mediation by the polyphosphoinositide system

In this model, ACTH and cAMP stimulate phosphatidylinositol synthesis leading to increased levels of polyphosphoinositides (240). The synthesis depends on calcium and on the formation of a labile protein factor and is blocked by cycloheximide. It is thought that the association of the inositol phospholipids with cytochrome $P450_{scc}$ increases the binding of inner motochondrial membrane-cholesterol to the enzyme. Labeth and co-workers (241) have demonstrated that such phospholipids as phosphatidyl choline and cardiolipin cause an increase in the binding of cholesterol to cytochrome $P450_{scc}$. They postulated that membrane phospholipids increase the affinity of cytochrome $P450_{scc}$ for cholesterol by binding to an effector site or sites on cytochrome $P450_{scc}$. Farese and associates also observed that both the polyphosphoinositides and cardiolipin stimulated pregnenolone formation in mitochondria of adrenals from control rats, but not in mitochondria of adrenals from ACTH-treated animals (242). This action by the phosphoinositides was accompanied by an increase in the amount of cytochrome $P450_{scc}$ which was in the high spin state -- indicative of the association of the cytochrome with cholesterol.

The speculation exists that Ca^{++} might also be influential at the mitochondrial level since the polyphosphoinositides strongly bind

Ca^{++}. Some evidence for this role of calcium is provided by the demonstration by Leier and Jungman (243) that ACTH-enhanced uptake of ^{45}Ca by rat adrenal slices leads to the localization of the ^{45}Ca primarily in the mitochondria.

E. Steroid Biosynthesis

1. Enzymatic reactions

The mitochondrial conversion of cholesterol to pregnenolone is mediated by the cholesterol side chain cleavage system and requires molecular oxygen and NADPH:

$$\text{Cholesterol} + 3O_2 + 3\text{NADPH} \rightarrow \text{Pregnenolone} + \text{isocaproic aldehyde} + 3H_2O + 3\text{NADP}^+$$

The cholesterol side chain cleavage system consists of 3 redox proteins that transfer electrons from NADPH to molecular oxygen (See Fig. 5).

a. an FAD-containing flavoprotein, "NADH: adrenodoxin reductase",

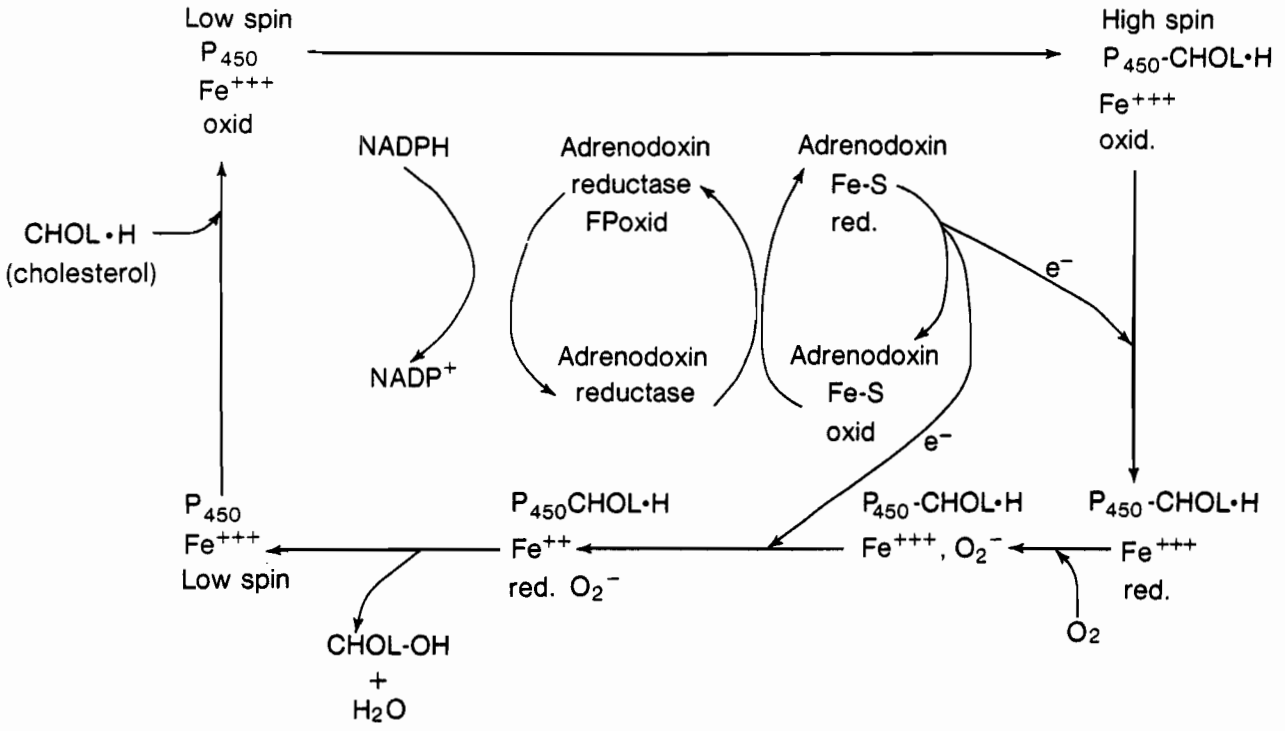
b. an Fe_2-S_2 type iron-sulfur protein known as "adrenodoxin", and

c. the terminal oxidase, cytochrome $P450_{scc}$, a hemoprotein mixed-function oxidase.

The complete pathway from cholesterol to the corticosteroids are presented in figures (5,6,7) and other synthetic routes for the synthesis of corticosterone are possible. For instance, Kraulis and Birmingham (244) demonstrated the conversion of progesterone to corticosterone

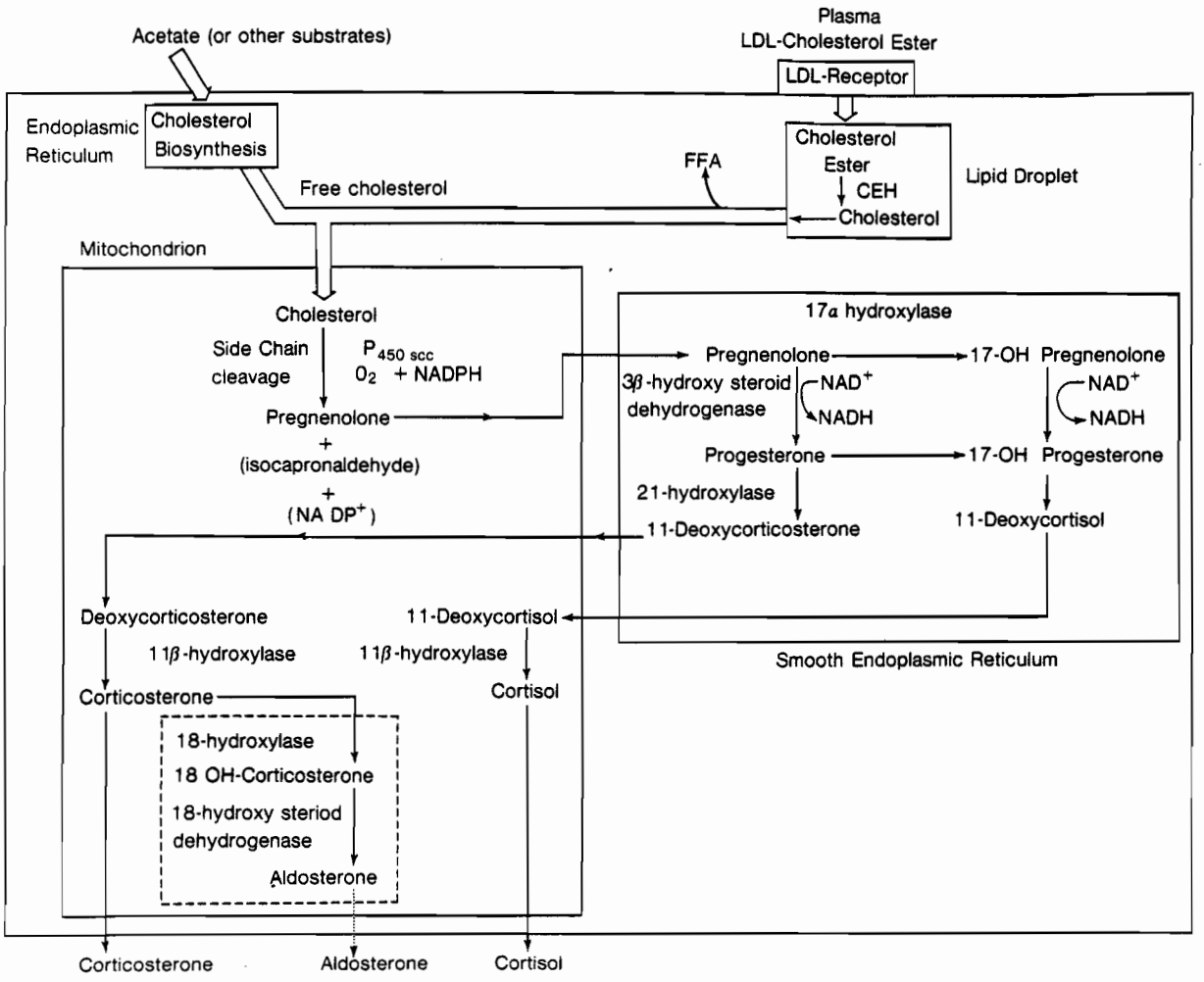
through 11-hydroxyprogesterone. This was based on evidence for the

FIG 5



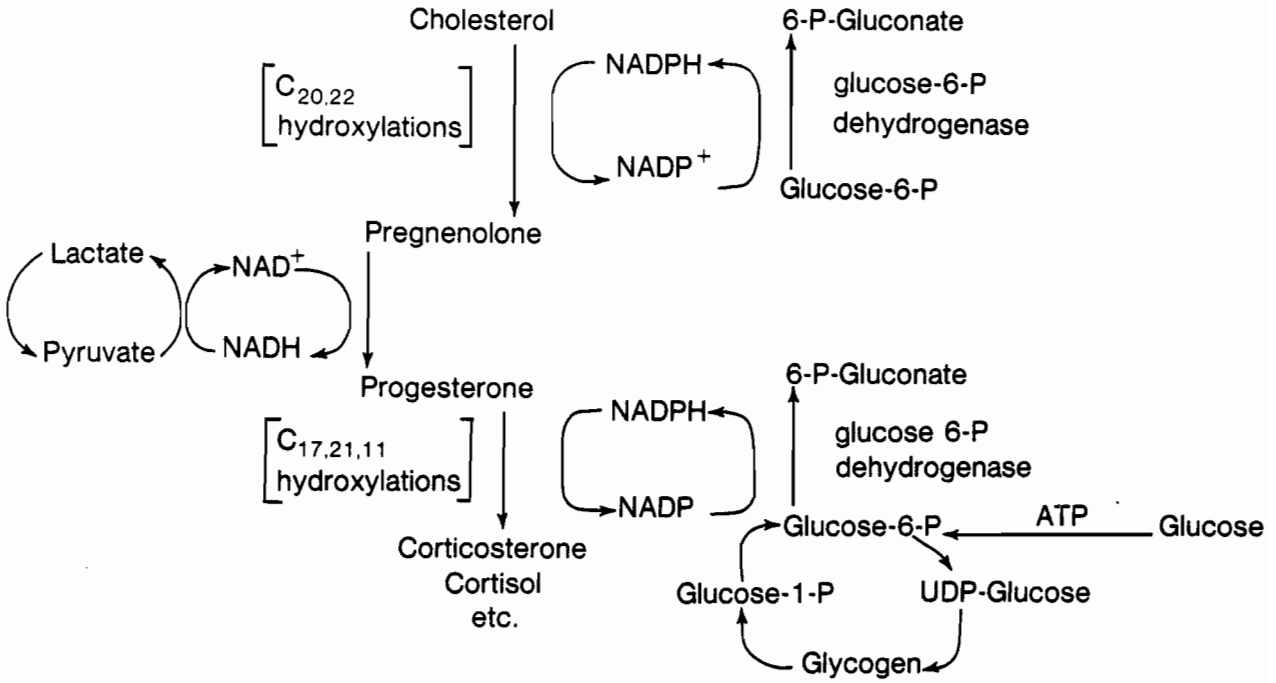
Cholesterol side-chain cleavage enzyme system and Redox

FIG 6



Intracellular steroid biosynthesis

FIG 7



Steroid biosynthesis: Cofactors, substrates and intermediates

endogenous production of 11-hydroxyprogesterone as well as the ready conversion of 11-hydroxyprogesterone to corticosterone by adrenals from different species (244).

The precise sequence of events in the side chain cleavage process is still not clear particularly because postulated intermediates are not easy to isolate. One popular mechanism suggests that there are first two successive hydroxylations of cholesterol, at C-20 and C-22, catalyzed by the same enzyme and giving rise to 20 α, 22R - diOH cholesterol, which is then acted on by a 20α, 22-C-27 desmolase in a concerted reaction, breaking the 20-22 carbon-carbon bond and releasing the C_{21} pregnenolone and a C_6 isocaproic unit:

cholesterol $\rightarrow$ 20α-OH cholesterol $\rightarrow$ 20α, 22R-diOH-cholesterol $\rightarrow$ Pregnenolone + Isocaproic aldehyde.

The reaction, Pregnenolone $\rightarrow$ Progesterone, proceeds in two enzymic steps: The 3β-hydroxysteroid dehydrogenase and Δ^{4-5} isomerase reactions which convert the 3β-hydroxyl group to a ketone and shift the double bond from the 5-6 position to the 4-5 position.

The conversion of pregnenolone to cortisol involves cytochrome P450 enzymes distinct from the $P450_{scc}$. Of these cytochrome $P450_{11\beta}$, catalyses hydroxylation at carbon 11 and is located in the inner mitochondrial membrane as is $P450_{scc}$. In contrast $P450_{17}$ and $P450_{21}$, which catalyse hydroxylation at carbon 17 and 21, respectively, are localized in the endoplasmic reticulum and are reduced by NADPH via NADPH-cytochrome P450 reductase, also present in the endoplasmic reticulum.

2. Chronic ACTH action

Whereas the acute action of ACTH involves the mobilization of cholesterol into the enzymatic pathway, the chronic action involves the regulation of the levels of the biosynthetic enzymes. Addition of ACTH to bovine adrenocortical cell culture increased the rates of synthesis of cytochromes $P450_{scc}$, $P450_{11}$, $P450_{21}$and adrenodoxin and adrenodoxin reductase (245).

In the case of cytochromes $P450_{scc}$ and $P450_{11}$ and adrenodoxin, the ACTH-induced increases reached a maximum at 36 hours. These ACTH-mediated elevations have been suggested to result partly from an increase in the levels of specific mRNA's. In addition ACTH chronically modulates the levels of these steroidogenic enzymes by altering their rates of turnover for instance by increasing their half-lives (246). This long term effect of ACTH can be mimicked by analogs of cAMP with respect to induction of synthesis of steroidogenic enzymes in bovine adrenocortical cells in culture (247).

3. Adrenal steroid products

The adrenal cortex produces 3 groups of steroid hormones: the glucocorticoids, the mineralocorticoids, and the sex steroids. However, under normal circumstances the adrenal production of the sex steroids is less significant than gonodal production of androgens and estrogens.

In early studies by Birmingham and co-workers (248,249,250) the rat adrenal was found to secrete corticosterone, 18-hydroxycorticosterone, aldosterone, and a novel steroid identified for the first time, as 18-hydroxy-11-deoxycorticosterone (18-OH-DOC). These investigators also observed the absence of 17α-hydroxylation in the

mouse. However, they noticed that the secretion of 18-OH-DOC was produced uniquely by the rat, but not the mouse (although the product is formed endogenously also by man and various other animals (263, 264). They demonstrated that ACTH increased manyfold the secretion of 18-OH-DOC by rat adrenals.

The human adrenal on the other hand secretes mainly cortisol (via 17α-hydroxylation). In fact cortisol is the major product of all primate adrenals.

The discovery of 18-OH-DOC was followed by the observation that the steroid was produced in high amounts by adrenals of rats with adrenal regeneration hypertension (253). In addition the substance was found to be capable of evoking hypertension in the saline-fed, imitatorally nephrectomized rat (254), and to have an anti-diuretic and sodium retaining action in the adrenalectomized rat, without increasing potassium excretion (255).

F. Aerobic Glycolysis

"Aerobic glycolysis" refers to the metabolism of glycogen or glucose to lactic acid under aerobic conditions.

Mammalian cells usually make ATP either anaerobically through glycolysis and substrate level phosphorylation, or aerobically by oxidoreductase reactions that feed electrons into the electron transport chain (respiration). This latter pathway is the source of most of the ATP in mammalian cells, with only very few cell types relying on anaerobic production of ATP and lactic acid. Glycolysis for most cells only precedes the complete respiratory oxidation of glucose, and lactate production is usually practically undetectable in aerobic conditions (256). However, certain cell types deviate from this rule by producing lactate even under aerobic conditions.

1. ACTH-induced aerobic glycolysis

Birmingham and colleagues (257) found that mouse adrenal glands exhibited aerobic glycolysis under the influence of ACTH. The hormone caused a 3 to 6-fold increase in lactic acid output. This effect was also elicited by cAMP. The stimulation by this nucleotide and ACTH was dose-dependent and the response paralled the steroid production by the gland (258). In the above studies aerobic glycolysis was demonstrated also in human adrenals (258).

a. Requirements for the stimulation of adrenal aerobic glycolysis by ACTH:

To further elucidate the mechanism of ACTH and cAMP-induced aerobic glycolysis in mouse adrenals, Birmingham and associates investigated the influence of glucose, ouabain, potassium and

anaerobiosis (76). Low concentrations of ouabain elevated both lactate and steroid output whereas depolarizing concentrations of potassium (60mM) tripled steroidogenesis without affecting lactate production. Anaerobiosis stimulated glycolysis but inhibited steroid synthesis. Glucose supply was essential for ACTH-induced glycolysis but not for steroidogenesis. In another study, the role of calcium in ACTH-induced aerobic glycolysis was assessed (200). It was found that exogenous calcium is required for the glycolytic response to ACTH. However, the study demonstrated that cAMP or dibutyryl cAMP-induced glycolysis did not require calcium, suggesting the primary site of action of calcium to be at the level of ACTH-induced adenyl cyclase activity, i.e. preceding the formation of cAMP. The authors concluded further that ACTH-induced aerobic glycolysis and steroidogenesis could occur independently as well as in a parallel-fashion under certain circumstances.

2. Is ACTH-induced aerobic glycolysis steroid-mediated?

The studies by Birmingham and associates (272) indicated the following:

(a) Aerobic glycolysis and steroidogenesis can both be increased in a parallel fashion by ACTH, e.g. in the presence of glucose or by addition of ouabain.

(b) Aerobic glycolysis can be inhibited without any effect on steroidogenesis, e.g. inhibition of glycolysis by omitting glucose.

(c) Steroidogenesis can be stimulated without any effect on aerobic glycolysis, e.g. stimulation of steroid output by potassium.

(d) Glycolysis and steroidogenesis can be altered in opposite directions, as by anaerobiosis, which increases glycolysis but inhibits

steroid production.

Other studies demonstrated some steroid-mediated glycolysis (259): aerobic glycolysis was stimulated in vitro by corticosterone, 11β-hydroxyprogesterone, deoxycorticosterone, and progesterone. Corticosterone was applied at a concentration that led to an adrenal steroid content similar to that induced by ACTH. Steroids that could not be converted to corticosterone by the adrenal gland were inactive at stimulating aerobic glycolysis.

Additional evidence for steroid-mediated glycolysis came from studies with inhibitors of steroidogenesis. Aminoglutethimide (which blocks cholesterol side cleavage) inhibited ACTH-induced aerobic glycolysis by 63% while completely blocking steroidogenesis. Metopirone (which inhibits 11β-hydroxylation and other sites on the steroidogenic pathway) caused a small reduction of ACTH-induced lactate production.

From these studies, Birmingham and co-workers postulated that ACTH-induced aerobic glycolysis is largely, but not exclusively, steroid-mediated.

3. Aerobic glycolysis in other tissues

(a) Ovary

Aerobic glycolysis is increased in the ovary by LH and FSH (274).

(b) Brain

In the brain, aerobic lactic acid formation is evoked by electrical stimulation (260), by ouabain (261), or by cyclic AMP (262). Benjamin and Verjee (263) carried out studies to show that the neuron is the major site of stimulated aerobic glycolysis in the brain. They incubated slices of rat brain cortex with the drug protoveratrine, which

stimulates the ATP-dependent sodium pump causing enhanced neuronal influx of Na^+ and efflux of K^+. Protoveratrine stimulated aerobic glycolysis in the brain slices. It was concluded that the drug's action was due to the decrease of neuronal ATP levels and the increases of ADP and AMP, which could stimulate the rate-limiting phosphofructokinase thereby enhancing aerobic glycolysis.

Similarly, the stimulation of brain aerobic glycolysis by high K^+, NH_4+, or Ca^{++}-deprivation was considered to be partly due to direct stimulation of key glycolytic enzymes and partly due to a fall in the tissue ATP concentration (263).

The retina is one of the most active tissues exhibiting aerobic glycolysis (264) and has been used as an experimental tissue of choice because of the ease with which the aerobic condition can be maintained in the tissue.

(c) Proliferating tissues : (tumors, embryos, lymphocytes, and fibroblasts

It was 60 years ago when Warburg discovered that tumor cells, especially those showing a very high growth rate, formed lactate even under aerobic conditions (265). He postulated that defective tumor cell respiration was the cause of the phenomenon. This reason has not been supported by subsequent studies. The aerobic production of lactate by cancer cells has also been observed <u>in vivo</u>. The phenomenon has also been noted in normal proliferating tissues such as fibroblasts during the period of maximum cell division (266).

The mitogen phytohemagglutinin was shown to stimulate aerobic glycolysis in human lymphocytes (267). Lactate production increased in

chick embryo and skeletal muscle fibroblasts during log phase growth (282). Such an apparent relationship between aerobic glycolysis and cell growth has been studied in detail by Wang et al. (283). They provided evidence that in normal lymphocytes aerobic glycolysis was not only associated with cellular proliferation, but more specifically was related to DNA synthesis and lactate production. During enhanced glycolysis, glucose transport increased about 30-fold as did glucose utilization.

The mechanism governing this glycolysis-DNA synthesis link has still not been determined. It was initially thought that aerobic glycolysis occurred as a result of placing cells in culture (284). It appeared that all cells would produce lactate anaerobically and glycolysis would increase when cell density began to interfere with oxygenation. However, studies by Wang and co-workers ruled out such in vitro artefacts as the cause of aerobic lactate production (283).

Despite the lack of any clear-cut explanation for the phenomenon, it has been proposed that aerobic glycolysis might be a general characteristic of dividing cells, and that the Warburg effect rather than defective respiration might be a normal accompaniment of cell division.

(d) Vascular smooth muscle

Lactic acid is the main product of vascular smooth muscle (VSM) in spite of fully oxygenated conditions and an adequate oxidative capacity. Paul and associates (285) have provided evidence that such aerobic glycolysis in VSM is specifically coupled to sodium and potassium transport processes, whereas oxidative metabolism is associated with contractile energy requirements.

Their evidence excluded metabolic failure as the cause of the

phenomenon. In the above studies stimulation of Na-K transport by KCl and inhibition by ouabain paralleled change in aerobic glycolysis in VSM. The above authors proposed the following model: The vascular smooth muscle has to rely on aerobic glycolysis for energy to drive membrane transport processes. For the contractile force is transmitted by attachments of the myofilaments to dense bodies on the plasma membrane, and a distribution of mitochondria suitable to the energy demands of membrane transport processes might not be possible in the smooth muscle cell. The exact nature of the coupling of aerobic glycolysis to Na-K transport related processes is still not clear.

Aerobic glycolysis also occurs in erythrocytes and the coupling of glycolysis to Na-K transport via membrane-bound glycolytic enzymes has been postulated for erythrocytes (286).

4. Does aerobic glycolysis play any functional role?

To date there is no definite answer. The ATP generated by glycolysis could be used for energy-required processes, for instance steroid synthesis, in the case of adrenal cortex.

Increased lactate production <u>in vivo</u> may cause vasodilation, increasing blood flow and facilitating steroid secretion (288).

Intracellular pH and gap-junctional conductance:

Cell-to-cell communication is facilitated by the existence of gap-junctions (289) that allow the exchange of nutrients and signal molecules between cells without loss of material into the intercellular space. The gap-junctions are channels created by membrane proteins that span the lipid bilayer. The channel traverses the plasma membranes of two opposed cells. David Spray demonstrated that a decrease in

intracellular pH could profoundly decrease electrical conductance through gap junctions. He suggested that such closure of gap-junction channels at low pH can be brought about by acidification of the cell interior by weak acids such as lactate which is membrane permeant in undissociated form (275). Such an effect has been shown to be obtainable even under aerobic conditions (275).

SECTION B

MATERIALS AND METHODS

I. INTRODUCTION

The primary objective of this study was the measurement of the variations in steroidogenic and glycolytic activities due to differences in peptide chain length or structural modifications of ACTH. It was therefore necessary to develop a cell-suspension preparation free of proteases and other hydrolytic enzymes, in order that potency differences might not merely be a reflection of degrees of peptide degradation.

High sensitivity was required in order to detect responses of the cells to certain fragments and analogs and a major effort has been devoted to the development of a procedure that provides viable mouse adrenal cells with functionally active ACTH receptors of high sensitivity. Optimal conditions have been established following a lengthy period of trials and preliminary experiments.

The small incubation volumes and low cell concentrations called for micro-determination techniques for the measurement of cyclic AMP, corticosterone and lactic acid.

CHAPTER 5 MATERIALS

A. Animals

For each experiment, 70-100 24-day old male Swiss albino mice of the strain CD-1 were purchased from Charles River Canada Inc., St. Constant, Quebec. The animals were housed in a room and allowed to acclimatize for one week, during which time they were fed ad lib on Purina mouse chow and tap water. Light and darkness were controlled by means of a 12-hour cyclic switch.

B. Chemicals and assay kits

All chemical reagents were certified A.C.S. and purchased from Fisher Scientific Co., unless stated otherwise.

1. Cell suspension medium

a. Krebs-Ringer bicarbonate buffer with 0.01M glucose

b. Bovine serum albumin (Sigma A-7030) RIA grade; 99% pure; essentially fatty acid and globulin free; from Sigma Co., St. Louis, Missouri

2. Digestive enzymes

a. Collagenase: - Worthington type CLS lot 41A264 from Millipore Corporation, Freehold, NJ

b. Deoxyribonuclease I-Grade II: - from Boehringer-Mannheim Canada, Dorval, Quebec

3. Histological Stains

a. Trypan Blue, 0.5% in isotonic saline

b. Neisser's Stain A

4. Lactic acid assay kit

from Sigma Co., St. Louis, Missouri, containing

a. Glycine buffer, pH 9.2

b. NAD

c. Lactate Dehydrogenase

d. Lactate Standard

5. Reagents for corticosterone assay

Dichloromethane

Petroleum Ether

Concentrated H_2SO_4

Absolute Ethanol

0.IN NaOH

6. Cyclic AMP radioimmunoassay kit

from New England Nuclear (NEN), Lachine, Quebec.

a. Succinyl cyclic AMP tyrosine methyl ester- ^{125}I i.e. (ScAMP-TME)

- 0.75 μCi in 1ml of n-propanol: water solution (1:1)

b. Cyclic AMP antiserum complex

- solution of pre-reacted, first and second antibody in 0.1M sodium phosphate buffer, pH 6.2, containing sufficient antibody to bind approximately 40-50% of the labeled antigen in the absence of unlabeled antigen.

c. Normal Rabbit Serum

- Added to ScAMP-TME ^{125}I to give a working tracer solution containing ScAMP-TME ^{125}I 1% normal rabbit serum and 0.05M sodium acetate buffer, pH 6.2

d. Acetylating Reagents

- Acetic anhydride and triethylamine

6. Cyclic AMP- ^{3}H Recovery marker:

- 5 μCi of CAMP - ammonium salt $^{3}H(G)$ in 1 ml ethanol: water (1:1)

7. Glycolytic substrates

Fructose, Glycerol, Pyruvate, Glucose -- from Fisher Scientific. Fructose-6-P, Fructose-1,6-diphosphate (trisodium salt), Glucose-6-P (disodium salt) -- from Boehringer-Mannheim Co.

8. Nucleotides and nucleosides

Adenosine-5'-monophosphate -- from Boehringer-Mannheim Co.

From Sigma Co., Sodium salts of:

Adenosine 3':5'-cyclic monophosphoric acid

Guanosine 3':5'-Cyclic monophosphoric acid

Guanosine 5'-monophosphoric acid

$N^{6},O^{2'}$-dibutyryl adenosine-3':5'-Cyclic monophosphoric acid

$N^{2},O^{2'}$-dibutyryl guanosine-3':5'-Cyclic monophosphoric acid

Adenosine -- from Nutritional Biochemicals Corporation, Cleveland, Ohio.

9. Peptides

a. from BACHEM Co., Torrance, California:

ACTH (1-4), ACTH (1-17), ACTH (4-11), ACTH (7-38), ACTH (11-24), ACTH (1-14), ACTH (5-10), ACTH (4-10), αMSH, $\alpha(Nle^{4})$-MSH, $\alpha(Nle^{4}, D\text{-}Phe^{7})$ MSH

b. Donated by CIBA-Geigy:

ACTH (1-24) - Biological Reference Standard in all experiments,

ACTH (3-18)NH_2, ACTH (1-16)

c. Synthesized in the laboratories of:

- Dr. J. Ramachandran, Hormone Research Lab., California

NPS-ACTH (O-nitrophenyl sulphenyl)-ACTH

- Dr. K. Hoffman, Pittsburgh

(Ala^9) ACTH (1-24), (Phe^9) ACTH (1-24), (Lys^8, Phe^9) ACTH (1-24)

- Dr. K. Inouye, Shinogi Co., Japan

β-Ala^{10} ACTH (1-18), D-Ala^{10} ACTH (1-18), L-Ala^{10} ACTH (1-18), αAib^{10} ACTH (1-18), ACTH (1-18), ACTH (1-18)NH_2

- Dr. J. Goverde, Netherlands

ACTH (7-13)NH_2, ACTH (5-14)

- Dr. Peter Shiller, Clinical Research Institute of Montreal

ACTH (1-10)

10. Gas supply

95% O_2-5% CO_2 mixture from Medigaz Co., Quebec

11. Instrumentation

Fluorimetry: Turner Fluorimeter (model 110)

Radioimmunoassay: LKB 1275 Mini-Gamma Counter/Apple IIe Computer

Histology: Zeiss Microscope, Nauebauer Hemacytometer

Cell Incubations: Dubnoff Metabolic Incubator with shaker

CHAPTER 6 METHODS (I): THE ISOLATED ADRENAL CELL SUSPENSION SYSTEM

A. Rationale for the choice of mice

To date the rat has been the usual choice for obtaining isolated adrenal cells for hormonal studies. The size of the rat adrenal (20-50 mg) allows easy separation of the capsule from the gland. Furthermore the cell yield is quite great therefore allowing the investigator to start with just a few animals.

The mouse adrenal on the other hand is smaller (1.5-2.5 mg), and large numbers of mice are required to obtain enough adrenals for a cell suspension concentration of 100,000 cells per ml. However, with regard to adrenal aerobic glycolysis, the mouse is the animal of choice, for rat adrenal cells, unlike the mouse (276) secrete 18-OH DOC which is known to inhibit aerobic glycolysis (191). Mouse adrenal cells on the other hand respond to ACTH with 1.5 to 3-fold production of lactic acid. Triller and Birmingham (277) had further demonstrated that ACTH is far more effective in stimulating *in vitro* steroidogenesis, per unit gland weight, in the mouse adrenal than the rat adrenal. As for the various problems with cell yield the development of a procedure was undertaken for obtaining viable and sensitive mouse adrenal cells in sufficient yield.

B. Advantages of cell suspensions over tissue slices, intact glands, and *in vivo* studies

The *in vivo* testing of ACTH peptides could result in loss of their potencies due to non-specific adsorption to tissues, dilution by blood, enzymatic inactivation, and urinary excretion.

The use of whole adrenals or adrenal quarters in in vitro studies require relatively high concentrations of ACTH, probably due to diffusion barriers inherent in tissue preparation.

Isolated adrenal cell systems show high sensitivity to ACTH because of increased accessibility of the peptide to the cells (278), the optimal contact of the cells with the surrounding medium, and the decreased metabolism of ACTH by the adrenocortical tissues (279, 280). Also inter-animal variation within one experiment is eliminated, as adrenal cells from several animals are mixed in one large pool which is divided over the incubation flasks. Thus isolated adrenal cells when incubated in an in vitro system approximate the sensitivity to ACTH and the secretory capacity of adrenal cells in vivo, and were therefore considered most appropriate for studying the direct action on the target cells of ACTH fragments and analogs many of which are available only in microgram amounts.

C. Development of a procedure

It became evident from preliminary studies that published dispersion procedures for the rat adrenal (281,282,283,284) were not suitable for the mouse, probably because of greater fragility of the mouse adrenal cells towards the digestive enzymes and to tissue disaggregation methods. Thus much effort was spent to develop an effective procedure for obtaining sensitive and pure mouse adrenal cells in high yields. To achieve this goal the following factors had to be considered.

1. Choice of dispersing enzymes

Collagenase is effective against fibrous intercellular elements whereas trypsin and hyalurodinase have been considered to be, respectively, proteolytic and mucolytic against intercellular ground substance. Crude collagenase contains both proteinases and peptidases (285) and thus attack the ground substances as well as collagen fibers.

In the early trials trypsin was found unsuitable at any concentration. At the minimum concentration at which cells could be dispersed, the cells were un-responsive to ACTH, even in the presence of various concentrations of Lima Bean Trypsin inhibitor. Lower concentrations failed to disperse the adrenal tissue and increased mechanical dispersion lead to extensive cell damage. After testing samples of collagenase from Boehringer-Mannheim Co., Sigma Co., and Worthington Labs, the latter type, Worthington CLS collagenase was found to be the most effective digestive enzyme for obtaining responsive mouse adrenal cells in significant yields. The optimal concentration was found to be 0.3%. Optimal responsiveness was achieved by washing off the enzyme from the tissue before mechanical dispersion of cells. Yields were further enhanced by the addition of deoxyribonuclease which facilitates dispersion by reducing the viscosity of the medium due to released nucleic acids.

Later studies were conducted to further optimize the procedure:

2. The importance of albumin

Preliminary experiments in which mouse adrenal cell suspensions were prepared at various concentrations of bovine serum albumin (BSA) indicated that the BSA concentration had an influence on cell viability,

extent of damage, and responsiveness to hormone. A concentration of 4% was found suitable for the present studies. A number of other investigators have similarly demonstrated the importance of albumin.Rodbell found that the rupture of isolated fat cells in a medium free from serum albumin can be prevented by the addition of BSA (286). Kono also observed in his experiments on the preparation of fat cell suspensions that approximately one-half of the cells in a medium free from serum albumin are ruptured within 30 minutes. He also demonstrated that this rupture of fat cells is prevented by the addition of BSA to the medium (287). Furthermore, Sayers noted that when BSA is omitted from the incubation medium the steroidogenic response of rat adrenocortical cells to ACTH is appreciably reduced. The exact mechanism by which BSA maintains the cell integrity is not known although protection of cells from extensive digestion, particularly digestion of cell surface receptors could be considered.

In contrast to the use of 4% BSA during digestion, dispersion of tissues and centrifugation of cells in this study, final incubation of cells with peptides was carried out at a BSA concentration of 0.3%, as was determined to be optimal for both the glycolytic and steroidogenic responses. Sayers (288) similarly observed that concentrations of albumin higher than 0.5% were associated with a reduced response by rat adrenal cells, evidently due to reduction of free calcium ion concentration as calcium is bound by albumin (288). This could further explain why adrenal cells are most responsive at a calcium concentrations

of 7.6 mM. The ACTH vehicle in this study also contained 0.05% BSA to inhibit adsorption of the peptide to the plastic surfaces of the incubation beakers (288).

3. Purification

This was a critical step for the present experiments. The presence of cell fragments and broken cells could influence the sensitivity of the adrenal cells to the peptides. ACTH may be bound to the receptors of damaged cells which will not result in corticosteroidogenesis (289). Secondly, enzymes derived from the broken cells may destroy the ACTH peptides (280, 290). Thus it is quite possible that a peptide might show an increased potency not due to a greater excitation of the receptor but rather because of lesser or slower degradation of the peptide. Bennett et al. (280) have investigated in detail the fate of ACTH in the isolated cell assay system and have demonstrated that cell purification can decrease inactivation of ACTH peptides. Their method has been modified in the present study in which the cells are purified by unit gravity sedimentation (290, 291) after layering on top of 5% BSA.

The isolated adrenal cells obtained by enzymatic digestion and dispersion are actually a mixture of cells from the glomerulosa and from the fasciculata-reticularis regions of the cortex. Sayers et al. (288) have demonstrated that the small proportion of cells derived from the medulla do not appear in the suspension, as they are destroyed during dispersion.

4. Pre-incubation

Following purification of the cells a further gain in sensitivity can be achieved by a pre-incubation step. This is believed to further inactivate proteolytic enzymes previously released into the medium by broken cells. It has also been suggested that the pre-incubation step is necessary for restoring the integrity of the receptors. Results from early trials suggested an optimal pre-incubation period of 90 minutes.

5. Experimental procedures

a. Surgery

The animals were rapidly sacrificed by decapitation. The adrenals were removed, freed of fat, and cut to smaller pieces with scissors. About 120 adrenals were obtained per experiment.

b. Enzymatic digestion

The adrenal pieces were pre-incubated in a 100 ml teflon beaker for 1 hr at 37^{o}C under an atmosphere of 95% O_2/5% CO_2 in an enzymatic solution (20 ml) made up of:

0.3% collagenase

0.025% DNase

4% Bovine serum albumin and Krebs-Ringer bicarbonate buffer with 0.01M glucose, (KRBG).

c. Dispersion

At the end of digestion, the enzyme solution was carefully decanted off, the adrenal pieces washed with KRBG-BSA solution. Fresh KRBG-BSA was added and tissue dispersion carried out by gently pipetting (5 strokes) of the adrenal pieces, allowing the fragments to settle,

removing 2 ml of the cell suspension and repeating the process until all pieces were dispersed into cells.

d. Purification

Slow centrifugation and Unit Gravity Sedimentation. The crude suspension was filtered on a nylon gauze into 2 polypropylene centrifuge tubes. Cells were twice washed by very slow centrifugation at 25g for 2 min. at room temperature. The final pellet was further purified by layering on 5 ml 5% BSA-KRBG and allowing sedimentation under gravity for 30 min. at $4^{o}C$.

d. Pre-incubation

This was carried out to sensitize cells, restore integrity of receptors, and to inactivate residual hydrolytic enzymes. Purified cell suspension (0.5 ml) was incubated in a 10 ml teflon beaker for 2 hours under 95% O_2:5% CO_2 at $37^{o}C$ with gentle oscillation.

Following pre-incubation, cells were twice washed with KRBG-BSA by slow centrifugation at 25g/2min.

e. Histology

Washed, purified, pre-incubated cells were diluted with KRBG so that they contained 0.3% BSA. A sample of the diluted suspension was stained with Trypan Blue (0.5%) transferred onto a hemacytometer and examined under a Zeiss Light Microscope for purity assessment and cell count estimation. A range of 300,000-500,000 cells per ml were normally obtained.

f. Incubation with peptides

Peptide Diluent: Prepared by dissolving 50 mg BSA in 100 ml 0.9

% NaCl after which the pH was adjusted to 3.5 with IN HCl. ACTH is most stable at that pH.

0.45 ml Aliquots of diluted cell suspension were added to 10 ml teflon beakers containing 0.05 ml peptide solution in diluent. The beakers were placed in the Dubnoff metabolic shaker and the cells incubated for 3 hrs at 37°C under an atmosphere of 95% O_2 and 5% CO_2. Incubation was terminated by rapidly freezing the cell suspension at -80°C until analysis. For lactate and CAMP analysis, aliquots of cell suspension were boiled in polypropylene micro-centrifuge tubes for 10 min. to coagulate proteins and inactivate enzymes.

CHAPTER 7 METHODS (II): ASSAY PROCEDURES

A. Lactate assay

1. Principle

The assay is based on the measurement of the NADH formed during the conversion of lactate to pyruvate. Lactate dehydrogenase (LDH) catalyses the oxidation of L-lactate by NAD as follows:

$$\text{L-(+)-Lactate} + NAD^+ \underset{\longleftarrow}{\overset{LDH}{\longrightarrow}} \text{Pyruvate} + NADH + H^+$$

In the UV method first reported by Hohorst (292) the formation of NADH is measured by the increase in absorbance at 340 nm. The equilibrium of the reaction lies far to the left and to drive the reaction forward an excess of NAD is added. To force the reaction to completion the formed pyruvate is trapped with hydrazine (which reacts with the carbonyl group of pyruvate):

$$\text{L-(+)-Lactate} + NAD^+ + \text{Hydrazine} \xrightarrow[\text{pH 9.0}]{LDH} \text{Pyruvate} + \text{Hydrazone} + H_3O^+ + NADH$$

The present work utilizes a highly sensitive micro-fluorimetric method in which the increase in NADH concentration is measured by the increase in fluorescence. A filter fluorimeter is employed with a primary filter for excitation having an optimum at 360 nm and secondary filter for emitted light at about 460 nm. The procedure requires just 25 μl of protein-free neutralized cell extract and the enzymatic reaction is complete in 15 minutes at room temperature. The procedure is able to detect lactate in the nanomolar range. All the reagents required are available as a kit from Sigma Co.

2. Components

a. Lactate Dehydrogenase (LDH): as a suspension of LDH, isolated from beef heart, in ammonium sulfate. Approx: 1000 units/ml

b. Glycine Buffer: contains glycine and hydrazine, pH 9.2, chloroform added as preservative.

c. NAD Preweighed vial: vial contains nicotinamide adenine dinucleotide, Grade III, 10 mg.

d. Lactic Acid Standard Solution: contains L-(+)-Lactic acid, 0.40 mg/ml (4.44 mmol/liter).

e. Perchloric acid (3M $HClO_4$)

f. Potassium Hydroxide (5N).

3. Procedure

A reagent mixture is prepared as follows: 5 ml Glycine buffer (containing hydrazine) is diluted to a vial containing pre-weighed NAD. After dissolving the NAD by gentle inversions, 0.3 ml LDH is added. 0.5 ml aliquots of this reagent mixture are added to polypropylene tubes containing 25 μl neutralized, protein-free cell extracts. The reaction is allowed to reach completion in 10 minutes at room temperature. Increased fluorescence by produced NADH is measured in a fluorimeter at 360 nm excitation wavelength and 460 nm emission wavelength.

B. Cyclic AMP Radioimmunoassay

1. Principle

The radioimmunoassay method described by Steiner et al. (293) was chosen. This method employs an iodinated (^{125}I-ScAMP-TME) marking antigen which reacts reversibly with a specific antibody to cyclic AMP to form a labeled antigen-antibody complex. The high sensitivity of the

method is a result of the cyclic AMP antibody's enhanced affinity for cyclic AMP succinylated at the 2'-O-position. The free carboxyl group of this derivative is conjugated to human serum albumin and antibodies to the conjugated protein are obtained from rabbits. The resulting assay based on the competition of labeled and unlabeled cyclic AMP for the antibody was reported to be sensitive to 1-2 picomoles of cyclic AMP (293).

2. The New England Nuclear (NEN) RIA kit

This kit provides a procedure adapted from the method of Steiner et al. (293). The labeled antigen is a succinyl tyrosine-^{125}I methyl ester derivative of cyclic AMP. Separation of bound from free antigen is achieved by the use of a pre-reacted primary and secondary antibody complex. The primary antibody was prepared in rabbits against a succinyl cyclic AMP albumin conjugate, while the second antibody was prepared in sheep against rabbit globulin.

3. The NEN RIA Procedure

The employed labeled antigen is the iodinated tyrosine-^{125}I-methyl ester derivative of cyclic AMP succinylated at the 2'-O-position and the free carboxyl group of this derivative conjugated to human serum albumin. Formation of a labeled antigen-antibody complex as well as separation of this complex from free antigen is achieved with the addition of an antiserum complex. This complex is actually a pre-reacted double antibody complex. The primary antibody was prepared in rabbits against a succinyl cyclic AMP-albumin conjugate, while the second antibody was prepared in sheep against rabbit globulin. Such a pre-reacted system reduces pipetting and eliminates the need for a second

incubation. Acetylation of standards and samples are carried out with acetic anhydride and triethylamine. The displacement reactions usually reach equilibrium in 16-18 hours at 2-8°C after which buffer is added, tubes are centrifuged and the precipitate counted in a gamma counter. With this kit, one can measure 0.0025-25 picomoles of cyclic AMP per assay tube.

4. Preparation of sample for assay

Following incubation, 200 microliters aliquots of cell incubates were transferred into stoppered polypropylene microcentrifuge tubes, and placed in boiling water bath for 10 minutes. The choice of protein denaturation by boiling over deproteinization with $HClO_4$ or trichloroacetic acid, was based, first on the small volumes of the samples, and secondly on the problem of complete removal of acid traces following acid precipitation. Incomplete removal of acid following protein denaturation of samples, could lead to some interference with the binding of antibody to cyclic AMP. Over-neutralization of samples is also undesirable because cyclic nucleotides are unstable when kept in alkaline solutions.

C. Corticosterone determination

1. Introduction

Studies by Triller and Birmingham (276, 277) have indicated that the predominant corticosteroid secreted by mouse adrenals response to ACTH is corticosterone, which fluoresces in sulfuric acid. They demonstrated that 70% of the total UV-absorbing lipid obtained in the presence of ACTH were corticosterone, 18-hydroxy corticosterone and aldosterone, of which only corticosterone fluoresces. These steroids

were found to be present in the ratios 78:13:9 respectively (277). Thus cortisol is not formed in a major quantity, if at all, and so does not interfere. Sayers too has shown that practically all the fluorescence of steroids obtained when rat adrenal cells were stimulated with ACTH was due to corticosterone (284).

In this study, corticosterone in cell suspensions was extracted with dichloromethane and measured by a fluorimetric method. This method was originally developed by Sweat (294) and has been extensively studied by Peterson (295), Guillemin et al. (296), Moncloa et al. (297), Mattingly (298) and van der Vies (299).

2. Principle

The reactions of steroids with dehydrating agents (300) such as sulfuric, phosphoric, formic, and tricholoro-acetic acid are known to result in the formation of complex molecules which fluoresce intensely (301,302,300). This is the Salkowski reaction (303) and has been observed for corticosteroids by Reichstein and Shoppee (304). Of the large number of corticosteroids they tested only three compounds -- corticosterone, cortisol, and 20β-hydroxycortisol were found to exhibit sulfuric acid-induced fluorescence. The presence of a hydroxyl group at position 11 of the steroid nucleus appears to be necessary for the production of a high degree of fluorescence. The α,β-unsaturated keto group in ring A also exerts a strong influence on fluorescence. Hydroxyl group at position 17 exerts a minor influence on fluorescence . Reichstein and Shoppee (304) also observed that steroids with an aromatic ring A, such as estrone, estradiol and estriol fluoresce far more intensely than do most other biologically important steroids. In fact,

estrogens possess native fluorescence, being the only steroids with an unsaturated ring and phenolic substitution. It is therefore necessary to remove any estrogens present in the extracts, and this is easily accomplished with a dilute alkaline solution. The acidic nature of the phenolic group provides a selective removal of estrogens with alakli.

Sweat (294) in 1954 described a highly sensitive technique for measuring the fluorescence of ethanolic sulfuric acid solutions of corticosterone and cortisol. He compared the intensity of the fluorescence produced by corticosterone with that produced by cortisol, and determined that the maximal degree of fluorescence of corticosterone is induced by light of wavelength in the 455 to 460 nm region, which is also the range of the absorption peak for the steroid. Cortisol, by contrast, exhibits a much higher maximum absorption at 475 nm. However, both steroids were found to exhibit identical emission spectra, in the region 520 to 530 nm (294).

3. Experimental Procedure

The extractions were carried out as follows: 250 μl aliquots cell suspensions were diluted to 500 μl and transferred to polypropylene microcentrifuge tubes of 1.5 ml capacity. The suspensions were then washed with 1 ml petroleum ether to remove non-steroid lipids, and 0.4 ml samples of the washed suspensions were then transferred to fresh tubes containing 1 ml dichloromethane. After extracting for 15 sec using a vortex mixer, the tubes were centrifuged at 15,000 g for 5 min. The aqueous phase was aspirated off and the dichloromethane extract washed once with 0.1N NaOH to remove endogenous estrogens, and then twice with H_2O to remove the alkali. The purified extract was then extracted with

3 ml sulfuric acid: ethanol reagent (70:30 v/v) in borosilicate tubes for 30 sec using a vortex m ixer. The phases were separated by centrifugation at 500 g for 5 min and 90 minutes later the fluorescence of the acid phase was measured at 535 nm using an excitation wavelength of 470 nm. The fluorescent values were in arbitrary units and so were corrected using a corticosterone standard.

SECTION C

RESULTS AND DISCUSSION

EXPERIMENTAL OUTLINE

Introductory Data:

The adrenocortical response to ACTH

Project A THE REGULATION OF ACTH-INDUCED AEROBIC GLYCOLYSIS AND STEROIDOGENESIS.

(Chapter 8) - Effect of Substrates and Nucleotide Derivatives

Project B ACTH: STRUCTURE-ACTIVITY STUDIES

Introductory Data:

ACTH dose-response relationship

(Chapter 9) - Studies with ACTH-fragments
(Relation of chain length to activity)

(Chapter 10) - Studies with ACTH-analogs
(Effect of structural alterations in the 4-10 region)

(Chapter 11) - Studies with structurally-related peptide hormones

(Chapter 12) - The role of essential regions in the stimulation of cAMP formation

(Chapter 13) - The role of the tryptophan residue in ACTH action
(Studies with O-nitrophenylsulphenyl-ACTH(1-24))

Introductory Data

The adrenocortical responses to ACTH

Fig. (8) shows the response of isolated adrenocortical cells to 10^{-8}M ACTH over a period of 4 hours. Cell suspension samples were removed every hour and the lactate and corticosterone levels measured.

As the time course indicates, ACTH caused a 2-fold increase in lactic acid production and a 15-fold elevation of corticosterone level, by the third hour. In the following studies an incubation period of 3 hours was chosen for experiments involving isolated cells. Initial studies as well as other reports (Chapter 6) have indicated that isolated cells obtained through enzymatic digestion of tissues require longer incubations to enable cells to recover and functionality restored. It was also borne in mind the dangers of lengthy incubations such as end product inhibitions, phosphodiesterase action on cAMP, peptide inactivation by traces of proteolytic enzymes, etc. Three-hour incubation was found optimal after several initial studies.

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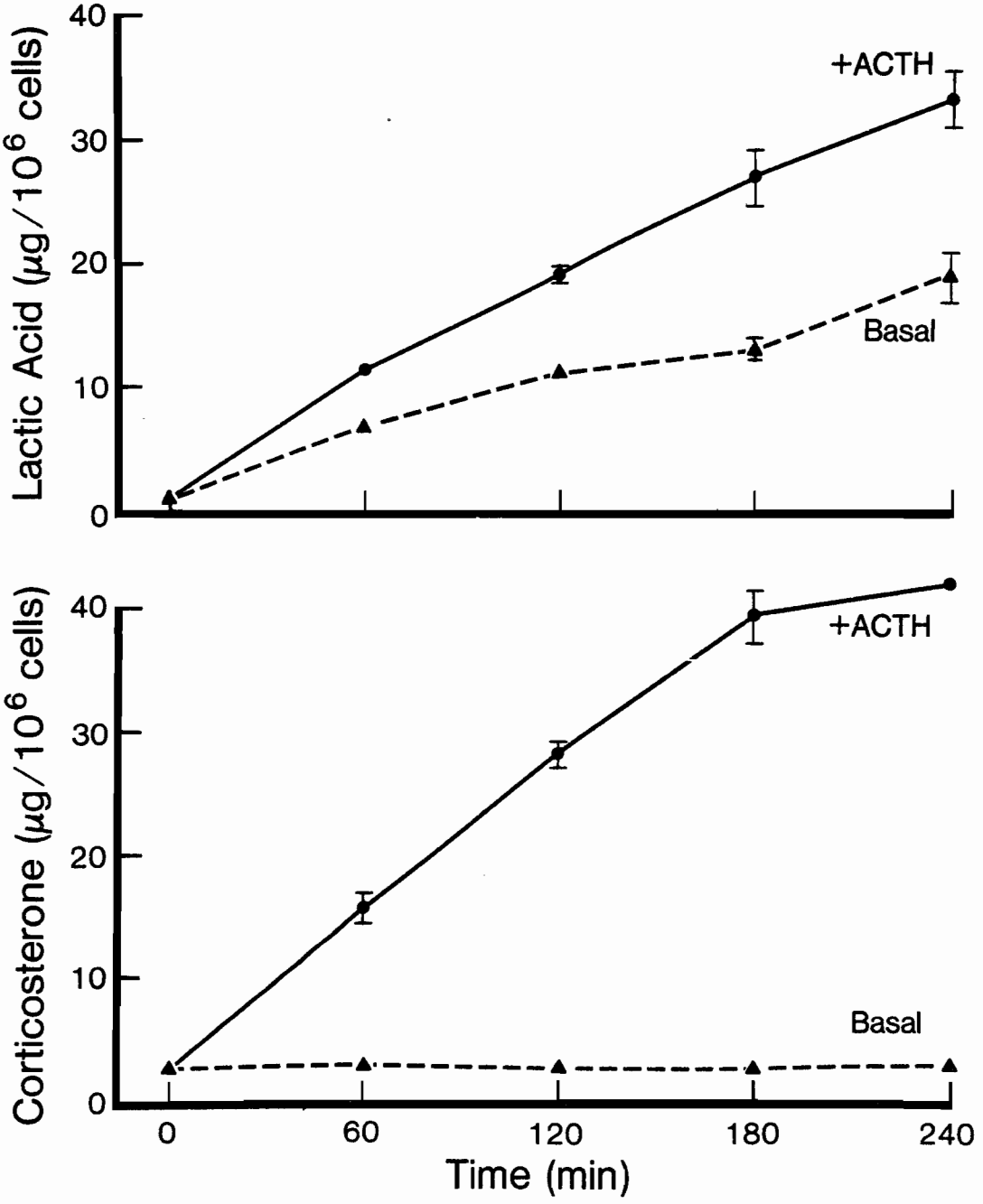
FIG 8

ACTH-Enhanced Aerobic Glycolysis and Stimulation of Steroid Production



PROJECT A

REGULATION OF ACTH-INDUCED AEROBIC GLYCOLYSIS AND STEROIDOGENESIS

CHAPTER 8

LACTIC ACID AND STEROID PRODUCTION BY INTACT MOUSE ADRENAL GLANDS AND CELL SUSPENSIONS: EFFECTS OF NUCLEOTIDE DERIVATIVES AND SUBSTRATES

(HINSON and BIRMINGHAM, (1984), Ref. 305)

I. INTRODUCTION

Intact mouse adrenal glands incubated in glucose-containing medium respond to adrenocorticotrophic hormone (ACTH) and cyclic AMP (cAMP) with a dose-dependent increase in steroid production and in aerobic glycolysis that may be partially steroid-mediated (76,257,259). Cyclic GMP was found to increase glycolysis of intact mouse adrenal glands only slightly and to inhibit steroidogenesis, in contrast to the stimulation it evoked in the rat adrenal gland (191). In the present work, the effects of nucleotide derivatives on these two responses have been studied in the mouse adrenal and substrate requirements have been further investigated. The dibutyryl derivatives rather than the parent compounds were utilized because of their anticipated resistance to phosphodiesterase activity and resulting increase in potency, considered desirable in studies on substrate specificity which might be rendered equivocal by a potential contribution of nucleotide-derived carbohydrate moieties. We have included cell suspensions to verify and extend the results obtained on intact and sectioned glands which are less sensitive to ACTH and more subject to diffusion barriers than are dispersed cells. Mouse, rather than rat adrenals were used to avoid complications in interpretation resulting from the inhibitory effect on glycolysis exerted by

18-hydroxydeoxycorticosterone (191), the second major adrenocortical steroid produced in response to ACTH by the rat, but not the mouse.

II. RESULTS

A. Effects of dibutyryl derivatives of cAMP and cGMP

In intact glands the dibutyryl derivative of cAMP (dbcAMP), tested at a concentration of 1 mmol/l, was more effective in stimulating lactic acid and corticosterone production than was cAMP at a concentration of 10 mmol/l (Fig. 9). The dibutyryl derivative of cGMP (dbcGMP) at a concentration of 1 mmol/l caused only a slight increase in corticosterone production, less than one-tenth that evoked by dbcAMP, and had no effect on lactic acid output. No effects were obtained with dbcGMP at a concentration of 0.5 mmol/l or with cGMP at a concentration of 10 mmol/l.

The responses of mouse adrenal cell suspensions to dbcGMP and dbcAMP were compared at concentrations of 0.01, 0.1 and 2 mmol/l (Fig. 10). In contrast to dbcGMP, dbcAMP caused highly significant, dose-dependent increases in both lactic acid and steroid production. The effects of dbcAMP and dbcGMP were compared with those of the free and uncyclized forms of the nucleotides at a concentration of 1 mmol/l (Fig. 11). Lactic acid production was increased by all the nucleotides examined but most effectively by dbcAMP. Although dbcGMP induced a statistically significant increase in lactic acid formation in this experiment, the response did not exceed that evoked by cGMP or GMP. Corticosterone production was not affected by AMP, GMP, cGMP and cAMP and only minimally enhanced by dbcGMP, compared to a 25-fold increase over the basal output observed with dbcAMP.

Figure 9.

Effects of cyclic AMP (cAMP), cGMP, dibutyryl cAMP (dbcAMP) and dbcGMP on lactic acid and corticosterone production in intact mouse adrenal glands in vitro. Values are means ± S.E.M. Broken lines indicate control values. ★$P < 0.05$, ★★$P < 0.01$, compared with control (Student's t-test). n = 4.

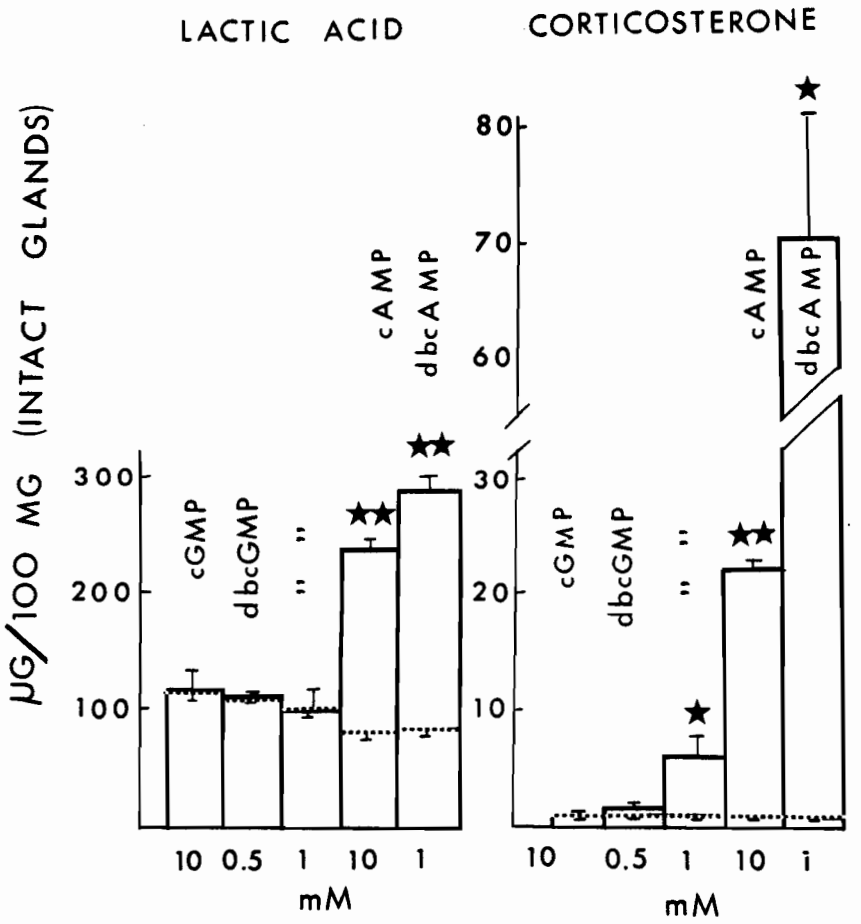


Figure 10.

Effects of dibutyryl cyclic AMP (dbcAMP) and dibutyryl cyclic GMP (dbcGMP) on lactic acid (—▲—) and steroid (--●--) production by mouse adrenal cell suspensions. Open symbols, production in the absence of added nucleotides. The average of triplicate samples from the same suspension are shown (± S.E.M. where it exceeded the size of the symbol).

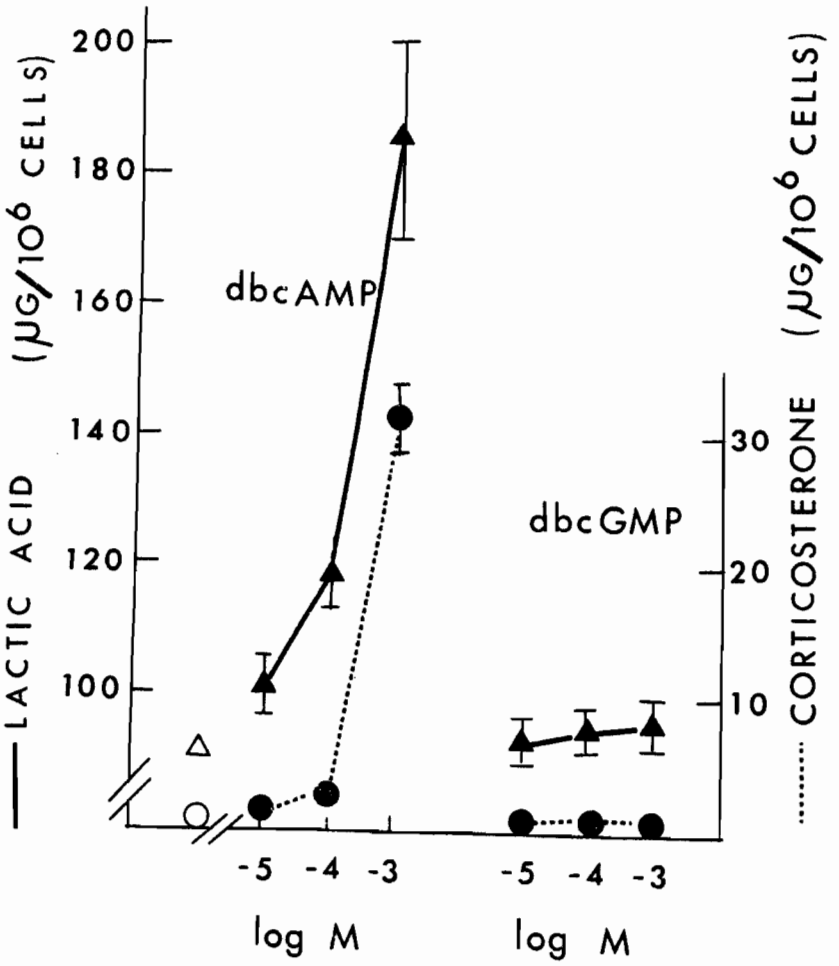
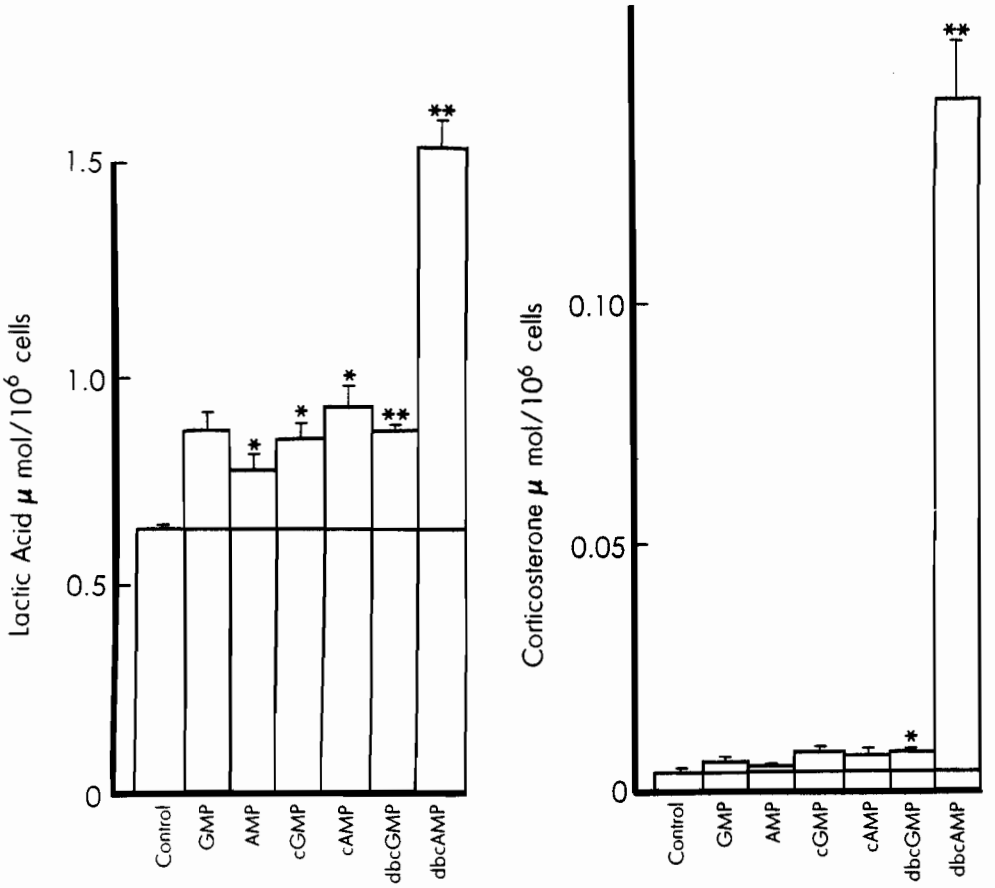


Figure 11.

Comparison of the responses to AMP, GMP and their cyclic (cAMP, cGMP) and dibutyryl (dbcAMP, dbcGMP) derivatives in mouse adrenal cell suspensions. Final concentration of all nucleotides, 1 mmol/l. The average of triplicate samples ± S.E.M. are shown.

*P < 0.05, **P < 0.01 compared with control (Student's t-test)



B. Effects of substrates

The substrates tested in unstimulated tissue and in the presence of ACTH or dbcAMP were glucose, glucose-6-phosphate, fructose, fructose-6-phosphate, fructose-1,6-diphosphate, all at a final concentration of 0.01 M and pyruvate and glycerol at a final concentration of 0.02 M. The concentration of carbon equivalents was thus equal for all substrates examined and corresponded to that most commonly used for glucose in vitro. Although all substrates were capable of stimulating lactic acid production when added by themselves, fructose-1,6-diphosphate was far the most effective in both intact and dispersed glands (Figs. 12 and 13). The glycolytic response to ACTH was, however, not affected by fructose-1,6-diphosphate but promoted best by glucose in cell suspensions and in intact glands. Pyruvate also enhanced ACTH-induced lactic acid production in intact glands, glucose increased the steroidogenic response to ACTH.

The effect of different substrates on dbcAMP-stimulated steroid production and glycolysis was examined in cell suspensions. Dibutyryl cAMP increased corticosterone production in substrate-free medium and none of the substrates potentiated this response significantly. Stimulation of glycolysis occurred only in the presence of glucose.

III. DISCUSSION

In intact mouse adrenal glands as well as in mouse adrenal cell suspensions, the glycolytic and steroidogenic responses to dbcGMP were minimal or absent in contrast to those elicited by dbcAMP. These findings are in keeping with earlier observations utilizing intact glands only and the free form of the cyclic

Figure 12

Effects of different substrates on basal and ACTH-stimulated corticosterone and lactic acid production by intact mouse adrenals *in vitro*. Left columns: basal output ± standard error of paired differences between the production in the presence and absence (broken line) of substrate. Right columns, net increase or decrease evoked by ACTH ± standard error of paired differences. No subs. = no substrate, GLY = glycerol, PYR = pyruvate, F1,6 = fructose 1,6-diphosphate, F6 = fructose-6-phosphate, F = fructose, G6 = glucose-6-phosphate, G = glucose. Asterisks over left columns denote significant effects of substrates on basal output (number of pairs = 11, 13, 8, 4, 13, 13, 19 for GLY, PYR, F1, 6, F6, F, G6 and G, respectively). Asterisks over right columns denote significant effects of ACTH (number of pairs = 15, 9, 9, 4, 2, 11, 9, 15 for no subs., GLY, PYR, F1, 6, F6, F, G6, G, respectively). *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ (Student's t-test for paired differences). Daggers denote a net response to ACTH significantly greater than the response to ACTH obtained in the absence of substrate. +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$, established by analysis of variance.

Fig 12

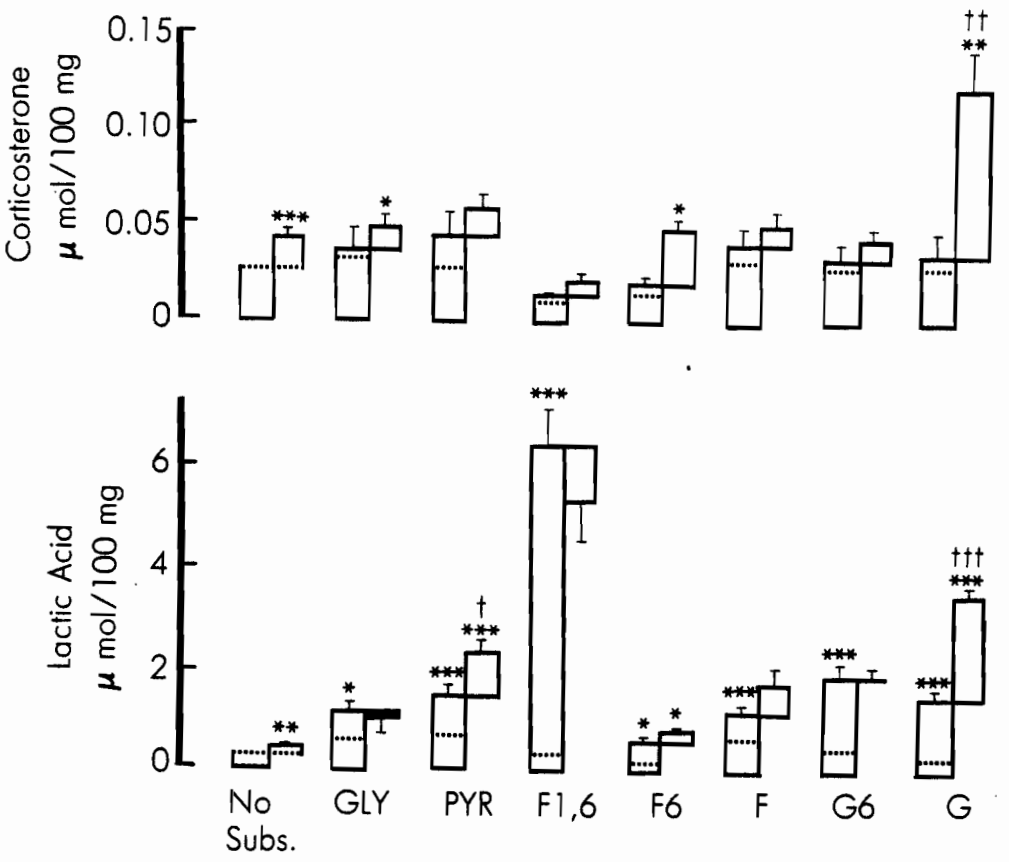
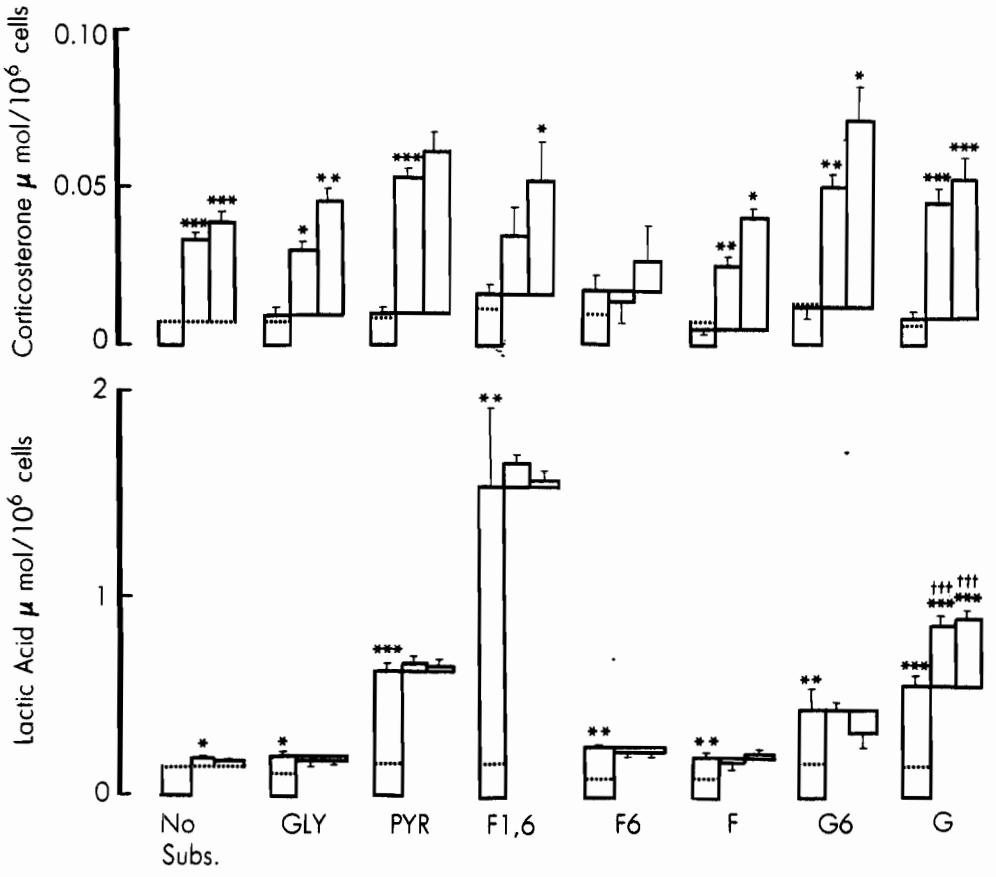

Corticosterone
μ mol/100 mg
0.15
0.10
0.05
0
Lactic Acid
μ mol/100 mg
6
4
2
0
No Subs.
GLY
PYR
F1,6
F6
F
G6
G

Figure 13

Effects of different substrates on basal, ACTH and dibutyryl cyclic AMP (dbcAMP), stimulated corticosterone and lactic acid production by mouse adrenal cell suspensions. Left columns: basal output ± standard error of paired differences between the production in the presence and absence (broken line) of substrates. Middle columns: net increase or decrease evoked by ACTH ± standard error of paired differences. Right columns: net increase or decrease evoked by dbcAMP ± standard error of paired differences. No subs. = no substrate, GLY = glycerol, PYR = pyruvate, F1,6 = fructose 1, 6-diphosphate, F6 = fructose-6-phosphate, F = fructose, G6 = glucose-6-phosphate, G = glucose. Asterisks over left columns denote significant effects of substrates on basal output (number of pairs = 6, 6, 12, 4, 6, 8, 28 for GLY, PYR, F1, 6, F6, F, G6 and G, respectively). Asterisks over middle and right columns denote significant effects of ACTH and dbcAMP (number of pairs = 16, 4, 4, 8, 4, 4, 4, 18 and 14, 4, 2, 7, 4, 2, 4, 16 for no subs., GLY, PYR, F1, 6, F6, F, G6 and G, respectively). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's t-test for paired differences). Daggers denote a net response to ACTH or dbcAMP significantly greater than the response to ACTH or dbcAMP obtained in the absence of substrate. ***$P < 0.001$.

Fig 13


Corticosterone μ mol/10⁶ cells
0.10
0.05
0
Lactic Acid μ mol/10⁶ cells
2
1
0
No Subs.
GLY
PYR
F1,6
F6
F
G6
G

nucleotides, which established a species difference between rat and mouse adrenals in their response to cGMP (191). Attention has been increasingly focused on the potential role of cGMP in mediating the action of ACTH (192,195,306,307). The difference in response to this nucleotide in rat and mouse adrenals emphasize the need for caution against species variability in the extrapolation of data. However, while exogenous cGMP has nearly the same potency as cAMP in the rat adrenal, endogenous cGMP is present in lower concentrations than cAMP (309).

The free form of cGMP, in contrast to cAMP, failed to stimulate mouse adrenal steroidogenesis in the present work, but it did not cause the inhibition of steroid output noted earlier, for unknown reasons.

The uncyclized nucleotides enhanced basal glycolysis in adrenal cell suspensions without affecting steroid production, in agreement with their behaviour in intact glands (191). They thus provided examples for the possible dissociation between the two events as did the increase in basal glycolysis but not steroidogenesis elicited by various substrates. Of these, fructose-1,6-diphosphate was by far the most effective. This is compatible with a rate-limiting step at the level of phosphofructokinase postulated for many tissues including the adrenal (309). Notably, neither ACTH nor dbcAMP enhanced the glycolytic responses to fructose-1,6-diphosphate but, in accord with earlier findings (76,200), both acted synergistically in the presence of glucose. Unexpectedly (76), glucose also enhanced the steroidogenic response to ACTH, but not in cell suspensions. The only other synergistically active substrate was pyruvate which was less potent than glucose and its action was confined to the glycolytic response in intact

glands. The results suggest that ACTH and dbcAMP directly or indirectly stimulate glycolysis at some site between the entry of glucose into the cell and the formation of fructose-1,6-diphosphate. Evidence for the activation of phosphofructokinase by ACTH has been obtained by Bell, Brooker & Harding (309) and by Kowal, Frenkel and Angee (310). Phosphofructokinase activity in the liver has recently been found to be hormonally regulated by the alteration in the level of fructose-2,6-biphosphate, a potent stimulator of this enzyme (311). Furthermore, vasopressin-induced glycolysis of hepatocytes is glucose-dependent (312). A role for fructose-2,6-biphosphate in the hormonal control of adrenal glycolysis should, therefore, be considered. The entry of glucose into the cell might also be controlled by ACTH analogous to the facilitation by leuteinizing hormone of glucose transport into granulosa cells of the ovary (313), another organ capable of responding to its trophic hormones by an increase not only of steroidogenesis, but also of lactic acid production (314,315,316). It is possible that endogenous steroids contribute to the glycolytic response since in the adrenal they can increase lactic acid production when added to the incubation medium at concentrations that raise the level of tissue corticosterone to that found in the ACTH-stimulated gland (259). Notably certain steroids have recently been shown to enhance glucose transport across the membrane (317).

PROJECT - B

ACTH: STRUCTURE-ACTIVITY STUDIES

GENERAL LEGEND

FOR DATA FIGURES

Isolated adrenocortical cells were incubated with varying concentrations of ACTH (1-24) and test peptide(s).

After 3 hours of incubation samples were frozen at -80°C until measurements. Lactic acid and corticosterone were measured as described in the Methods section (Chapter 7).

All values are expressed as a percentage of the maximal stimulation (unless indicated otherwise). One hundred percent response was that observed for maximal concentration of ACTH (1-24).

Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point.

INTRODUCTORY DATA

ACTH: dose-response relationship

The response of isolated adrenocortical cells to various concentrations of ACTH (1-24) is depicted in Fig. 14 over a concentration range of 10^{-12} to 10^{-6}M.

The cells responded to increasing ACTH concentrations with increasing production of lactic acid and corticosterone, reaching a maximum, in both cases, around 10^{-9}M ACTH.

At maximal ACTH concentrations, there was a 2-fold increase in lactic acid and a 20-fold increase in corticosterone over basal levels.

Equations

The dose-response curves approximate to a rectangular hyperbola. If one designates the dose of ACTH as D and the biological response of the cells as R, the dose and response can be related by the expression for a rectangular hyperbola,

$$\frac{R}{Rmax} = \frac{D}{(D + D_{50})}$$

where Rmax is the maximal rate of response and D_{50} is the dose at which half-maximal response is attained.

According to a model proposed by Ariens (318), Rmax is a measure of the intrinsic activity of a peptide, whereas D_{50} relates to its affinity for the receptor. In all the following structure-activity studies Rmax and D_{50} were estimated with a computer program that performed non-linear least-square fits to the data (Appendix A).

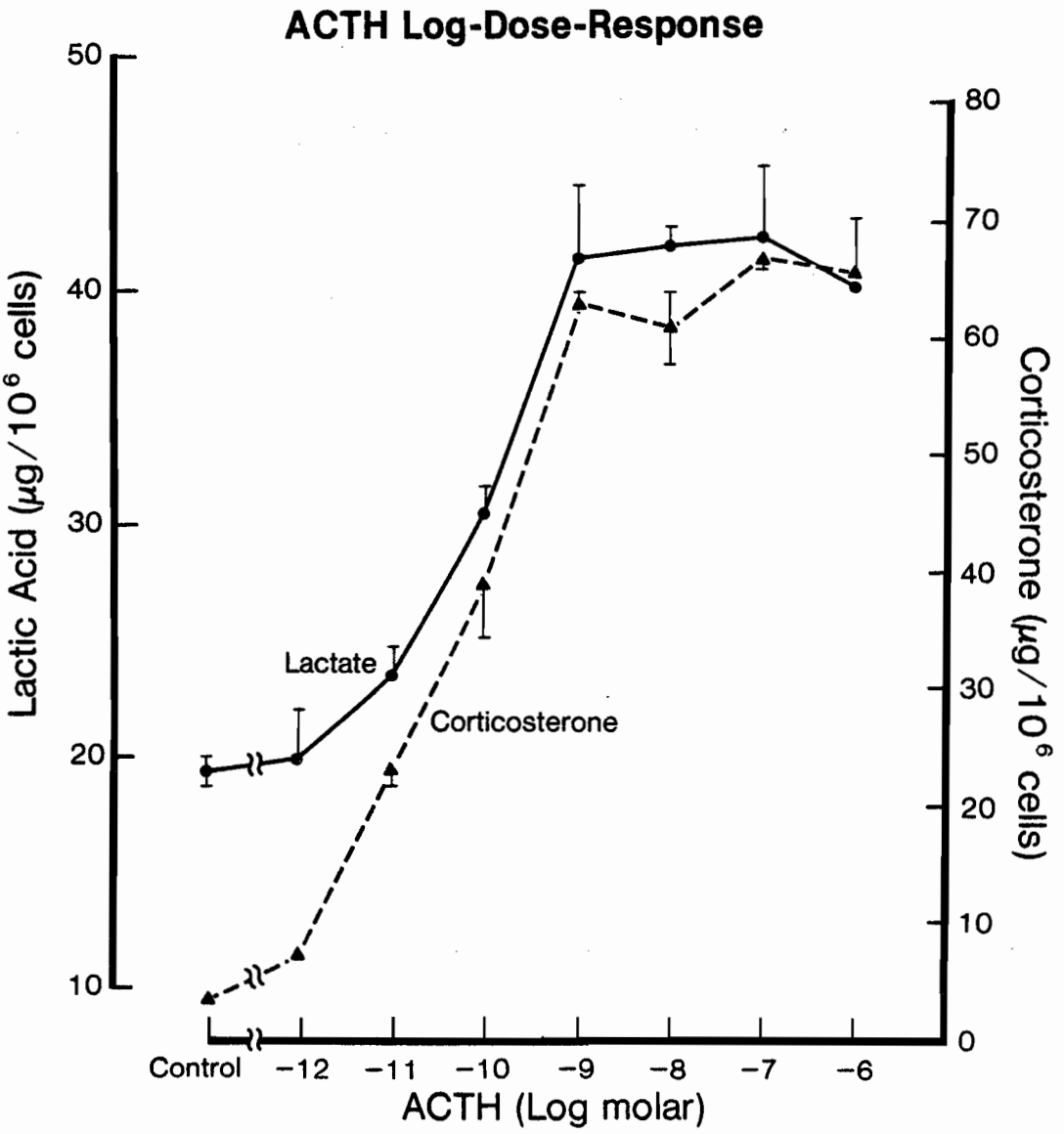


Figure 14

ACTH: Dose-response relationship

ACTH (1-24) was added to cells in concentrations ranging from 10^{-12}M to 10^{-6}M.

(See GENERAL LEGEND page 120)

Potency was derived as $\left[\frac{1}{D_{50}} \times 100\right]$ and % relative potency, as

$$\left[\frac{D_{50}\ \text{(ACTH 1-24)}}{D_{50}\ \text{(Peptide)}}\right] \times 100 \quad \%$$

CHAPTER 9

Studies with ACTH-fragments

(Relation of chain length to activity)

STUDY 9a. Effect of shortening from C-terminal end

The carboxyl-terminal half of ACTH (1-24) contains the message tripeptide: LYS^{11}-PRO^{12}-VAL^{13} as well as the address segment: LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}-PRO^{19}-VAL^{20}-LYS^{21}-VAL^{22}-TYR^{23}-PRO^{24} (Chapter 3).

The address region carries the basic tetrapeptide believed to be involved in the binding of the hormone to the receptor.

In order to assess the importance of the C-terminal region for the stimulation of glycolysis, a series of ACTH fragments were investigated in which the peptides had been shortened progressively from the C-terminal end of the molecule.

Fig. 15 shows the various log concentration-response curves for 6 C-shortened ACTH fragments, as well as for ACTH (1-24)(as a biological reference standard).

The fragments were tested at multiple concentrations, as indicated, for their ability to stimulate aerobic glycolysis (top figure) and their effectiveness as steroidogenic agents.

The behavior of the fragments can be described with reference to two parameters, maximal activity (Rmax) and potency (D_{50}).

Glycolysis

As the C-terminus was shortened, there was a progressive

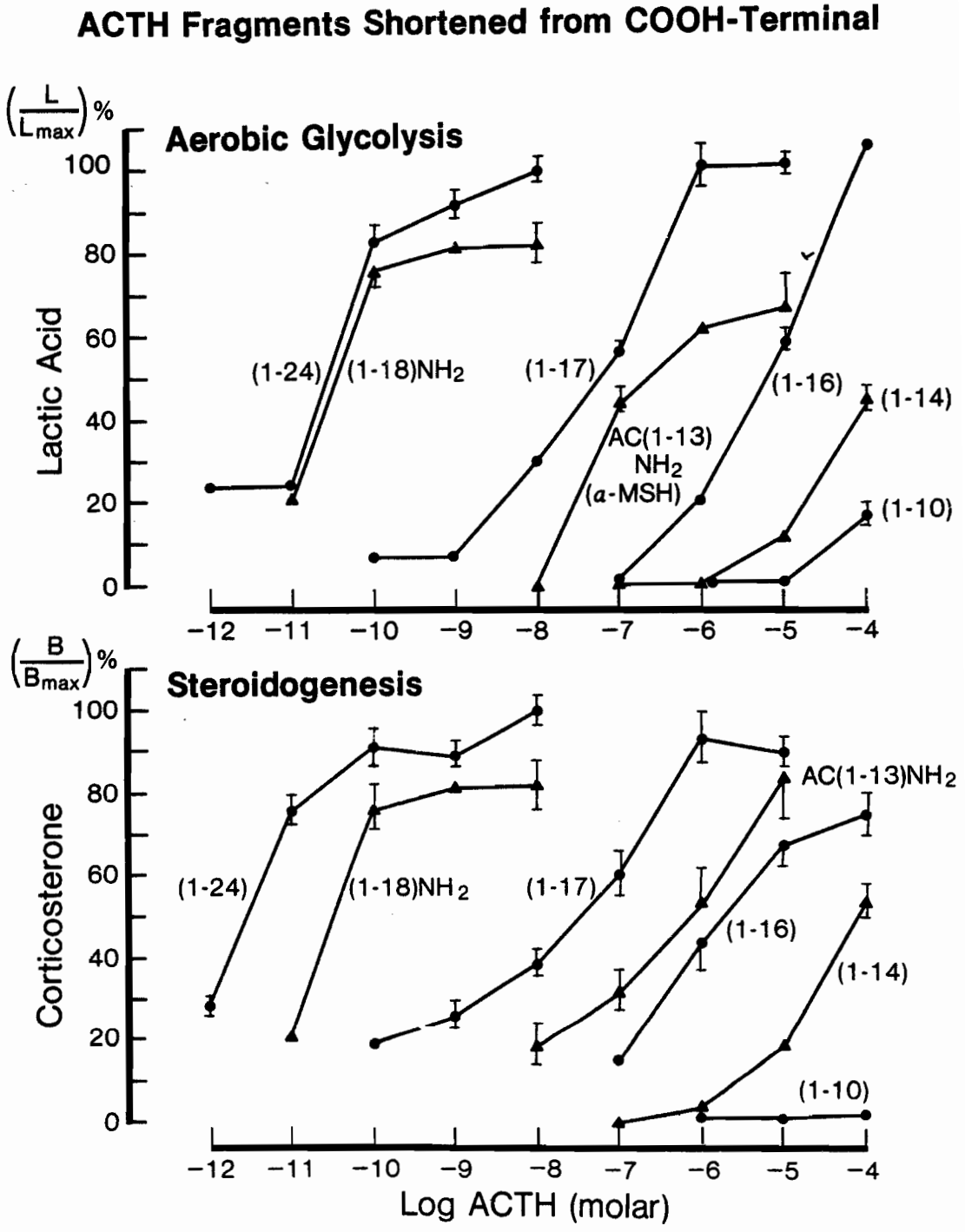


Figure 15

STUDY 9a: Effect of shortening from C-terminal end.

(See GENERAL LEGEND page 120)

displacement of the dose-response curves to the right, away from the curve for ACTH (1-24). This meant the affinities (or potencies) of the fragments were reduced as the C-terminal residues were removed from the hormone.

Thus, the curves were displaced to the right in order of decreasing peptide length as follows:
ACTH(1-24) > ACTH(1-18) > ACTH(1-17) > αMSH > ACTH(1-16) > ACTH(1-14) > ACTH(1-10).

An exception to the rule is α-MSH, which is ACTH(1-13) acetylated at the N-terminus and amidated at the carboxyl end. This peptide appeared more potent than ACTH (1-16) and ACTH (1-14). This could be explained in two possible ways. The apparent high potency could reflect resistance to amino- and/or carboxy peptidases. One could also argue that the fragment could exhibit optimal interaction with a specific melanotropic receptor on the adrenocortical cell.

Another noteworthy point was the observation that the curve for ACTH (1-18) almost coincided with that for ACTH (1-24). The two peptides differ only by the C-terminal hexapeptide, PRO^{19}-VAL^{20}-LYS^{21}-VAL^{22}-TYR^{23}-PRO^{24}. The data seemed to suggest that this segment was not essential for glycolytic potency. (The region contains a message for Mg^{++}-receptors in adipocytes) (Chapter 3). Further, ACTH (1-18) has the full complement of the basic tetrapeptide sequence, known to be essential for receptor affinity. As the basic residues were removed there was a drastic decline in potencies. The absence of ARG^{18} in ACTH (1-17) was associated with a fall in affinity by 4 orders of magnitude. Further absence of ARG^{17} resulted in an even lower potency, as seen

with ACTH (1-16). ACTH (1-14) sequence which lacks the entire basic tetrapeptide showed very little glycolysis-stimulating activity even at micromolar concentrations.

Thus, the basic tetrapeptide contributed to glycolytic potency.

ACTH (1-10) lacks both the basic tetrapeptide as well as a message sequence: LYS^{11}-PRO^{12}-VAL^{13}. This fragment had minimal activity at the concentrations employed.

Whereas the potencies varied almost in direct proportion to chain length, most of the peptides were capable of maximal stimulation of glycolysis if added at sufficiently high concentration. In this particular experiment ACTH (1-14) and ACTH (1-10) were not tested at concentrations exceeding 10^{-4}M and therefore their maximal activities are not evident in the graph.

Lmax is considered to be an index of the ability to excite the receptor whereas potencies reflected only the affinity of the fragments for the receptor. Therefore it can be concluded that the residues of the C-terminal half of ACTH (1-24) were not essential to the excitation of the receptor or receptors responsible for the stimulation of glycolysis, but contributed greatly to receptor affinity and consequently glycolytic potency.

The slightly lower Lmax for ACTH (1-18) NH_2 and α-MSH is not readily explicable, except for the fact that the 2 peptides were the only ones with amidated carboxyl ends. Perhaps a free carboxyl end is optimal for receptor activation.

Steroidogenesis:

The lower part of Fig. 15 depicts the effectiveness of the fragments as steroidogenic agents. The findings were nearly identical to those for the glycolytic response, except for the following observations: ACTH (1-18) was steroidogenically less potent than ACTH (1-24), unlike the identical potencies exhibited in the glycolytic actions. α-MSH elicited maximal steroidogenic activity albeit at higher concentrations.

At 10^{-4}M ACTH (1-10) induced a small glycolytic response but was without effect on steroidogenesis. These differences are an indication that glycolysis and steroidogenesis need not exactly correspond. Table 2 summarizes the relative potencies for the glycolytic and steroidogenic actions of the series of fragments studied. The observation that ACTH (1-18) was as potent as ACTH (1-24) in stimulating aerobic glycolysis but only half as potent steroidogenically would seem to suggest that the sequence PRO^{19}-VAL^{20}-LYS^{21}-VAL^{22}-TYR^{23}-PRO^{24} absent in ACTH (1-18) might be essential for maximum affinity, but does not contribute to glycolytic potency.

Another major discrepancy in potencies was observed for the small fragment ACTH (1-14). The peptide was more potent glycolytically than steroidogenically by as much as 4 orders of magnitude. Another small fragment α-MSH also exhibited 4-fold greater glycolytic than steroidogenic potency. It appears that the presence of the basic tetrapeptide (LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}) is more critical for steroidogenesis than for glycolysis. Hence it may be postulated that stimulation of aerobic glycolysis has different affinity requirements

TABLE 2. Summary

EFFECT OF SHORTENING FROM C-TERMINAL

		Relative Potency (%)	
		LA	STD
(1-24)	S Y S M E H F R W G K P V G K K R R P V K V Y P	100	100
(1-18)NH_2	S Y S M E H F R W G K P V G K K R R	99	50
(1-17)	S Y S M E H F R W G K P V G K K R	0.2	0.16
(1-16)	S Y S M E H F R W G K P V G K K	$1x10^{-3}$	$0.4x10^{-3}$
(1-14)	S Y S M E H F R W G K P V G	$0.5x10^{-3}$	$0.2x10^{-7}$
AC(1-13)	NH_2 S Y S M G H F R W G K P V	$40x10^{-3}$	$9x10^{-3}$

KEY: S= SER, Y= TYR, M= MET, E= GLU, H= HIS, F= PHE, R= ARG, W= TRP, G= GLY, K= LYS, P= PRO, V= VAL.

than that of steroidogenic activity.

Summary

1. With the loss of the basic tetrapeptide core (15-18) glycolytic and steroidogenic potencies were lowered, but Rmax remained virtually the same suggesting that there was loss of affinity but no loss of capacity to excite the receptor. Since the fragments were capable of eliciting the same Rmax as ACTH (1-24), they could be considered full agonists in the system.

2. The C-terminal hexapeptide (19-24) sequence was more important for steroidogenesis than for glycolysis. Thus, the data have shown that almost half of the peptide chain of ACTH (1-24) can be eliminated from the carboxyl end without loss of intrinsic glycolytic as well as steroidogenic activities (the ability to elicit Rmax). It was thus expected that the minimum essential structure for both glycolytic and steroidogenic activity was present in the first 14 residues of the N-terminal region of ACTH (1-24).

The next series of studies therefore examined the N-terminal amino-acid residues in an attempt to delineate a message sequence for glycolysis.

STUDY 9b

The effect of shortening from the N-terminal end

The N-terminal half of ACTH (1-24) is made up of SER^{1}-TYR^{2}-SER^{3}-MET^{4}-GLU^{5}-HIS^{6}-PHE^{7}-ARG^{8}-TRP^{9}-GLY^{10}-LYS^{11}-PRO^{12}-VAL^{13}.

As discussed in Chapter 3 the first 3 residues SER-TYR-SER have been shown to potentiate the steroidogenic activity in rat adrenocortical cells.

The importance of these potentiating residues in the stimulation of aerobic glycolysis was assessed by comparing ACTH (1-18) NH_2 with ACTH (3-18) NH_2. In the same experiment, ACTH (7-38) and ACTH (11-24) were also tested.

The 4 fragments elicited glycolytic and steroidogenic responses as presented on Fig. (16). The maximum rate of aerobic glycolysis elicited by ACTH (3-18) NH_2 was the same as that induced by ACTH (1-18) NH_2. However, the curve for ACTH (3-18) NH_2 was displaced to the right, indicating a higher D_{50} and hence a smaller affinity.

Further shortening of ACTH from the amino terminal to the 7th residue and beyond, led to complete loss of glycolytic activity. In fact, the peptides ACTH (7-38) and ACTH (11-24) suppressed lactate production to below basal level.

Similar results were obtained for steroidogenesis. The relative potencies were computed as presented below:

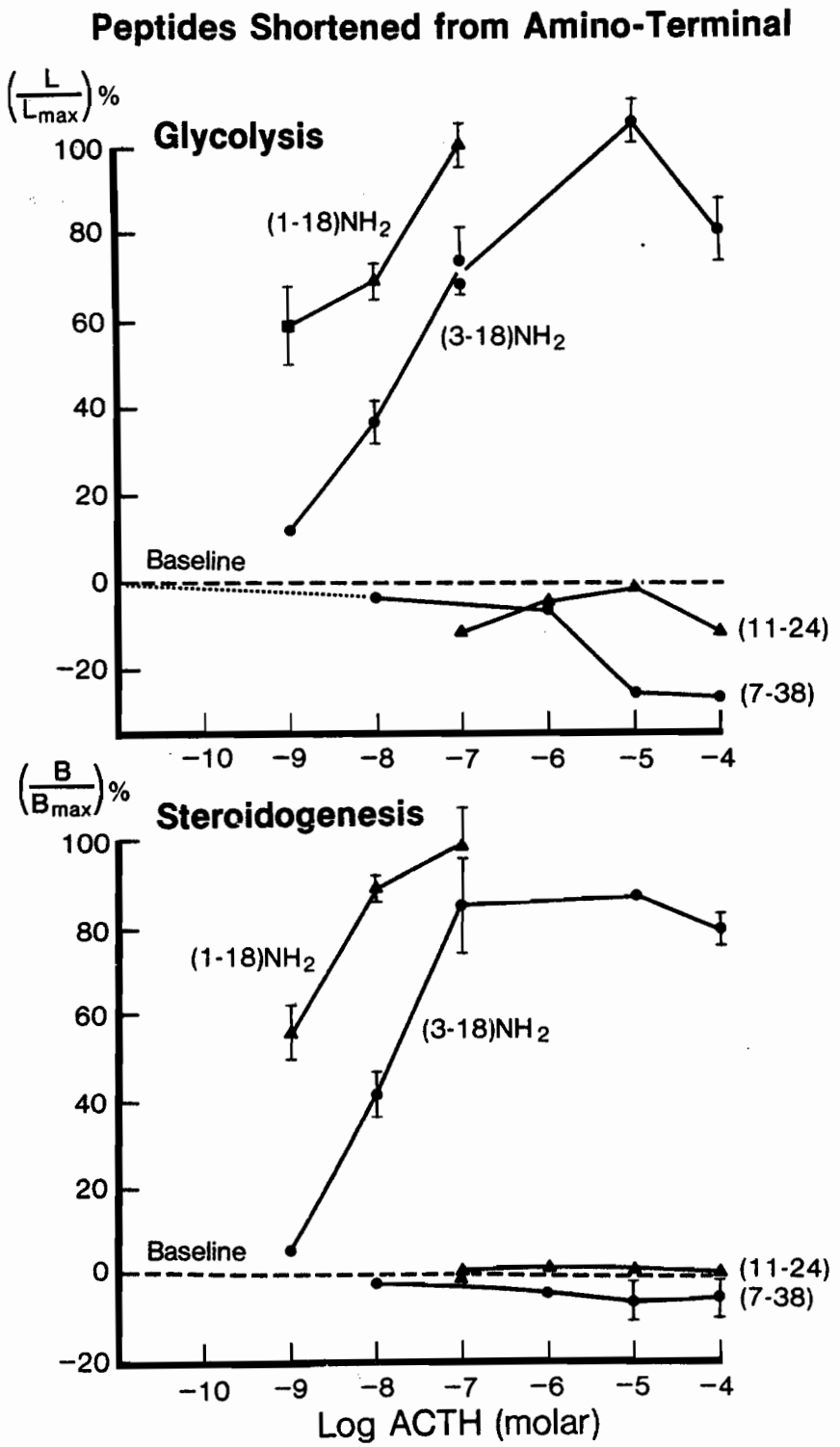


Figure 16

STUDY 9b: Effect of shortening from the N-terminus

(See GENERAL LEGEND page 120)

	Relative potency (%)	
	Glycolysis	Steroidogenesis
ACTH (1-18) NH_2	100.0	100.0
ACTH (3-18) NH_2	4.5	6.0

Summary

1. The first two N-terminal residues, SER^1-TYR^2, were not essential for the stimulation of glycolysis and steroidogenesis but their absence led to about 15-fold lowering of relative biological potency.

2. Fragments (7-38) and (11-24) were completely inactive and antagonized basal lactic acid production.

The possibility existed that ACTH (7-38) retained an affinity for the receptor yet failed to activate the receptor.

To test this, ACTH (7-38) was combined with ACTH (1-24) in the next study.

STUDY 9c

Inhibition studies with ACTH (7-38)

ACTH (7-38) is also referred to as Corticotropin Inhibiting Peptide. Its presence in the human pituitary was reported by Li et al. (319). The fragment carries the basic binding sequence (15-18) considered to be the address segment for steroidogenesis. It was therefore of interest to determine whether the fragment could influence binding of ACTH (1-24) to receptors associated with the stimulation of aerobic glycolysis.

The present study examined the inhibitory potential of ACTH (7-38).

As demonstrated in Fig. 17 ACTH (1-24) was added to isolated adrenocortical cells either alone or in combination with ACTH (7-38). Increasing concentrations of ACTH (7-38) from 10^{-8} to 10^{-6}M decreased rate of lactic acid and steroid production induced by 10^{-10}M ACTH (1-24). This inhibition was overcome by increasing the concentration of ACTH (1-24) from 10^{-10}M to 10^{-8}M.

Thus ACTH (7-38) fulfilled the three criteria for a competitive antagonism:

(1) The fragment did not exhibit any capacity to stimulate lactate or steroid production.

(2) ACTH (7-38) inhibited in a dose-dependent manner the action of ACTH (1-24).

(3) This inhibition was reversed by increasing doses of ACTH (1-24). It could be postulated that ACTH (7-38) could bind to receptors involved in both aerobic glycolysis and steroidogenesis.

So far, it has been evident that the first 13 residues of the

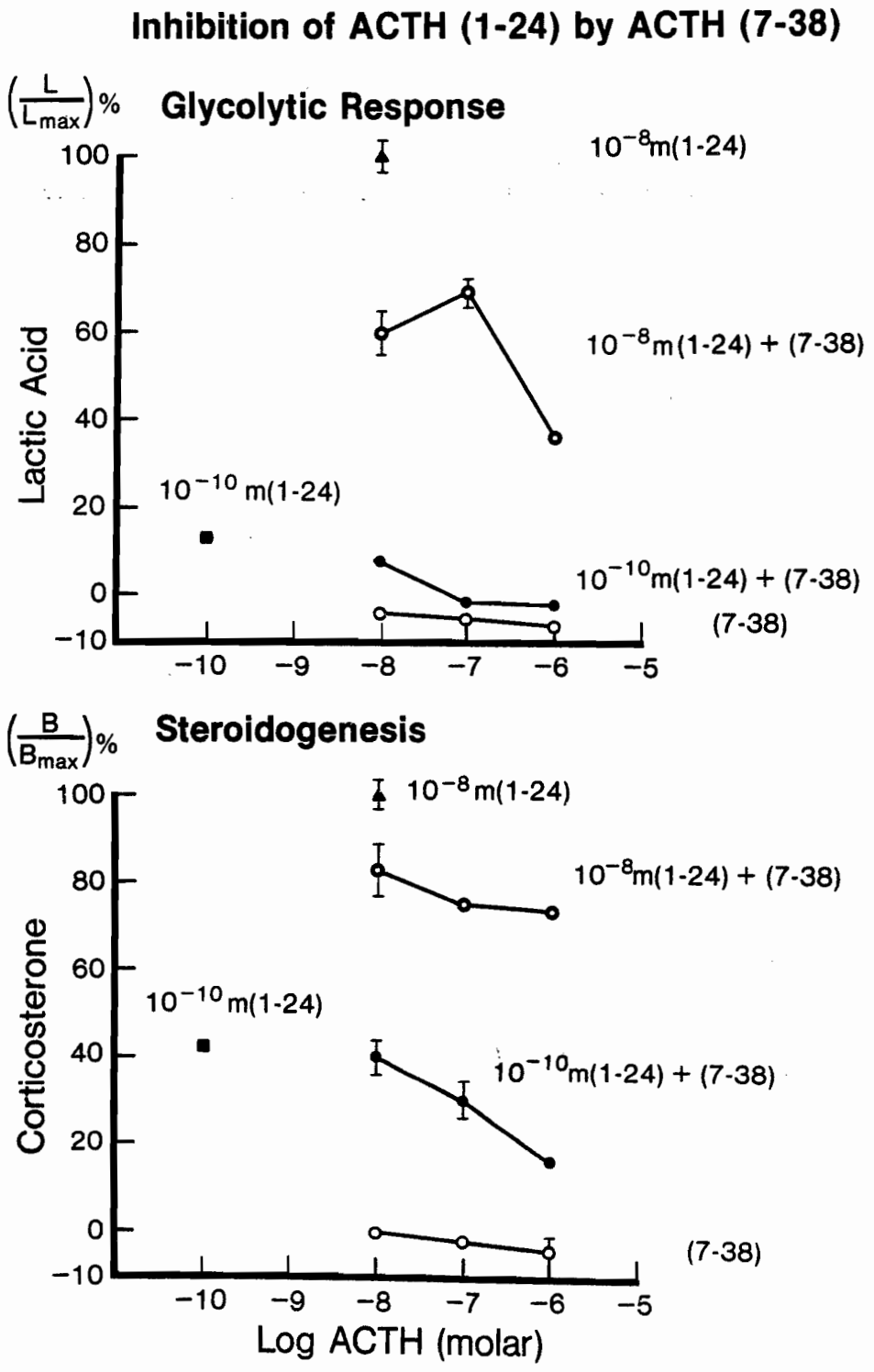


Figure 17

STUDY 9c: Inhibition studies with ACTH (7-38).

ACTH (1-24) and/or ACTH (7-38) were added to cells in various combinations of concentrations, as indicated.

(See GENERAL LEGEND page 120)

amino-terminal portion of ACTH contain crucial information for exciting receptors implicated in glycolytic and steroidogenic activities.

It has also been evident that in the absence of the basic address sequence (15-18) the potencies of ACTH fragments are very much reduced. However, at high enough concentrations, maximal responses identical to those by ACTH (1-24) can be elicited by short ACTH fragments.

Such a high concentration has been employed in the next study to examine short N-terminal ACTH fragments lacking the binding address sequence.

STUDY 9d

Effect of short N-terminal fragments

Eight short fragments were tested at high, 1mM, concentration in order to determine the minimum sequence required for inducing aerobic glycolysis and steroidogenesis in adrenocortical cells.

The peptides investigated were:
ACTH: (1-14), (4-10), (4-11), (5-10), (1-10), (7-13), (1-13), and (11-24). ACTH (1-24) was included as reference.

The absolute activities are displayed in the histogram in Fig. 18.

ACTH fragments (1-4), (5-10), and (11-24) did not elicit any glycolytic or steroidogenic activity. The remaining fragments including ACTH (4-10) were active and induced glycolysis and steroidogenesis to nearly the same maximal extent as ACTH (1-24).

It was rather surprising to find that ACTH (7-13) was active since it had already been shown that ACTH (7-38) was inhibitory, and furthermore, ACTH (5-10) was inactive.

The relation of primary sequence to relative activities is presented in Table 3.

Of the active fragments, ACTH (1-13) showed the greatest relative activities. This is hardly surprising since it was the longest N-terminal fragment of the 8 peptides studied.

Of significance were the following key findings:

(1) ACTH (1-10) was much less effective at stimulating glycolysis (65%) than steroidogenesis (86%).

(2) ACTH (1-10) elicited glycolysis to a lesser extent (65%) than glycolysis induced by ACTH (4-11) (83%). (The situation was opposite in

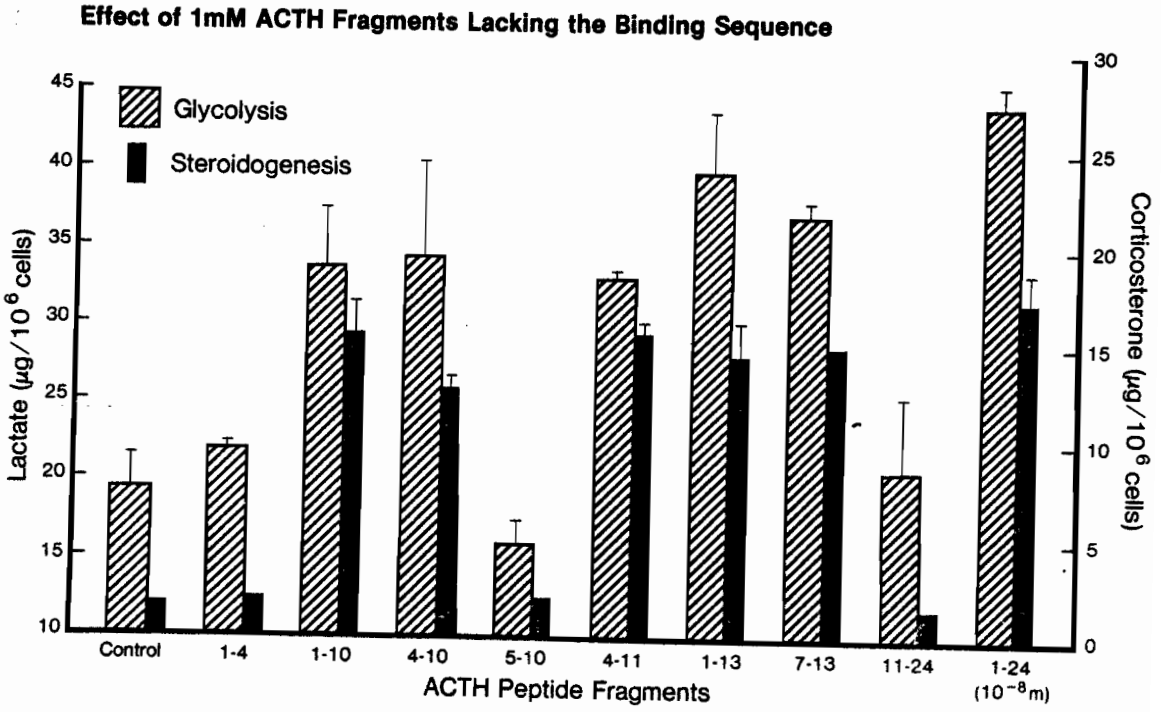


Figure 18

STUDY 9d: Effect of short N-terminal fragments

Histogram rectangles represent absolute values. Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each triplicate.

Lactic acid and corticoterone were measured as described in the Methods section (Chapter 7).

TABLE 3. Effect of short N-terminal fragments

RELATIVE ACTIVITIES at 1 mM

Glycolysis %	Steroid %		
94	82	(1-13)	S Y S M E H F R W G K P V
65	86	(1-10)	S Y S M E H F R W G
9	2	(1-4)	S Y S M
83	74	(4-11)	M E H F R W G K
72	67	(4-10)	M E H F R W G
0	0	(5-10)	E H F R W G
87	58	(7-13)	F R W G K P V
5	2	(11-24)	K P V G K K R R P V K V Y P
100	100	(1-24)	S Y S M E H F R W G K P V G K K R R P V K V Y P

KEY: S= SER, Y= TYR, M= MET, E= GLU, H= HIS, F= PHE, R= ARG, W= TRP, G= GLY, K= LYS, P= PRO, V= VAL.

the case of steroidogenesis: 86% by (1-10) and 74% by (4-11)).

(3) ACTH (7-13) was more effective at inducing glycolysis (87%) than steroidogenesis (58%).

(4) ACTH (7-13) stimulated glycolysis to almost the same extent (87%) as ACTH (1-13) (94%). Yet the steroidogenic activity induced by ACTH (7-13) was only 58%, compared to 82% induced by ACTH (1-13).

These observations can best be explained by postulating that:

(1) The sequence LYS^{11}-PRO^{12}-VAL^{13} was much more essential for aerobic glycolysis than for steroidogenesis (particularly the LYS^{11} residue). However in the absence of the hexapeptide NH-SER^{1}-TYR^{2}-SER^{3}-MET^{4}-GLU^{5}-HIS^{6}, the biological activity contributed by LYS^{11}-PRO^{12}-VAL^{13} is abolished by the presence of the binding address. Thus (7-13) is an agonist whereas (7-38) peptide is an inhibitor.

(2) The steroidogenic response depended more on residues closer to the N-terminus, e.g., SER^{1}-TYR^{2}-SER^{3}-MET^{4}-GLU^{5}-HIS^{6}-PHE^{7}-TRP^{8}. (Thus steroid production by ACTH (7-13) was much lower (58%) than ACTH (1-10) induced steroidogenesis (86%).

(3) In the absence of the binding address sequence LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}, methionine in position 4 was necessary for full steroidogenic potency (thus ACTH (4-10),(4-11),(7-13) are all less active than (1-10) or (1-13)).

(4) In the absence of LYS^{11}-PRO^{12}-VAL^{13} the methionine residue in position 4 is essential for both steroidogenesis and glycolysis. (Thus ACTH (4-10) was active but ACTH (5-10) completely lacked activity).

To further assess the importance of the position 4 methionine, as well as the role of LYS^{11}-PRO^{12}-VAL^{13}, the fragments, ACTH (5-14) and ACTH (1-14) were employed in the next study.

STUDY 9e

ACTH (5-14) vs ACTH (1-14)

ACTH (5-14) which has the sequence GLU^{5}-HIS^{6}-PHE^{7}-ARG^{8}-TRP^{9}-GLY^{10}-LYS^{11}-PRO^{12}-VAL^{13}-GLY^{14} lacks the N-terminal potentiator sequence NH-SER^{1}-TYR^{2}-SER^{3} as well as methionine in position 4. In this study, ACTH (5-14) was compared to ACTH (1-14) as shown in Fig. 19.

For interpretation, a different plot was used Fig. 20, based on a linear transformation of the log-dose-response equation. The transformation was as follows:

Linear Transformation of Dose-Response Equation

For a dose (D) and biological response, R, the equation for a rectangular hyperbola

$$\frac{R}{Rmax} = \frac{(D)}{(D) + (D_{50})} \qquad (1)$$

can be rearranged as follows:

$$\frac{(D)}{R} = \frac{(D) + (D_{50})}{Rmax} \qquad (2)$$

or

$$\frac{(D)}{R} = \frac{(D)}{Rmax} + \frac{(D_{50})}{Rmax} \qquad (3)$$

i.e., $$\frac{(D)}{R} = \frac{1}{Rmax}(D) + \frac{(D_{50})}{Rmax}$$

Therefore, if $\frac{(D)}{R}$ is plotted against (D), then $\frac{1}{Rmax}$ = slope

and $\frac{(D_{50})}{Rmax}$ = y-intercept.

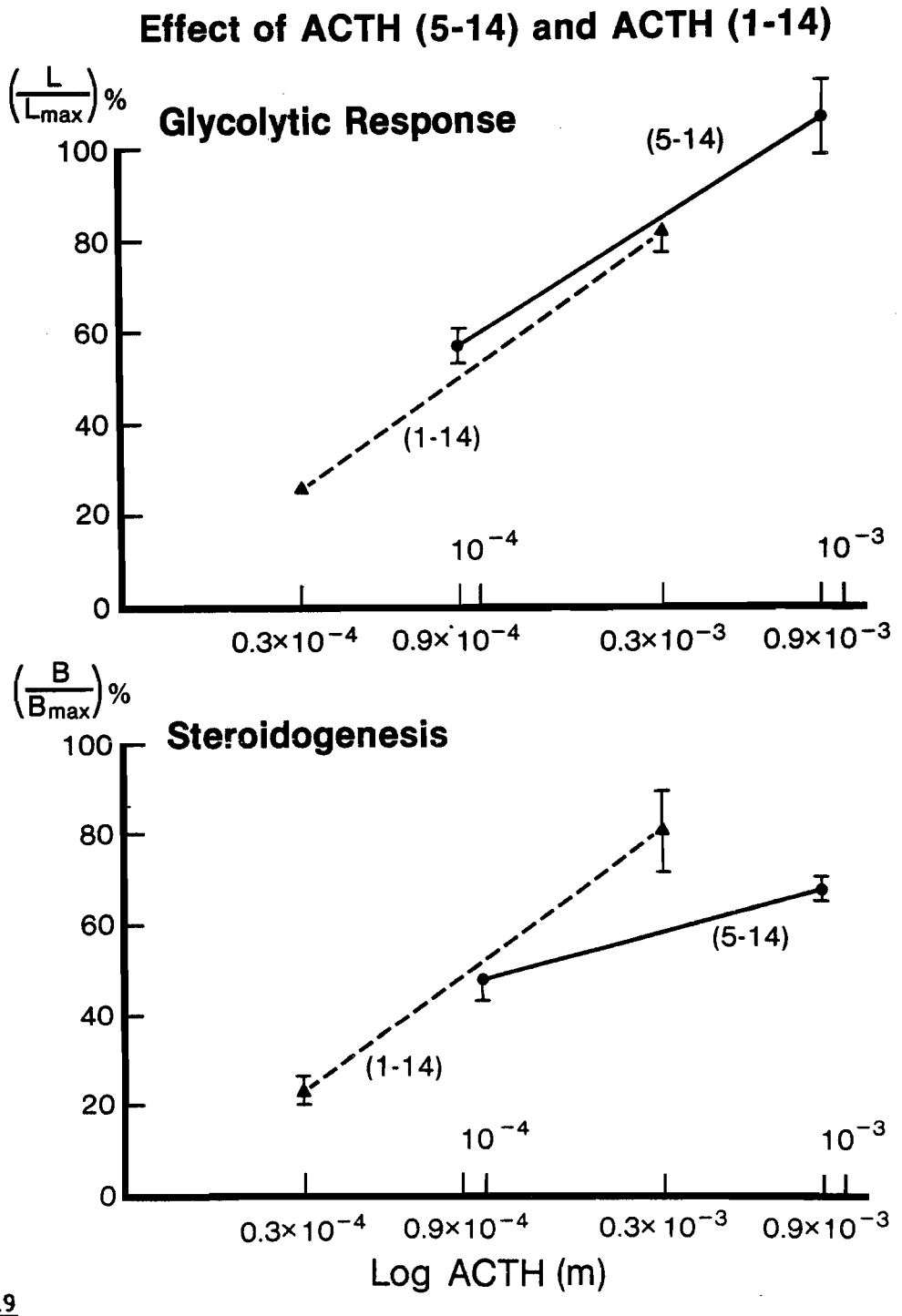


Figure 19

ACTH (5-14) vs ACTH (1-14)

Concentrations of peptides utilized were:

0.9×10^{-4}M, and 0.9×10^{-3}M ACTH (5-14);

0.3×10^{-4}M, and 0.3×10^{-3}M ACTH (1-14)

ACTH (1-24) was tested at 10^{-10}, 10^{-9}, and 10^{-8}M.

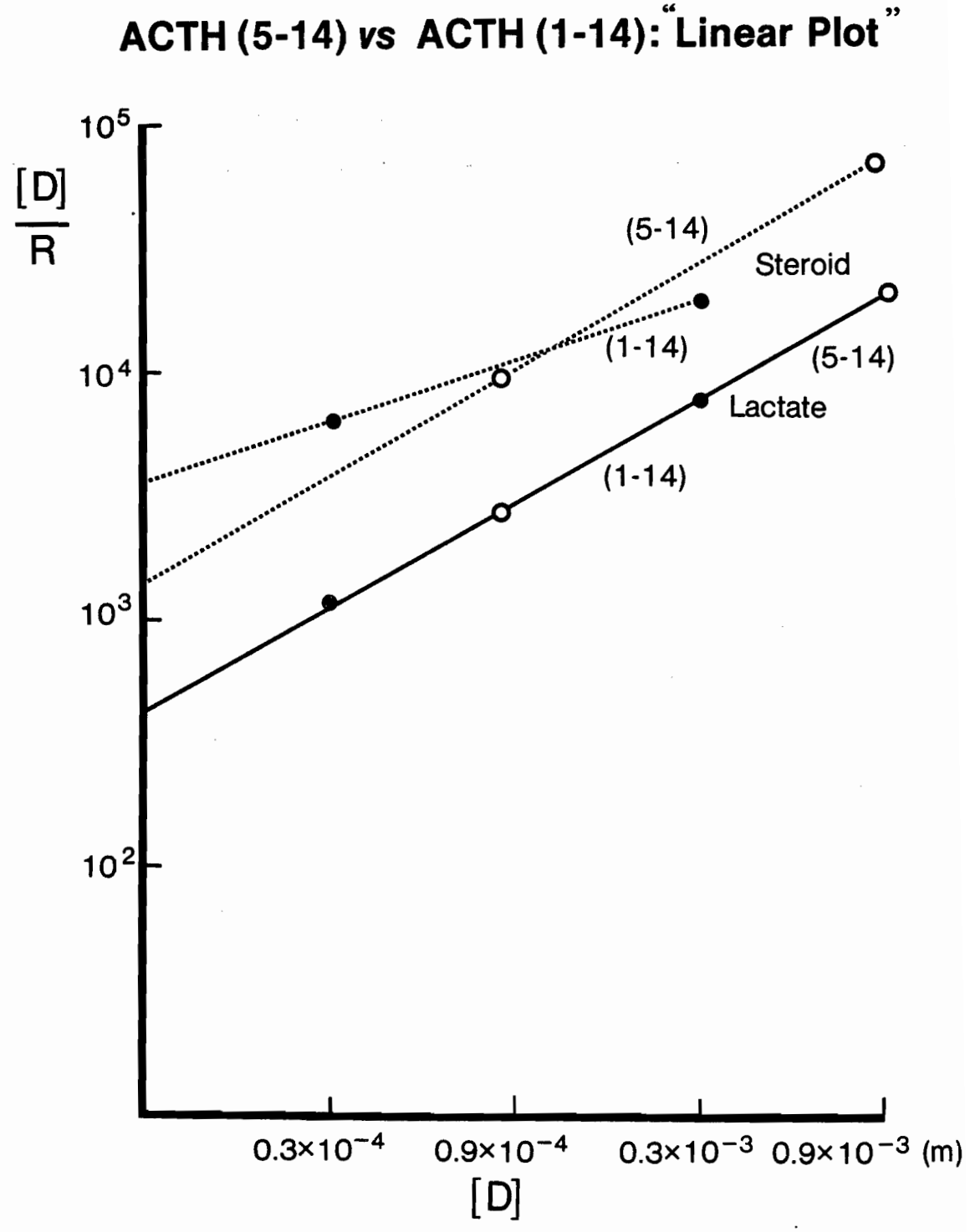

ACTH (5-14) vs ACTH (1-14): "Linear Plot"
10^5
[D]
R
10^4
10^3
10^2
(5-14)
Steroid
(1-14)
(5-14)
Lactate
(1-14)
0.3×10^{-4}
0.9×10^{-4}
0.3×10^{-3}
0.9×10^{-3} (m)
[D]

FIG 20

Hence, the greater the slope, the lesser the Rmax. In Fig. 20, "Linear Plot" actually refers to a plot of a linearly-transformed equation of the type $y = mx + c$ (where m = slope and c = intercept).

Such a transformation enables the estimation of Rmax and D_{50}, for in this experiment not enough points were available for computer estimation of Rmax and D_{50}.

Fig. 20 shows that for lactate production, ACTH (1-14) and ACTH (5-14) exhibit identical slopes and y-intercept, meaning, the two peptides have identical glycolytic potencies and maximal activities.

In contrast, different patterns of steroidogenic action are elicited by ACTH (1-14) and ACTH (5-14). ACTH (1-14) has the smaller slope and therefore greater Rmax. ACTH (5-14) has smaller D_{50} and therefore greater potency, but smaller Rmax than ACTH (1-14). This would suggest ACTH (5-14) had greater affinity for the steroidogenic receptor but less capability to excite the receptor. Thus, methionine at position 4, might be essential for the activation of the steroidogenic receptor but non-essential for the stimulation of glycolysis. The reason for the seemingly higher potency af ACTH (5-14) is not apparent.

In a study with rat adrenocortical cells, Seely et al. (320) found that ACTH (5-24) elicited the same maximal rate of steroidogenesis as ACTH (1-39) and the fragment was considered a full agonist. In the absence of the basic sequence (15-18), it appears that different types of receptors are activated, as by ACTH (5-14), which in the present study was found to be a partial activator of the steroidogenic receptor, (compared to ACTH (1-14)), but exhibited the same ability as ACTH (1-14) to stimulate glycolysis.

The fact that ACTH (5-24) has been found to be a full agonist for steroidogenesis (320) would seem to support the earlier postulate that in the absence of the binding address sequence (as is the case with ACTH (5-14)), the presence of methionine in position 4 is necessary for full intrinsic steroidogenic activity. Thus ACTH (5-14) was only a partial agonist for steroidogenesis. However, ACTH (5-14) was a full agonist for the stimulation of aerobic glycolysis. This also agrees with the current proposal that in the presence of LYS^{11}-PRO^{12}-VAL^{13}, methionine in position 4 is not important for the stimulation of aerobic glycolysis.

This concludes the description of the studies done with ACTH fragments.

STUDIES WITH ACTH FRAGMENTS

OVERALL SUMMARY

(1) Shortening of ACTH (1-24) from the carboxy-terminal end greatly reduces affinity when the binding address sequence (15-18) is removed, yet is without any effect on the intrinsic ability to stimulate maximally glycolysis or steroidogenesis.

(2) Elimination of the first 2 residues from the amino-end also lowers affinity without affecting intrinsic activities. Removal of methionine at position 4 reduces intrinsic steroidogenic activity without any effect on maximal stimulation of glycolysis. Removal of amino terminal residues from ACTH (1-24) beyond the first 6 residues completely removes biological activity. ACTH (7-38) can competitively inhibit ACTH (1-24) in the stimulation of glycolysis and steroidogenesis.

(3) In the absence of the binding address sequence (15-18) full steroidogenic potency requires the presence of methionine at position 4. MET^{4} is not essential for maximal glycolytic potency if LYS^{11}-PRO^{12}-VAL^{13} is present (even in the absence of the binding address sequence).

CONCLUSIONS

1. Both aerobic glycolysis and steroidogenesis

(a) The address sequence LYS-LYS-ARG-ARG is required for maximal affinity.

(b) Messages are located near N-terminal.

(c) The fragment GLU-HIS-PHE-ARG-TRP-GLY by itself, is inactive.

(d) The 2 minimum sequences for both activities are

MET^4-GLU^5-HIS^6-PHE^7-ARG^8-TRP^9-GLY^{10}, and
PHE^7-ARG^8-TRP^9-GLY^{10}-LYS^{11}-PRO^{12}-VAL^{13}.

2. Aerobic glycolysis

(a) Potentiated by the sequence LYS^{11}-PRO^{12}-VAL^{13}.

(b) In the absence of MET^4-GLU^5-HIS^6, the binding site LYS^{15}-LYS^{16}-$ARG^{17}ARG^{18}$ exerts negative influence (negative address).

(c) In the presence of LYS^{11}-PRO^{12}-VAL^{13}-, MET^4-GLU^5-HIS^6 is not essential for activity.

(d) The essential region for glycolytic action is: GLU^5-HIS^6-PHE^7-ARG^8-TRP^9-LYS^{11}-PRO^{12}-VAL^{13}.

3. Steroidogenesis

(a) Potentiated by the sequence SER^1-TYR^2-SER^3-MET^4-GLU^5-HIS^6.

(b) Presence of methionine residue always important.

(c) Essential region for activity is: SER^1-TYR^2-SER^3-MET^4-GLU^5-HIS^6-PHE^7-ARG^8-TRP^9.

The next series of studies focussed on the individual amino acid residues in the 4-10 region of ACTH. ACTH analogs were utilized to investigate the effects of structural modification of critical amino acid residues.

CHAPTER 10.

Studies with ACTH-analogs

(Effect of structural alterations in the 4-10 region)

INTRODUCTION

The high affinity of peptides for their receptors is contributed by the sum of the individual affinities of the amino-acid side chains. These side-chains are capable of interacting with the hormonal receptors in 4 ways:

(1) ionic interactions (between positive and negative charges)

(2) hydrophobic bonds (between lipid-like side chains)

(3) aromatic-aromatic interactions (stacking of aromatic rings in peptide hormones with similar ones in the receptor proteins), and

(4) hydrogen-bond formation (between carbonyl group oxygen and amine or hydroxyl groups).

The use of synthetic analogs, in which a particular residue is replaced or modified, provides a means of investigating the role of a particular amino-acid side chain. This approach was adopted in evaluating the roles of MET^4, PHE^7, ARG^8, TRP^9, and GLY^{10}, in ACTH-induced glycolysis and steroidogenesis. (GLU^5 and HIS^6 have not been specifically investigated in the present study because of unavailability of suitable analogs).

STUDY 10a

Effect of amino-acid substitution at positions 4 (MET) and 7 (PHE)

Two α-MSH analogs were utilized in this study, as well as the unmodified hormone.

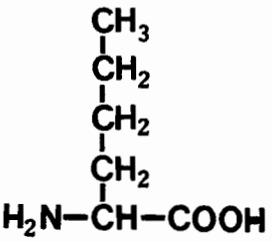
In one analog, methionine residue in position 4 was replaced by norleucine. The other analog had D-Phenylalanine instead of the normal L-amino acid, at position 7. The abilities of these analogs to stimulate glycolysis and steroidogenesis were determined. Because of the low potency of α-MSH, higher concentrations (10^{-6},10^{-5}, and 10^{-4}M) of peptide were employed. ACTH (1-24) was included as reference standard.

Fig. 21 and Table 4 summarize the relative activities of the peptides. The values presented in Table 4 reveal the following:

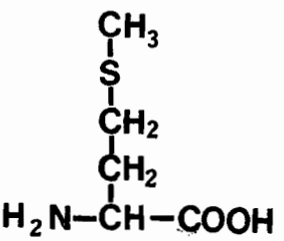
(1) α(Nle^4, D-Phe^7) MSH showed only slight glycolytic activity, and virtually no steroidogenic activity.

(2) α(Nle^4) MSH was the most potent of the 3 melanotropic peptides. Its effect was more pronounced for steroidogenesis than for glycolysis.

The above observations can be explained by considering the following structures:



NORLEUCINE



METHIONINE

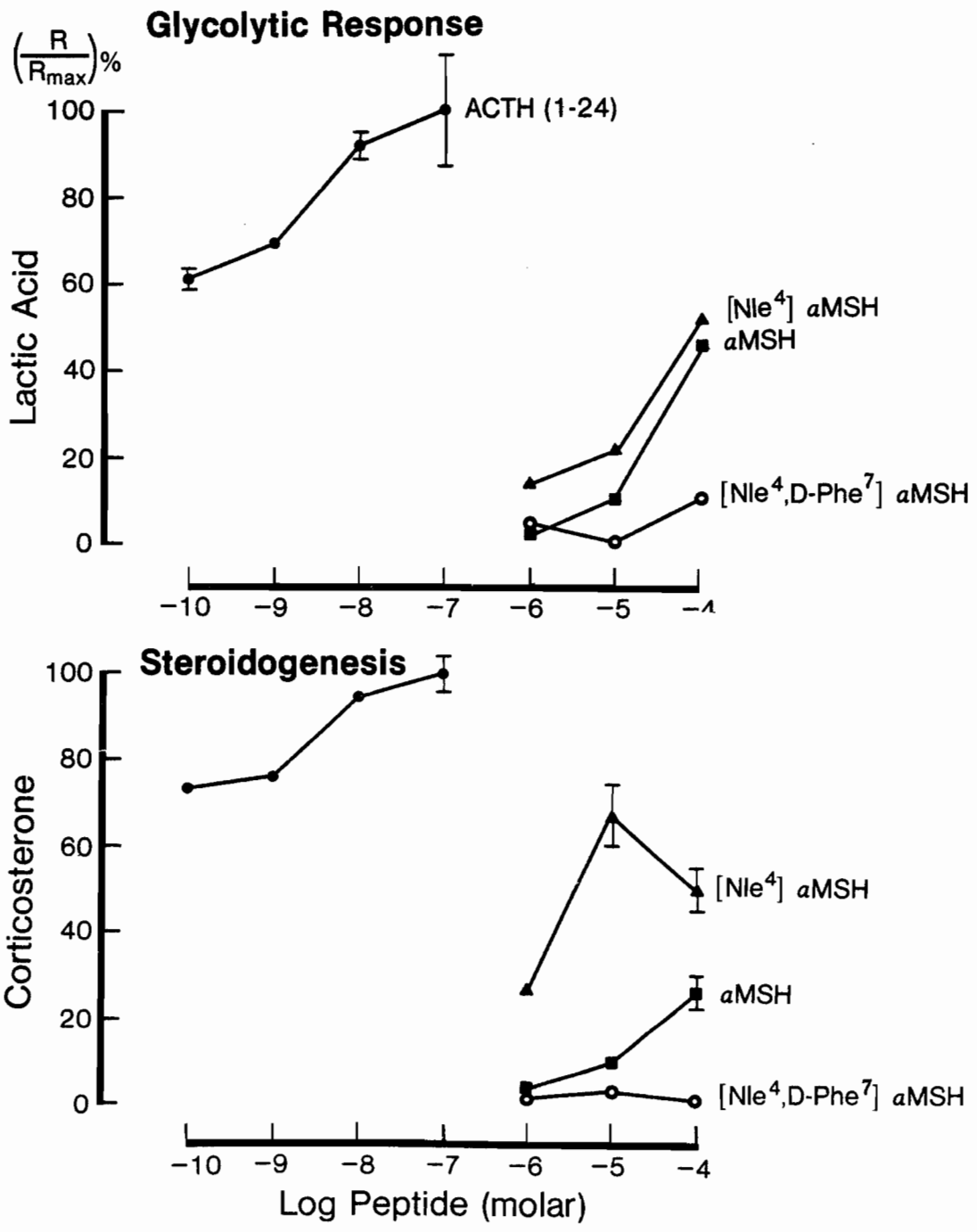


Figure 21

STUDY 10a: Effect of amino-acid substitutions at positions 4 (MET) and 7 (PHE)

αMSH analogs were tested at 10^{-6}, 10^{-5}, and 10^{-4}M

(See GENERAL LEGEND page 130)

TABLE 4.

RELATIVE ACTIVITIES (%)

	Aerobic glycolysis			Steroidogenesis		
	10^{-6}M	10^{-5}M	10^{-4}M	10^{-6}M	10^{-5}M	10^{-4}M
[Nle^4]αMSH	14	21	52	26	67	50
[Nle^4, D-Phe^7]αMSH	5	0	11	1	2	0
αMSH	4	10	46	2	9	26

The side chain of methionine is known to be a potent hydrophobic binding site in many biological systems involving specific peptide-protein interactions (321). Replacement of the thioester linkage present in methionine by a methylene group as in norleucine would be expected to lead to greater hydrophobicity. The results indicate that the hydrophobicity of methionine at position 4 was essential for activity, especially for steroidogenesis. The fact that (Nle^4, $D\text{-}Phe^7$)αMSH showed virtually no activity suggests that the L-configuration is essential for activity. Introducing a D-stereoisomer would be expected to disrupt the α-helical nature of the region. Thus the ability to form an α-helix can be postulated to be essential for the proper orientation of the side-chains in the interaction with specific receptors.

Similar studies have been conducted by Sawyer et al. (322) on amphibian melanophores. They showed that (Nle^4) αMSH was more potent than α-MSH in stimulating melanosome dispersion. However, in their studies, the analog (Nle^4, $D\text{-}Phe^7$) α-MSH was found to be 26-times more potent than α-MSH in melanotropic activity (323).

STUDY 10b

Effect of substitutions in the 8th and 9th positions

In the ACTH molecule the 8th amino-acid from the N-terminal is Arginine and the 9th position is occupied by Tryptophan.

The roles of the guanidino side chain at position 8 and the indole group at position 9 of ACTH were investigated employing 3 analogs:

Analog I: ALA at position 9

II: PHE at position 9

III: LYS at position 8 and PHE at the 9th.

The results are displayed in Fig. 22.

Substitition at position 9

As the figure shows, replacement of TRP^9 with an aliphatic amino-acid such as Alanine results in drastic loss of activity the loss being more pronounced for the steroidogenic activity.

Replacement of TRP^9 by PHE^9 leads to intermediate activity for both lactate and steroid production.

The order of activity for position 9 residues can be summarized as $TRP^9 \rangle PHE^9 \rangle ALA^9$.

Thus both the glycolytic and steroidogenic potencies were proportional to the aromaticity and hydrophobicity of the side chain at position 9. Tryptophan and Phenylalanine differ in that the indole group of tryptophan is capable of forming H-bonds through the indolyl-NH group. The lower activity of PHE^9 substitution could be related to this loss of H-bonding potential. Furthermore, the indole group is much more efficient as a charge donor than the benzene side chain of

phenylalanine. In fact, the primary importance of the electron-rich tryptophanyl residue in biologically active molecues has been attributed to its role as an electron donor. Thus in certain biologically active compounds the modification of TRP residues with substituents that tend to deactivate the aromatic indole nucleus by reducing its electron density results in a reduction in biological activity (324,325,326,327).

The PHE side chain is known to be poorly electron-donating (325). Whereas the hydrophobicity of residues in position 9 might be important for receptor binding the electron-donor property could be implicated in the excitation of the receptor. Thus, Hofmann et al. (328) found that analogs of ACTH in which TRP is replaced by PHE are almost devoid of biological activity and yet exhibit high affinity for ACTH receptors.

Substitution at position 8

Replacement of ARG^8 by Lysine (with PHE at position 9) led to striking differences in response between glycolysis and steroidogenesis.

As Fig. 22 indicates, the analog (LYS^8, PHE^9) ACTH was glycolytically more active than even (ARG^8, PHE^9) ACTH, and yet steroidogenic activity was very low for (LYS^8 PHE^9). This consitutes a marked dissociation between the two responses and might suggest that different regions of ACTH are involved.

As discussed in Chapter 3, the guanidino side-chain of Arginine is unique in the sense that it can form multiple H-bonds at the same time, a process that is considered to be "recognition" (in analogy to DNA base-base interactions).

It can therefore be postulated that the guanidyl group at position

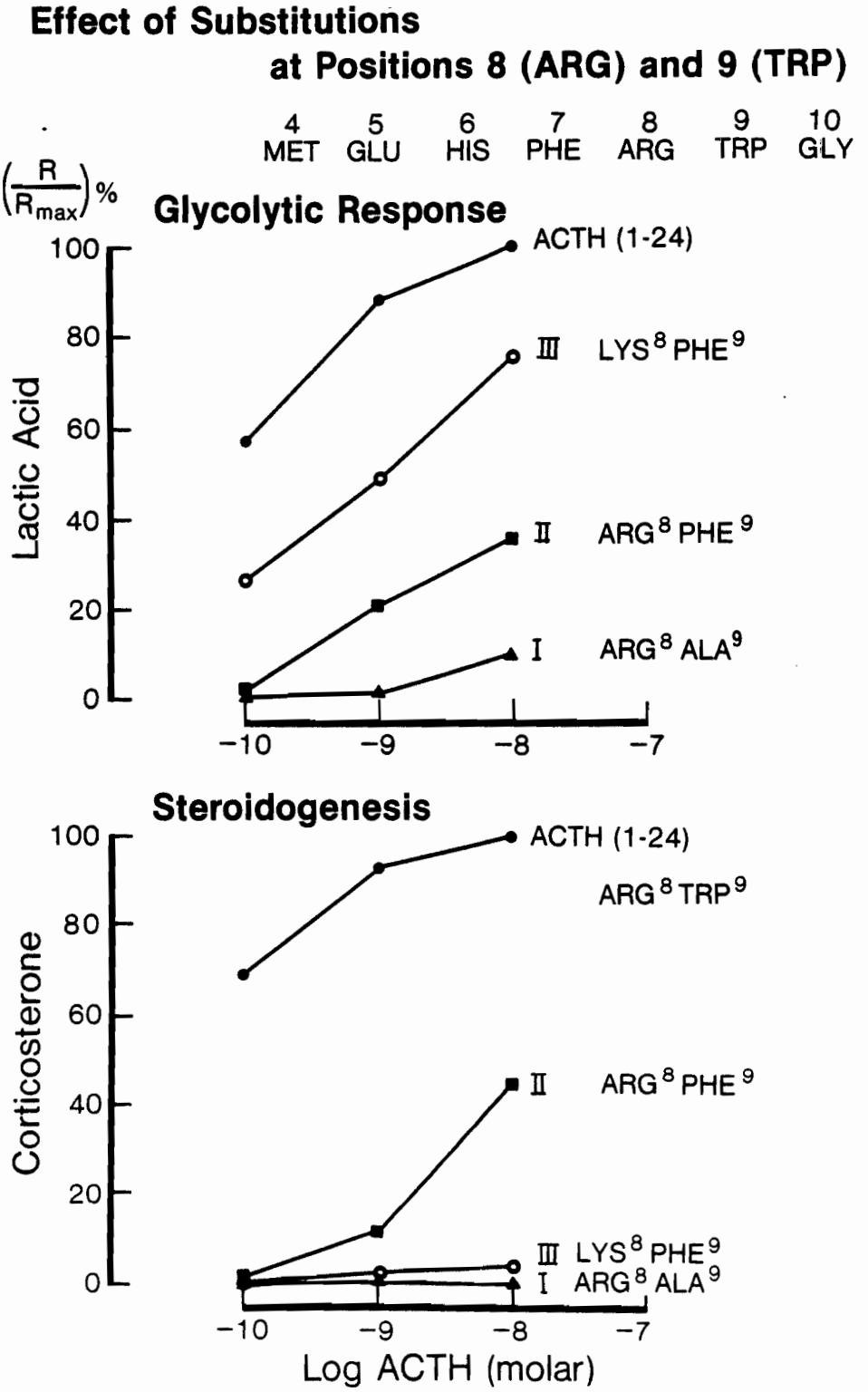


Figure 22

STUDY 10b: Effect of substitutions at the 8th and 9th positions.

8 might be implicated in the steroidogenic receptor activation but might not be essential for the stimulation of receptors involved in ACTH-induced glycolysis. The results also suggest that the basicity of the residue at position 8 was the important factor for the stimulation of glycolysis whereas for steroidogenesis the characteristic features of the guanidino group were more important.

The results are consistent with the findings from previous studies (steroidogenic action of ACTH) by Finn et al. (329). They studied the behavior of bovine and rat adrenocortical cells towards three analogs the same as those used in this study. They too found that half-maximal rates for steroidogenesis decreased in the order TRP $\rangle$ PHE $\rangle$ ALA.

In summary, the indole ring at position 9 was essential for the stimulation of both aerobic glycolysis and steroidogenesis whereas the guanidyl side chain of Arginine at position 8 was necessary for the steroidogenic action of ACTH. Steroidogenesis was inhibited by replacing ARG^{8} with Lysine, whereas stimulation of glycolysis increased with the same amino acid replacement.

The next and final study in this series with analogs focussed on the 10th position.

STUDY 10c

Effect of substitutions in position 10

Glycine occupies the 10th position (from the amino end) of ACTH, and is considered to be important for the adoption of the biologically-active conformation of the ACTH molecule. This is postulated to be due to the fact that glycine allows free rotation along the peptide bond and due to the location of glycine, joining the N-terminal segment of messages (1-9) with the binding address segment (11-18). Furthermore GLY^{10} connects two message sequences: MET^{4}-GLU^{5}-HIS^{6}-PHE^{7}-ARG^{8}-TRP^{9} and LYS^{11}-PRO^{12}-VAL^{13} (see Chapter 3). Position 10 is therefore considered to be a conformationally and functionally-sensitive locus. GLY^{10} always appears with the highly conserved hexapeptide sequence ($ACTH^{4-9}$) as found in α , β, and γ-MSH, as well as in ACTH.

It was of interest to determine how glycolytic and steroidogenic potencies would be influenced by imposing steric restrictions at position 10, to ascertain whether steroidogenesis would be affected as much as glycolysis, and to establish whether structural modifications at position 10 influence the ability of LYS^{11}-PRO^{12}-VAL^{13} to contribute to the stimulation of glycolysis.

Studies were therefore conducted on a series of analogs of ACTH (1-18) with various substitutions at position 10.

The four analogs of ACTH (1-18) investigated had the following residues in place of L-glycine at position 10:

(1) L-Ala

(2) D-Ala

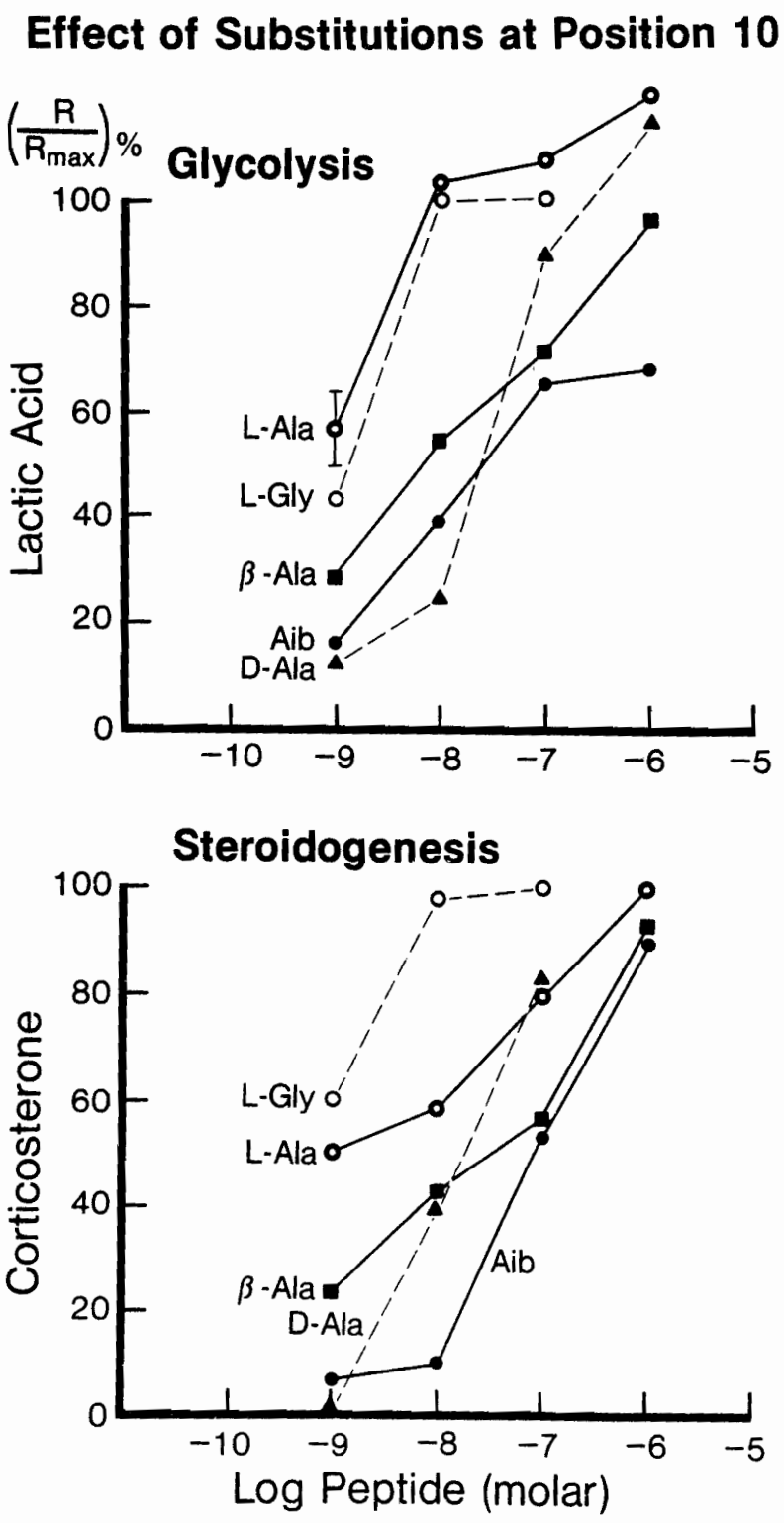
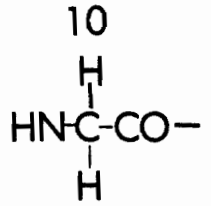
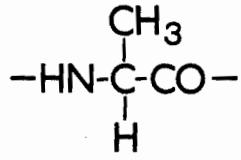
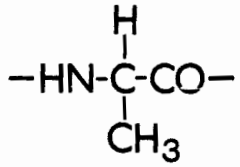
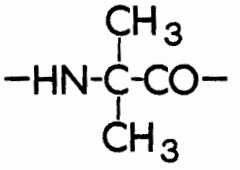
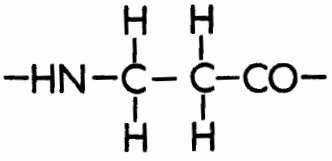


Figure 23

STUDY 10c: Effect of substitions at position 10.

(See GENERAL LEGEND page 120)

TABLE 5

	Relative Potencies (%)	
	Lactate	Steroid
1 2 3 4 5 6 7 8 9 10 S-Y-S-M-E-H-F-R-W- 	100	100
L-ALA 	**100**	75
D-ALA 	5	17
a AIB 	10	2
β-ALA 	34	21

(3) β-Ala

(4) α- amino isobutyric acid (AiB).

Their structures are presented on Table 5. Fig. 23 indicates variations in potencies for both glycolysis and steroidogenesis gave L-ALA and L-GLY identical glycolysis curves, but for steroidogenesis, L-GLY exhibited a higher potency than L-Ala.

The differences are more readily discernible in Table 5 where computed relative potencies are presented. Replacement of L-GLY by L-ALA did not affect the glycolytic potencies but slightly lowered the ability to stimulate steroidogenesis. Substitution with β-Ala lowered glycolytic potency by 60% and steroidogenic potency by 80%. When L-GLY^{10} was replaced by D-ALA, there was a drastic lowering of glycolytic potency, and steroidogenic activity was reduced by 17%. The reversed situation was obtained with α-AiB substitution. Glycolytic potency was depressed to 10% and only 2% for steroidogenesis. These results seem to suggest that substituents at position 10 that would not disrupt the α-helical conformation of the region did not affect glycolysis and steroidogenesis to any great extent. Freedom of rotation about the peptide bond seemed essential. Thus the α-helix breaker D-ALA had very diminished potencies.

αAiB, with 2 bulky groups attached to the α-carbon, would be expected to hinder free rotation as well as to disrupt the α-helix.

β-ALA resembles glycine except for the inserted methylene group. Replacement of glycine by β-ALA led to a lowering activity, but not as pronounced as that observed with D-ALA or αAiB.

Substitution by D-ALA affected glycolysis more than

steroidogenesis. Perhaps, the active sequence for glycolysis was closer to GLY^{10} and hence more sensitive to α-helix disruption in that area.

In summary, the glycolytic potency was affected in the following decreasing order: L-ALA > β-ALA > αAiB > D-ALA.

Steroidogenesis was influenced according to the following decreasing order of potencies: L-ALA > β-ALA > D-ALA > αAiB.

This would suggest that rotation around the peptide bond is more critical than α-helix conformation for steroidogenic activity but less important for glycolysis.

STUDIES WITH ANALOGS

CONCLUSIONS

Position 4 (MET):	Hydrophobicity essential for glycolytic and steroidogenic actions. Sulfur atom unimportant.
Position 7 (PHE):	L-configuration essential for activity.
Position 8 (ARG):	Basicity essential for glycolysis. Guanidyl group unimportant for glycolysis but essential for steroidogenic action.
Position 9 (TRP):	Aromaticity important for affinity. Indolyl-NH group essential for receptor excitation. Electron-charge donating ability important for activity.
Position 10 (GLY):	Essential for affinity. Important for bioactive conformation (α-helix, rotational flexibility).

More evidence for the importance of these amino acid residues has been sought by investigating other peptide hormones that share sequence homology with ACTH.

CHAPTER 11

Effect of structurally-related peptide hormones

STUDY 11a

Effect of vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide hormone, occurring in the small intestine, as well as various tissues in the mammalian body. It is known to stimulate adenylate cyclase activity in ileal (330), liver and fat (331) cell membranes.

Just recently, VIP-like molecules have been found in chromaffin cells of the rat (332) and human (333) adrenal gland, and several studies have shown that VIP could stimulate steroidogenesis in mammals (334,335). However, the concentration of VIP required to stimulate steroidogenesis have been found to be much higher than those of ACTH (334,335). Kowal et al. found the steroidogenic activity of VIP comparable to that of ACTH (1-16) and ACTH (5-14) fragments, but they observed that the ability of VIP to generate CAMP was much lower than that of ACTH fragments (334). They noted that the potency of VIP closely resembled that obtained for NPS-ACTH (334). They attributed the ACTH-like action of VIP to sequence homology with ACTH. Table 6 shows the alignment of VIP and ACTH for maximum homology. However except for PHE^{7} of ACTH there was no apparent homology in the message-rich (5-10) region. It was therefore found appropriate to determine if VIP was capable of stimulating glycolysis in mouse adrenal cells, despite the limited homology.

As displayed in Fig. 24, VIP was virtually inactive at stimulating

ACTH (1-24) *vs* Vasoactive Intestinal Peptide

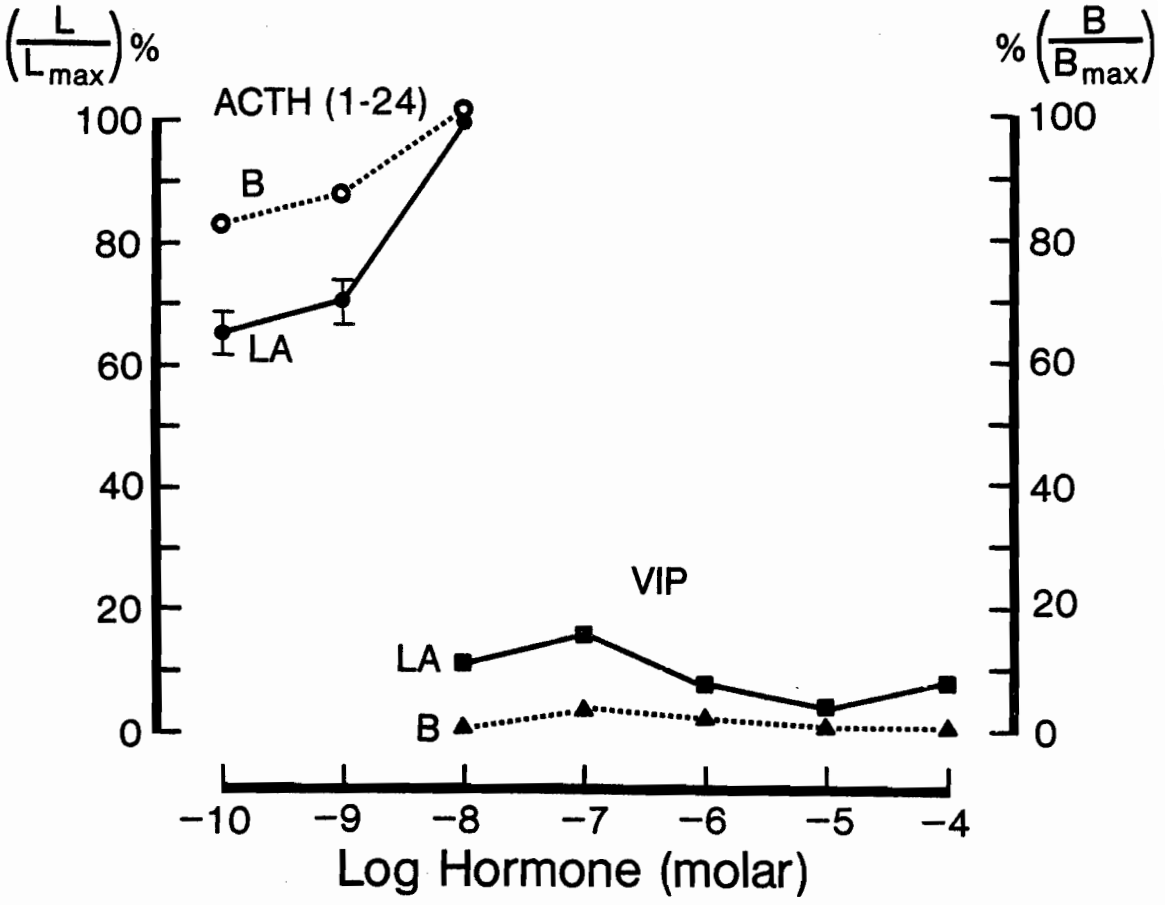


Figure 24

STUDY 11a: Vasoactive intestinal peptide vs ACTH (1-24).

(See GENERAL LEGEND page 120)

glycolysis or steroidogenesis in the concentration range available.

The present finding differs also from the results recently obtained by Leboulenger et al. (336). They demonstrated that VIP was able to stimulate corticosteroid secretion in amphibians and that microfilaments were involved in the VIP-induced adrenocortical stimulation. They further showed that neither prostaglandins, nor calcium were involved in the VIP action. (They employed a VIP dose of 10^{-5}M).

Table 6 also shows the sequence of Parathyroid Hormone (PTH) arranged against ACTH to illustrate homology. The next study examined the effect of PTH on adrenocortical cells.

TABLE 6. Sequence homology among ACTH, VIP, and PTH.

VIP		1	HIS	SER	ASP	ALA	VAL	PHE	THR	ASP	ASN	TYR	THR	ARG	LEU	ARG	LYS	GLN	MET 1
ACTH	1	SER	TYR	SER	MET	GLU	HIS	PHE	ARG	TRP	GLY	LYS	PRO	VAL	GLY	LYS	LYS	ARG	ARG 1
hPTH	15	LEU	ASN	SER	MET	GLU	ARG	VAL	GLU	TRP	LEU	ARG	LYS	LYS	LEU	GLN	ASP	VAL	HIS 3

STUDY 11b

Effect of hPTH

Parathyroid Hormone (PTH, Parathormone) is a single-chain 84-amino acid peptide hormone of the parathyroid gland, and is involved in calcium regulation, bone mineral resorption and renal phosphate and calcium excretion (337). The biological activity of the hormone has been found to reside in the first 27 amino-terminal residues.

Comparison of the amino-acid sequence of the biologically active region of the molecule, PTH (15-32) with the first 18 residues of ACTH reveals a region of significant homology (Table 6). The (1-11) sequence of ACTH shows five positions of homology with PTH (15-32). This homology of PTH with the message sequence of ACTH prompted Rafferty et al. (338) to investigate the steroidogenic action of bovine and human PTH on rat adrenocortical cells. They observed that human PTH (1-34) elicited steroidogenesis over the dose range 10^{-7}M to 10^{-5}M and the degree of stimulation was the same as that elicited by ACTH over the range 10^{-11} to 10^{-9}M.

Following the above report, human PTH (hPTH) was chosen as a probe for a further study on the roles of SER^3, MET^4, GLU^5, and TRP^9 in ACTH-induced aerobic glycolysis and steroidogenesis. (Those 4 residues are present in the amino terminal region of hPTH.)

The results are presented in Fig. 25. The left portion of the figure is a plot of the linearly-transformed dose-response equation, used to estimate Rmax and D_{50}.

From the right-hand side dose-response plots one may notice that PTH is capable of stimulating aerobic glycolysis to almost the same

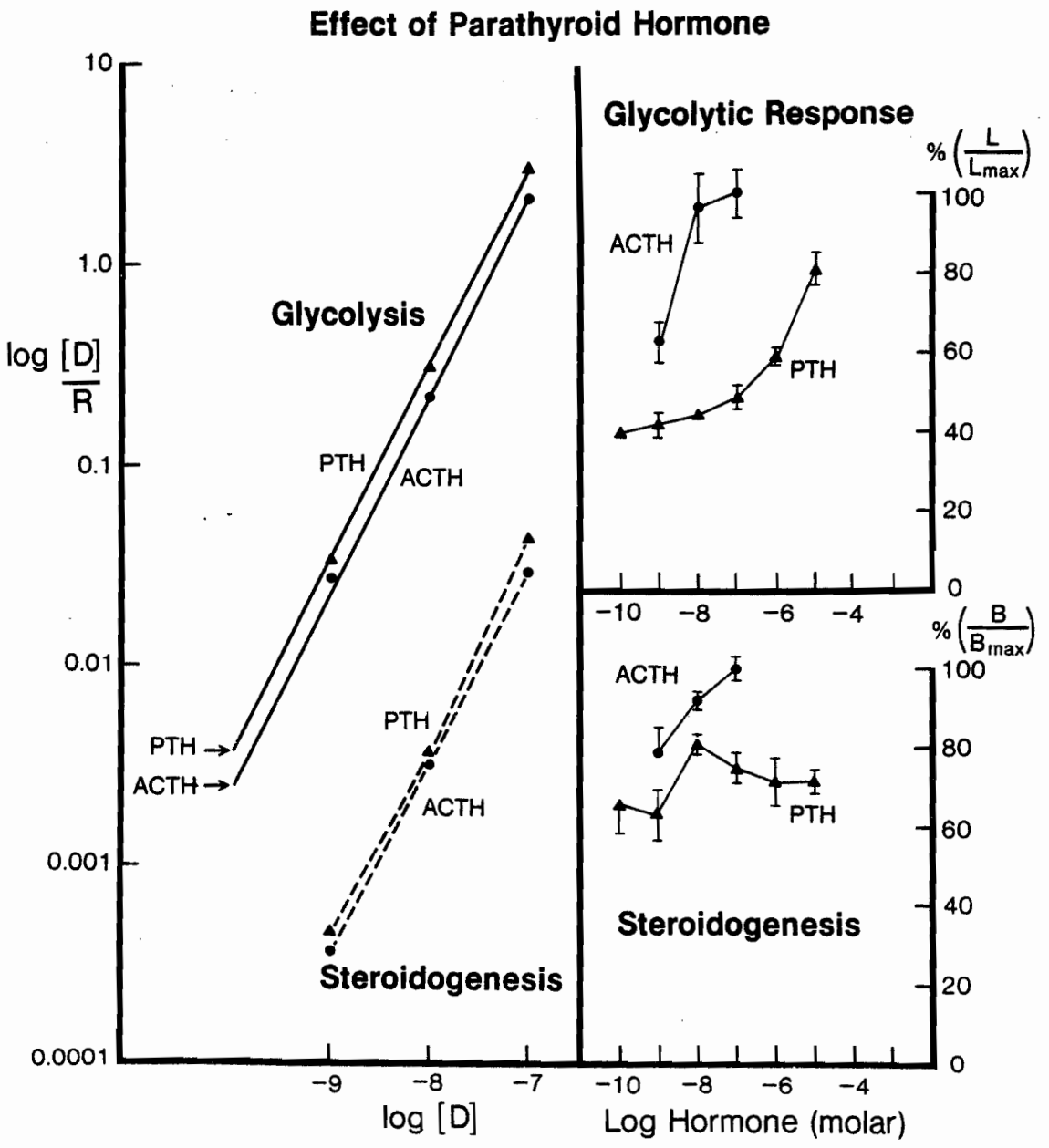


Figure 25

STUDY 11b: Parathyroid hormone vs ACTH (1-24).

Left-hand portion is "linear-plot" of right-hand graphs.

(See GENERAL

maximal values as ACTH (1-24), although, with much lower potency. On the other hand, a somewhat biphasic steroidogenic response was elicited by PTH, even though all concentrations of PTH stimulated steroidogenesis well above 50% of that induced by ACTH (1-24).

With the aid of the linear equation plot (on the left-handed side), it can be seen that the lines are parallel for glycolysis, meaning identical Rmax for both peptides. The y-intercept values show that ACTH has greater affinity than PTH for the adrenocortical receptor.

With regard to steroidogenesis, a complex pattern was exhibited, and the two lines converge and diverge. Nevertheless they were nearly parallel although the y-intercepts would be much more difficult to determine.

It can be stated that hPTH exerted a glycolytic action in a manner different from its steroidogenic action.

The fact the Lmax was identical for both PTH and ACTH means PTH contained residues capable of fully eliciting a glycolytic response much like ACTH.

The homology between PTH and the ACTH hexapeptide core i.e., ACTH (4-9), is made up of SER^{2}, MET^{4}, GLU^{5}, and TRP^{9}.

These residues therefore appear to be adequate for the triggering of adrenocortical receptors involved in the stimulation of aerobic glycolysis.

The absence of ARG in that region of PTH did not seem to influence the glycolytic action.

The picture obtained for steroidogenesis is not easily explicable.

The results from the VIP study would seem to suggest that more

than SER^2 and PHE^7 in ACTH is required for adrenocortical action.

(VIP carries only SER and PHE of the ACTH message region - Table 6.)

GENERAL SUMMARY

Effects of ACTH fragments, analogs, and related peptide hormones

(1) In the presence of the binding address sequence (LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}), GLU^{5}-HIS^{6} is required in addition to PHE^{7}-ARG^{8}-TRP^{9} for both glycolytic and steroidogenic activities. (Thus ACTH (7-38) is inactive.)

(2) In the absence of LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}, MET^{4} is non-essential for glycolysis so long as LYS^{11}-PRO^{12}-VAL^{13} is present.

In the absence of LYS^{11}-PRO^{12}-VAL^{13}, MET^{4} is necessary for biological activity. (Hence, ACTH (5-10) is inactive.)

(3) LYS^{11}-PRO^{12}-VAL^{13} contributes to glycolytical action more than steroidogenesis. The presence of MET^{4} is more critical for steroidogenesis than for glycolysis.

(4) ARG^{8} is essential for steroidogenesis but non-essential for glycolysis.

(5) The presence of MET^{4}, GLU^{5}, and TRP^{9} are adequate for the stimulation of glycolysis (as demonstrated with hPTH).

It does seem that there are no distinct messages specifically for glycolysis or steroidogenesis, but that different messages exist, each capable, to somewhat different degrees, of stimulating glycolysis and steroidogenesis.

Thus, the different messages give rise to different receptor affinities but each is capable of triggering a receptor.

In the presence of LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18} the message for glycolysis and steroidogenesis would be GLU^{5}-HIS^{6}- - - TRP^{9}

(message 2).

In the absence of the basic tetrapeptide, another sequence, (message 1), MET^{4}- -PHE^{7}-ARG^{8} - - - LYS^{11}-PRO^{12}-VAL^{13} is responsible for steroidogenesis and glycolysis, with MET^{4} as a probable binding residue for the steroidogenic activity, whereas LYS^{11}-PRO^{12}-VAL^{13} is a binding site for glycolytic action.

The question arose as to which of these amino acid residues were important for adenyl cyclase activation?

The following study was concerned with the interrelationship between steroidogenic and glycolytic activities of ACTH-related peptides and their ability to stimulate cyclic AMP formation.

CHAPTER 12

The role of essential regions in the stimulation of CAMP formation

STUDY 12a

CAMP: Time course and dose-response relationship

Fig. 26 shows the effect of incubation time on ACTH-induced CAMP formation when ACTH (1-24) was added at a concentration of 10^{-8}M. Aliquots of cells plus medium were removed at 0, 15, 60, and 180 minute intervals.

Fig. 26 indicates that ACTH elevated CAMP levels as early as 15 minutes after addition of the peptide to 2.5 times the basal level.

Maximal levels were reached at 60 minutes and stayed constant for 3 hours. Notably, basal levels also increased in the absence of ACTH to a maximum at 60 minutes and then declined to a value of one half that obtained in the presence of ACTH at 3 hours.

The stimulation of CAMP production by ACTH occurred in a dose-dependent manner as illustrated in Fig. 27.

ACTH was added to isolated adrenal cells at concentrations ranging from 10^{-12}M to 10^{-8}M and CAMP levels were measured at the end of a 3 hour incubation period.

In this experiment, 4-fold production over control was achieved with ACTH at 10^{-8}M.

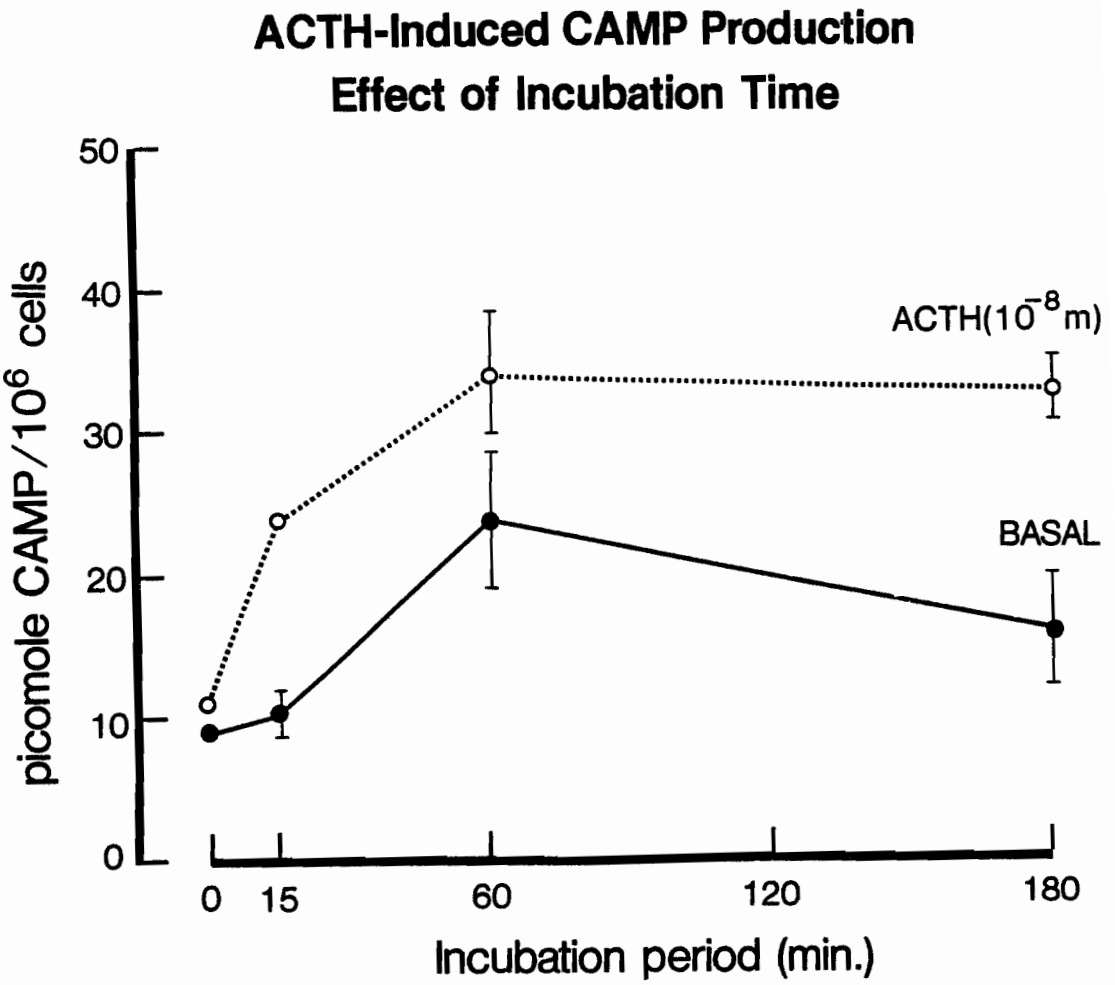


Figure 26

STUDY 12a: CAMP: Time Course

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point.

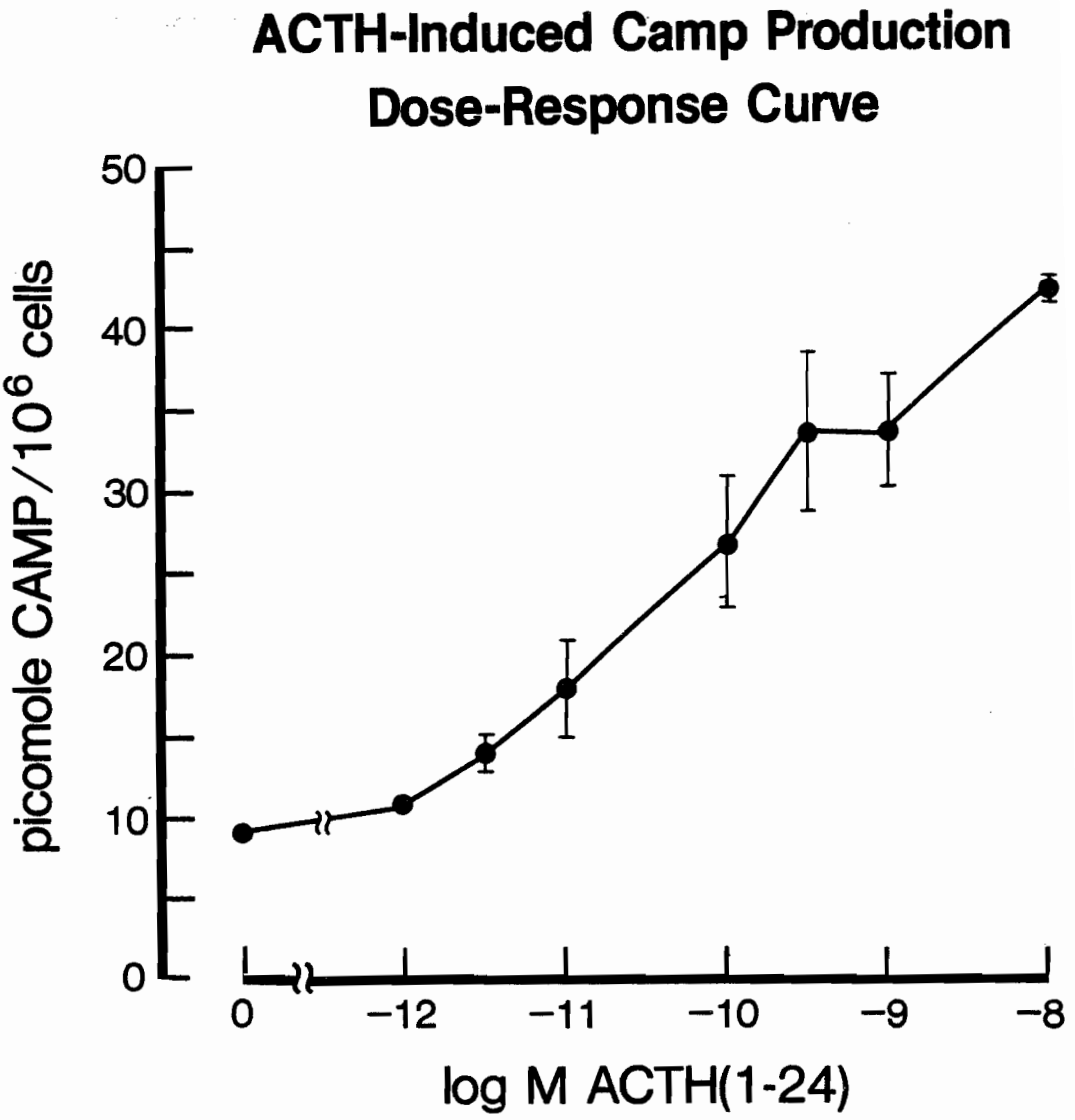


Figure 27

STUDY 12a: CAMP: Dose-response relationship

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point.

STUDY 12b

The roles of MET^4, GLU^5, ARG^8, TRP^9 and LYS^{11}-PRO^{12}-VAL^{13} in ACTH-induced CAMP formation

The following peptides were used for this study:

ACTH (1-14)	(ALA^9) ACTH (1-24)
ACTH (5-14)	(PHE^9) ACTH (1-24)
ACTH (4-10)	(LYS^8, PHE^9) ACTH (1-24)
ACTH (4-11)	
ACTH (7-13)	Parathyroid Hormone, hPTH (1-34)
ACTH (1-10)	

Four experiments were conducted:

(i) The role of MET^4 was evaluated by comparing ACTH (1-14) with ACTH (5-14)

(ii) MET^4, GLU^5, TRP^9 were investigated by the action of hPTH on CAMP formation

(iii) The short N-terminal fragments, ACTH (4-10), ACTH (4-11), ACTH (1-10) and ACTH (7-13), were studied together to assess the role of the tripeptide LYS^{11}-PRO^{12}-VAL^{13} in CAMP formation.

(iv) The importance of ARG^8 and TRP^9 in CAMP formation by ACTH was determined by studying the analogs (ALA^9) ACTH (1-24), (PHE^9) ACTH (1-24), and (LYS^8, PHE^9) ACTH (1-24).

STUDY 12b(i)

Role of MET^4: ACTH (5-14) vs ACTH (1-14) (Fig. 28)

ACTH (5-14) was added in two concentrations, 0.9×10^{-3}M and 0.9×10^{-4}M and for ACTH (1-14) concentrations of 0.3×10^{-4}M and 0.3 x

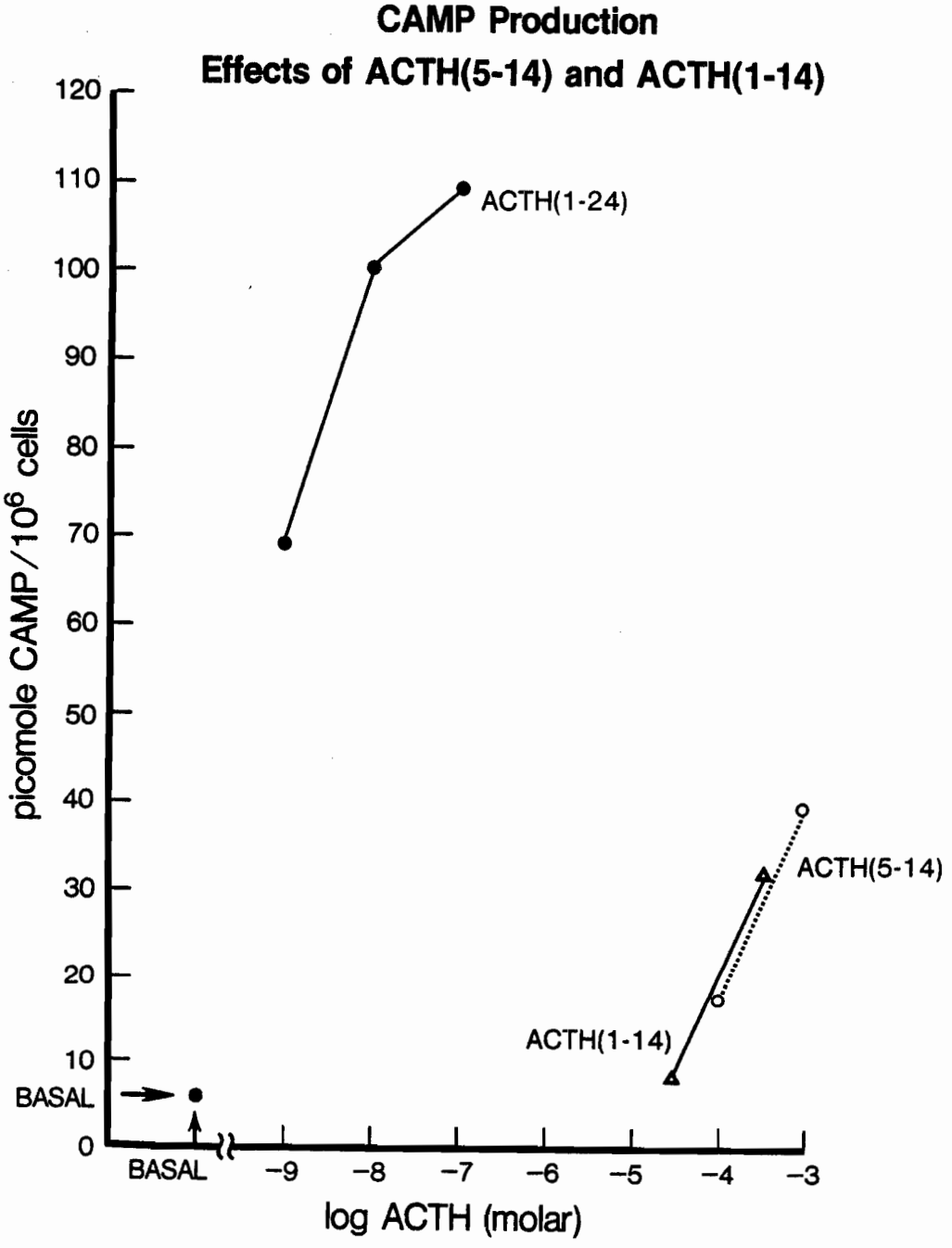


Figure 28

STUDY 12b(i): Role of MET^4: ACTH (5-14) vs ACTH (1-14).

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point

10^{-5}M were employed. The results indicate identical rates of CAMP formation for the two peptides, although the fragments had much lower potencies than ACTH (1-24).

This contrasts to the results obtained for steroidogenesis (Fig. 19) and agrees more with the glycolytic response pattern in that study.

Thus it appears MET^4 is not critical for CAMP formation, although its absence affected maximal steroidogenic activity.

STUDY 12b(ii)

CAMP formation and the roles of MET^4-GLU^5-TRP^9 as present in human parathyroid hormone

hPTH (1-34) and ACTH (1-24) were investigated as illustrated in Fig. 29. Despite maximal glycolytic capability, hPTH failed to stimulate CAMP production at concentrations at which it enhanced aerobic glycolysis (Fig. 25). There was a slight increase in CAMP at a PTH concentration of 10^{-5}M.

STUDY 12b(iii)

The significance of LYS^{11}-PRO^{12}-VAL^{13} in ACTH-induced CAMP formation

Effects of short N-terminal fragments, ACTH (4-10), ACTH (4-11), ACTH (1-10) and ACTH (7-13)

The results for this study are displayed in the histogram in Fig. 30. The short ACTH fragments were tested at a high concentration of 1mM. All the 4 fragments stimulated CAMP formation above control values, in the following order of increasing effectiveness: (4-10) $<$ (1-10) = (4-11) $<$ (7-13). This suggests that LYS^{11}-PRO^{12}-VAL^{13} is an

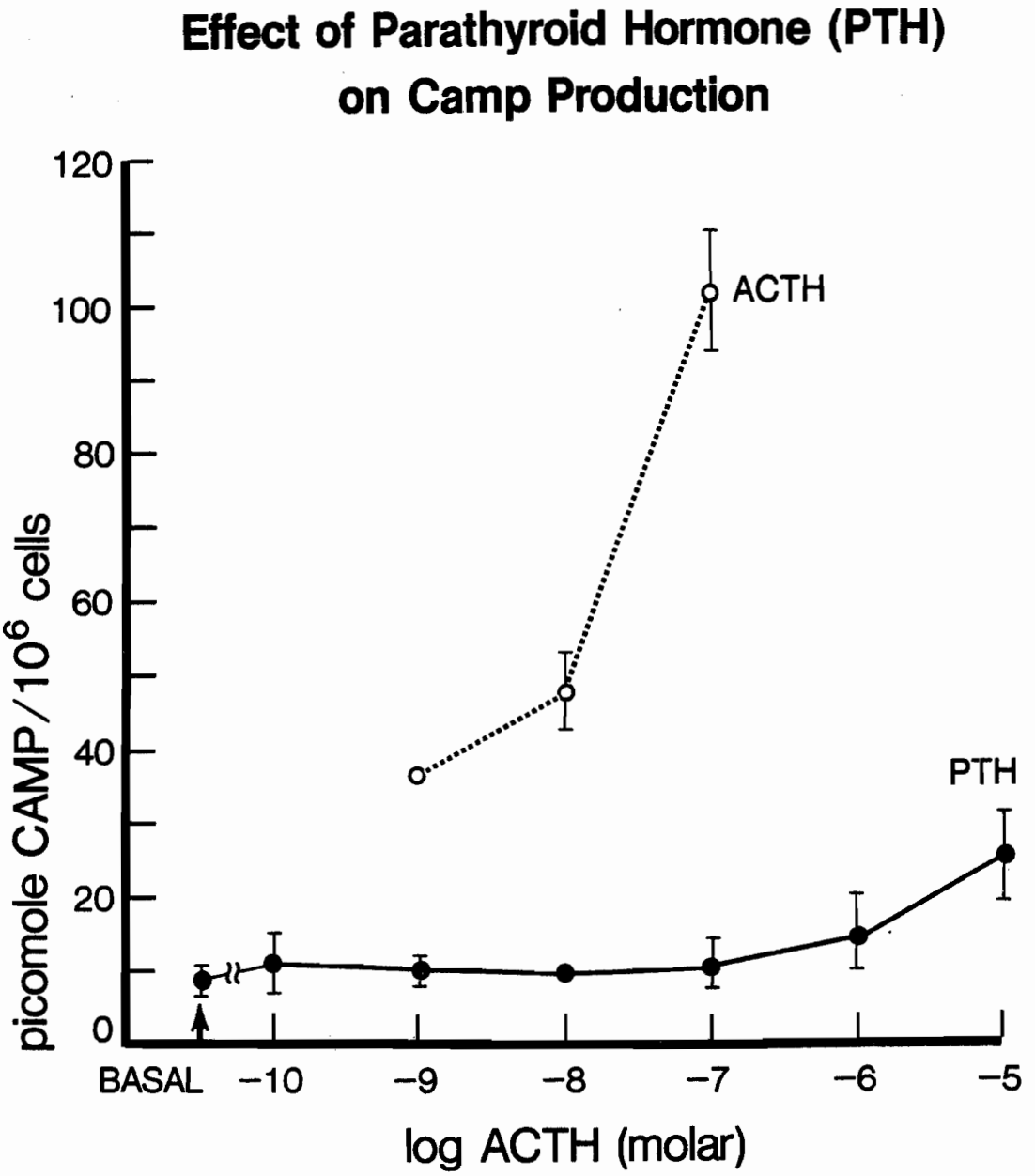


Figure 29

STUDY 12b(ii): CAMP formation and the role of MET^4-GLU^5-TRP^9 as present in hPTH

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point.

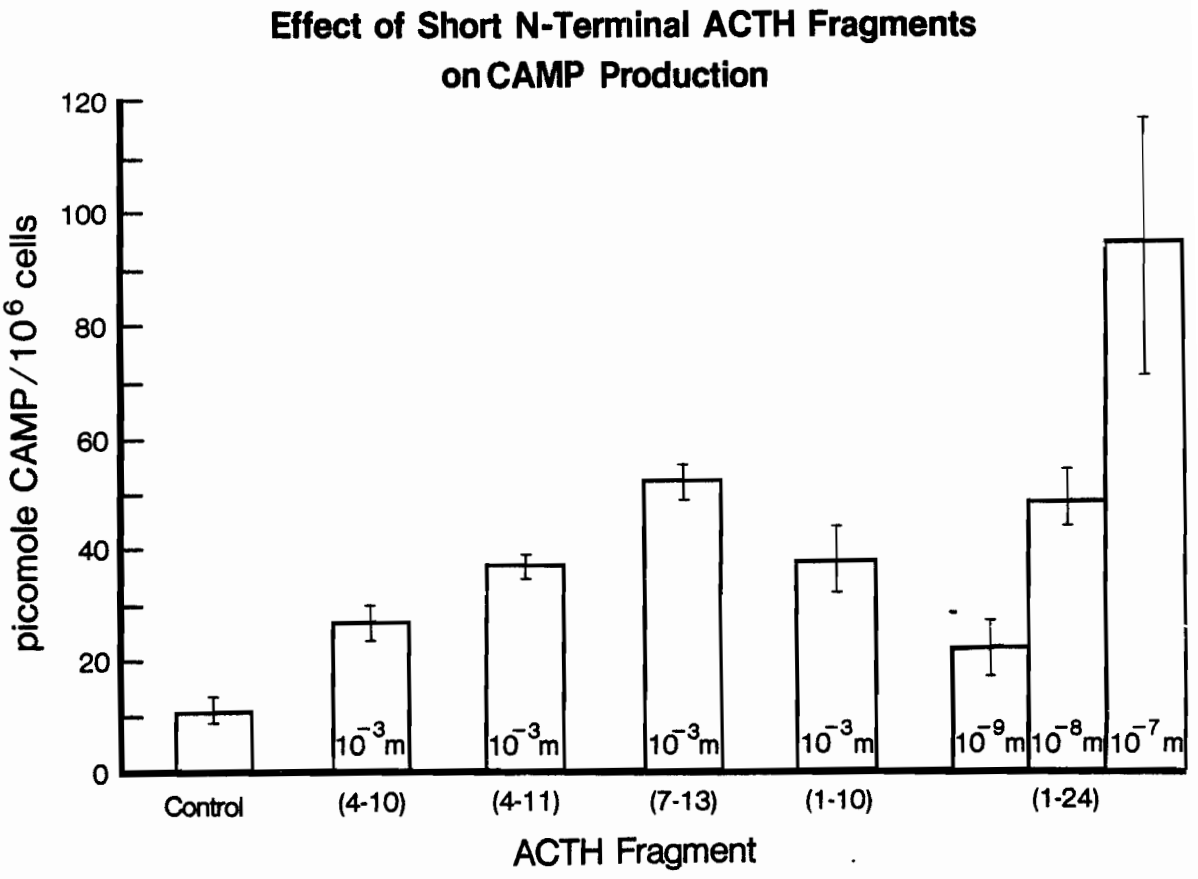


Figure 30

STUDY 12b(iii): The significance of LYS^{11}-PRO^{12}-VAL^{13} in ACTH-induced CAMP formation

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate analyses of each of triplicate incubations at each point.

important factor for CAMP formation. The fact that (4-11) was more potent than ACTH (4-10) and equipotent to ACTH (1-10) indicated that the presence of LYS^{11} contributed greatly to adenyl cyclase activation.

STUDY 12b(iv)

The roles of ARG^{8} and TRP^{9} in CAMP formation

Effects of (ALA^{9}) ACTH (1-24) I

(PHE^{9}) ACTH (1-24) II

and (LYS^{8}, PHE^{9}) ACTH (1-24) III

The results are plotted in Fig.31 All the 3 analogs were much less effective than ACTH (1-24) in stimulating CAMP production, although there were some differences in activities.

Arranged in order of increasing effectiveness they ranked as follows: ALA^{9}(I) < PHE^{9}(II) < $LYS^{8}PHE^{9}$(III) < ACTH (1-24).

These results suggest the TRP^{9} is essential for CAMP stimulation: When TRP^{9} was replaced by PHE^{9} it made little difference whether position 8 was occupied by LYS or ARG. These results differ from those obtained for lactic acid and steroid formation. $LYS^{8}PHE^{9}$ was a full agonist for glycolysis but a poor one for steroidogenesis.

This study, as well as several others previously described, implicate TRP^{9} as the single most important amino-acid residue for ACTH action. The final study was devoted to a closer examination of the role of Tryptophan in ACTH action, employing the analog, O-nitro phenyl sulphenyl-TRP^{9}-ACTH (or NPS-ACTH).

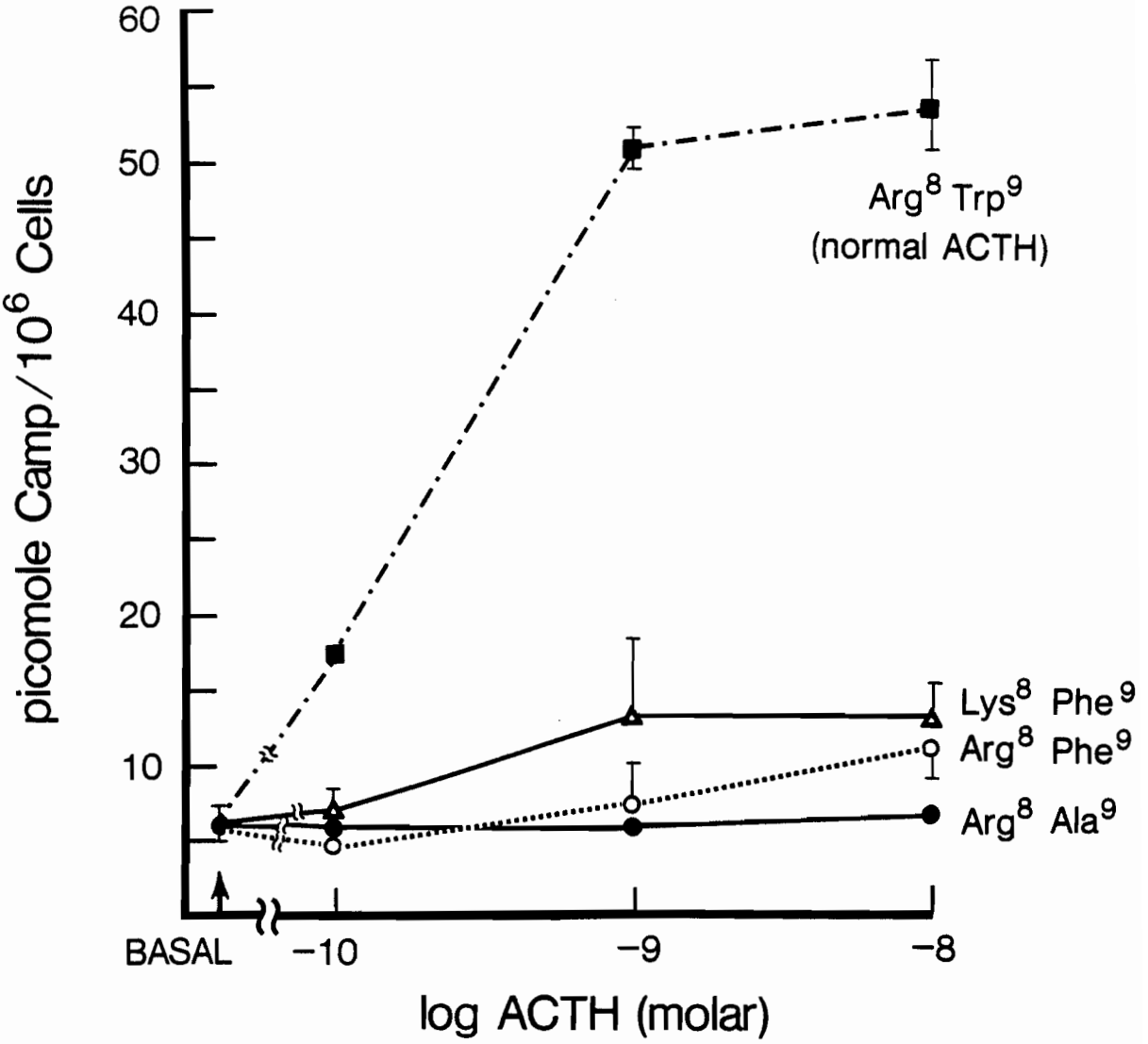


Figure 31

STUDY 12b(iv): The roles of ARG^8 and TRP^9 in CAMP formation

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate

CHAPTER 13

The role of tryptophan^9 residue in the interaction of ACTH with its receptor

INTRODUCTION

The tryptophan residue is rarely found in naturally occurring biologically active peptides but when it does occur, it has been noted to play an important role to elicit full biological activity. Through structure-function studies, TRP has been found to be essential for the exertion of full biological action of pancreozymin and cholecystokinin (339), gastrin (340) and luteinizing hormone releasing hormone (LHRH)(341). The 3 physicochemical properties that endow tryptophan with its unique role in hormone action are: (i) hydrogen bonding, (ii) charge-donor capacity, and (iii) lipophilicity.

For example the charge-donor capacity has been found to be of primary importance for the activity of pancreozymin.

Ramachandran and co-workers modified the tryptophan residue of ACTH by reacting with O-nitrophenylsulphenyl chloride and found a complete loss of lipolytic activity in rat fat cell (342) but not in those from rabbits (343). These findings were confirmed by Sayers et al. (344) who emphasized the importance of TRP^9 in the process involved in the excitation of receptors by corticotrophic peptides.

In this final study, NPS-ACTH was utilized to gain more insight into the role played by the indole side chain in ACTH-induced aerobic glycolysis, steroidogenesis, and CAMP formation in mouse adrenocortical cells.

STUDY 13a

Effect of NPS-ACTH on aerobic glycolysis and steroidogenesis

The analog NPS-ACTH is formed by substituting O-nitrophenyl sulphenyl at position 2 of the indole ring of tryptophan.

NPS-ACTH was added to adrenocortical cells at concentrations that ranged from 10^{-9}M to 10^{-5}M. Unmodified ACTH (1-24) was also tested from 10^{-10}M to 10^{-8}M.

The glycolytic and steroidogenic responses are depicted in Fig. 32.

The results revealed a striking disparity between the glycolytic response and the steroidogenic response to the analog.

Whereas NPS-ACTH at higher concentrations produced the same maximal steroidogenic response as the native hormone, the analog was not capable of maximal glycolytic stimulation at any concentration. The maximal glycolytic activity obtained with the analog was only about one-third the maximal glycolytic activity elicited by ACTH (1-24).

However the curves were shifted to the right by NPS-ACTH, for both glycolysis and steroidogenic and glycolytic receptors. However, whereas ability to excite the steroidogenic receptor was unchanged by the use of the analog, intrinsic glycolytic activity of the analog was greatly diminished.

Since the introduction of the bulky O-nitrophenylsulphenyl group to the TRP indole ring may be expected to (1) exert steric hindrance, and (2) interfere with H-bonding. It can be postulated that steric factors around position 9, and/or H-bonding through the indolyl-NH, can influence aerobic glycolysis, whereas charge donation and hydrophobic interaction (both unaltered in NPS-ACTH) could play a role in steroidogenesis.

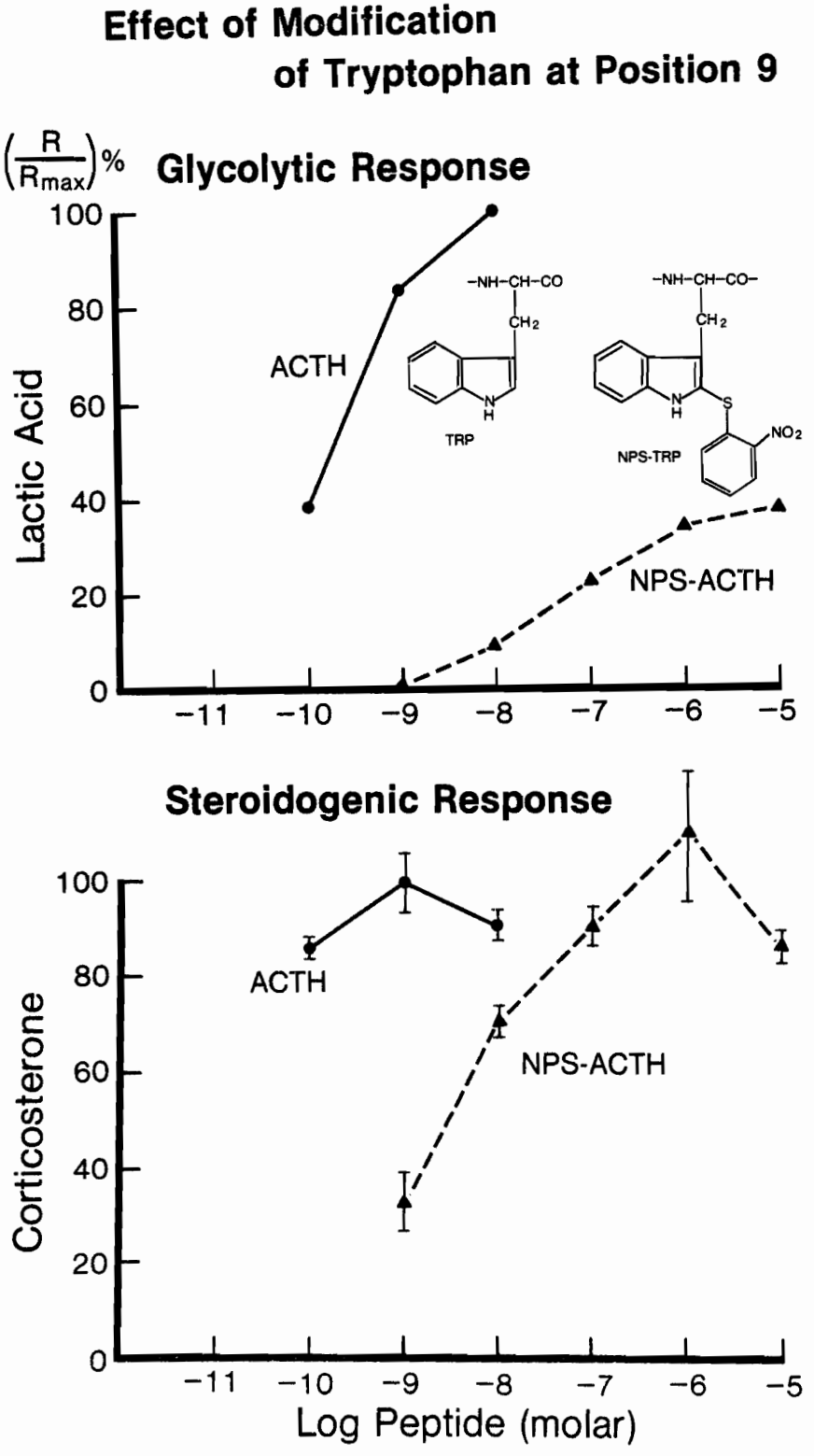


Figure 32

STUDY 13a: Effect of NPS-ACTH on aerobic glycolysis and steroidogenesis

(See GENERAL LEGEND page ...)

The results can best be explained by proposing that the indolyl-amino group is involved in H-bonding with the receptor thereby triggering the receptor, whereas hydrophobic interaction is important for steroidogenesis.

The next study evaluated the ability of NPS-ACTH to stimulate CAMP formation.

STUDY 13b

Effect of NPS-ACTH on CAMP formation

Two experiments were performed. In the first experiment (Fig. 33a), NPS-ACTH was tested at concentrations ranging from 10^{-10} to 10^{-7}M.

In the second experiment, much higher concentrations of NPS-ACTH were applied (10^{-9}M to 10^{-5}M).

In the first experiment, there was hardly any stimulation of CAMP production by the analog even at 10^{-6}M concentration of NPS, where maximal steroidogenic activity was attainable (Fig. 32).

At higher concentrations of 10^{-7} to 10^{-5}M (Fig. 33b) there was some enhancement of CAMP production ($\sim$ 2-fold), compared to 7-fold by ACTH (1-24). Yet at such high concentrations of NPS-ACTH maximal steroidogenic activities were elicited (Fig. 32). It can be argued that the 2-fold production of CAMP was adequate for maximal stimulation of steroidogenesis. Bristow et al. (345) in their study on the effect of ACTH (5-24) on steroidogenesis and CAMP production in rat adrenocortical cells found that ACTH (5-24) caused maximal stimulation of steroidogenesis at concentrations of the fragment that elicited no discernible cyclic AMP production. Two hypotheses (discussed in Chapter 4) had been used to explain the discrepancies.

According to the Dual Receptor Concept (Chapter 4), an acute action of ACTH at low physiological concentrations could stimulate steroidogenesis either by a pathway completely independent of cyclic AMP, or through a mechanism utilizing pre-existing background amounts of cyclic AMP (Compartment Guidance Hypothesis)(Chapter 4).

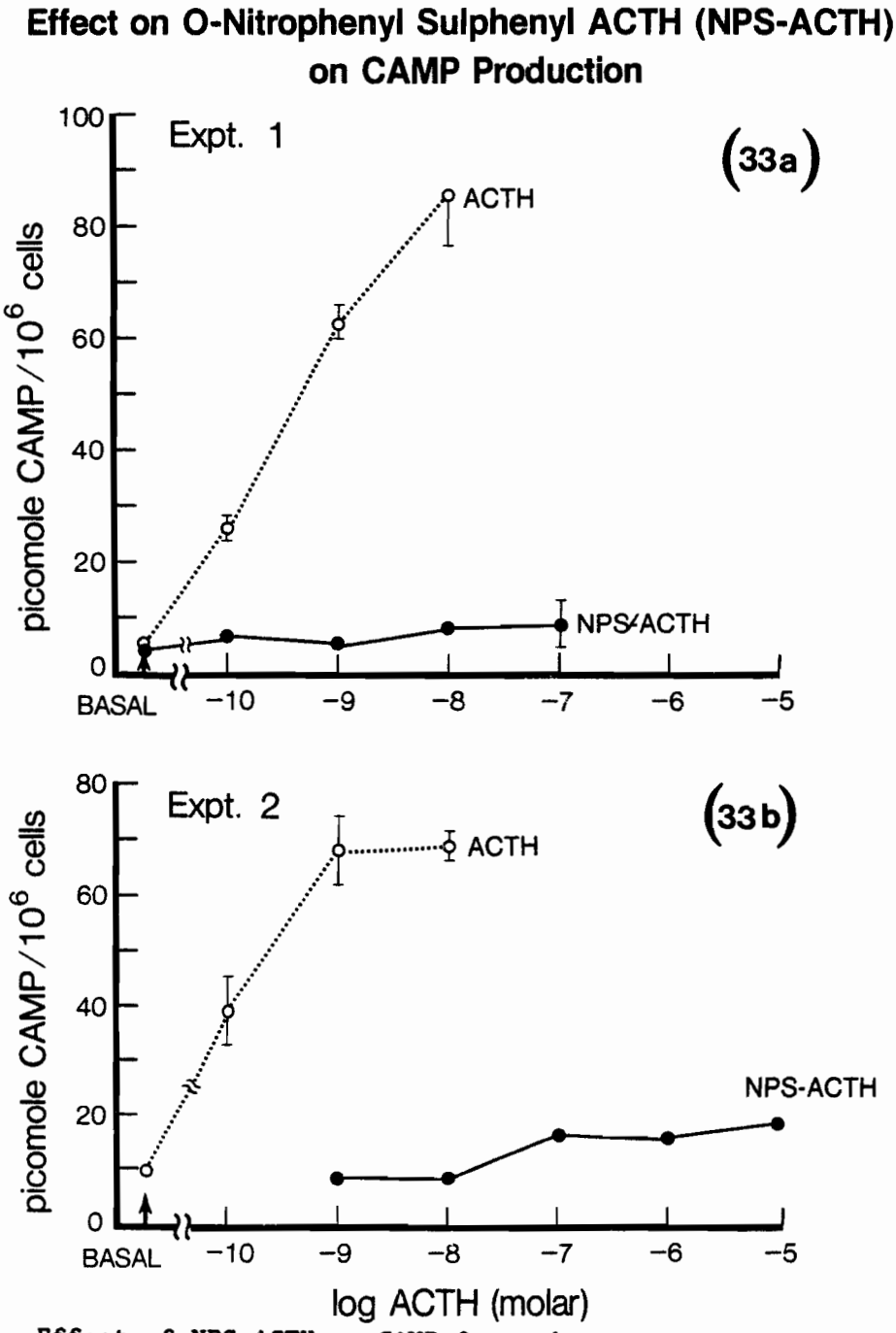


Figure 33

STUDY 13b: Effect of NPS-ACTH on CAMP formation

Expt. 1 - NPS-ACTH was added to cells at concentrations ranging from 10^{-10} to 10^{-7}M.

Expt. 2 - Concentrations of NPS-ACTH employed were 10^{-9} to 10^{-7}M.

Plotted points represent absolute values of CAMP measured in boiled cell extracts by R.I.A.

Vertical bars extend to the limits of the S.E.M. for duplicate

In any case, this study has demonstrated that the indole side chain of tryptophan is very essential for the stimulation of glycolysis.

The question now is which of the physicochemical properties of the indole group was altered by the O-nitrophenyl sulphenyl substitutions? For steroidogenesis, NPS-substitution only affected the D_{50} (affinity). The analog was capable of eliciting the same Rmax as native ACTH, suggesting that NPS-ACTH was capable of fully exciting the steroidogenic receptor. This could be explained by proposing that the NPS-substituent only affected affinity due to the bulky group interfering with tight binding to the receptor. It can therefore be postulated that the role of the indole group in steroidogenic action is to promote binding to the receptor.

In the case of aerobic glycolysis, tryptophan seemed to be primarily involved in receptor excitation, in addition to facilitating binding to the receptor.

Substitution with NPS-TRP reduced Rmax by about 60%. Such a diminished receptor excitability could be brought about by interference of the NPS-substituent with H-bonding between the indolyl-NH group and a group on the receptor, assuming such an interaction is responsible for receptor activation.

The results of this study with NPS-ACTH strongly suggest that ACTH-induced aerobic glycolysis and steroidogenesis might be mediated through either different receptors or through a common receptor associated with two different mechanisms.

The finding further indicates that the extent of ACTH-induced glycolysis does not always correlate with ACTH-induced steroidogenesis.

For the two processes might be quite independent.

An attempt will now be made to integrate all the findings in this research work into a suitable model that could elucidate how the corticotropin molecule instructs adrenocortical cells to increase production of lactic acid and corticosteroids.

CHAPTER 14

GENERAL DISCUSSION

Two aspects of the mechanism of ACTH-induced aerobic glycolysis have been studied.

(a) the substrate and nucleotide requirements (Project A);

(b) the structural requirements (Project B).

Project A has been fully discussed in Chapter 7, so the present chapter will briefly review all the data obtained from the various stucture-activity studies in Project B.

ACTH peptides are considered full agonists if at a suitable concentration they will produce the same maximal response (Rmax) as the native hormones (i.e., capable of fully exciting the receptor).

The various peptides studied have been grouped according to whether they were fully or partial agonists or whether they were antagonists. When modification leads to greater potency, the analog is called superagonist.

(1) Studies with fragments

For both aerobic glycolysis and steroidogenesis the following fragments were full agonists: (ACTH) (1-18), (1-17), (1-16), (1-14), (1-13), (1-10), (4-10), (7-13), and (3-18).

Whether or not ACTH (5-14) is a full agonist has not been conclusively determined for lack of sufficient material. ACTH (7-38) and ACTH (11-24) were antagonists.

(2) Studies with related peptide hormones

PTH was a full agonist for glycolysis, and could be a partial agonist for steroidogenesis - the results were not easily explicable. VIP was inactive.

(3) Studies with analogs

(a) Position 4 Methionine: Substitution with Norleucine leads to superagonism (i.e., compared to α-MSH). Position 7 Phenylalanine: Replacement by D-Phe leads to inactivity.

(b) Position 8 Arginine and 9 Tryptophan:

(Arg^8 Ala^9) ACTH: Very low activity

(Arg^8 Phe^9) ACTH: Full agonist

(Lys^8 Phe^9) ACTH: Full agonist for glycolysis antagonist for steroidogenesis.

(4) Studies with O-NPS-ACTH

NPS-ACTH - Partial agonist for glycolysis,
Full agonist for steroidogenesis

MODEL

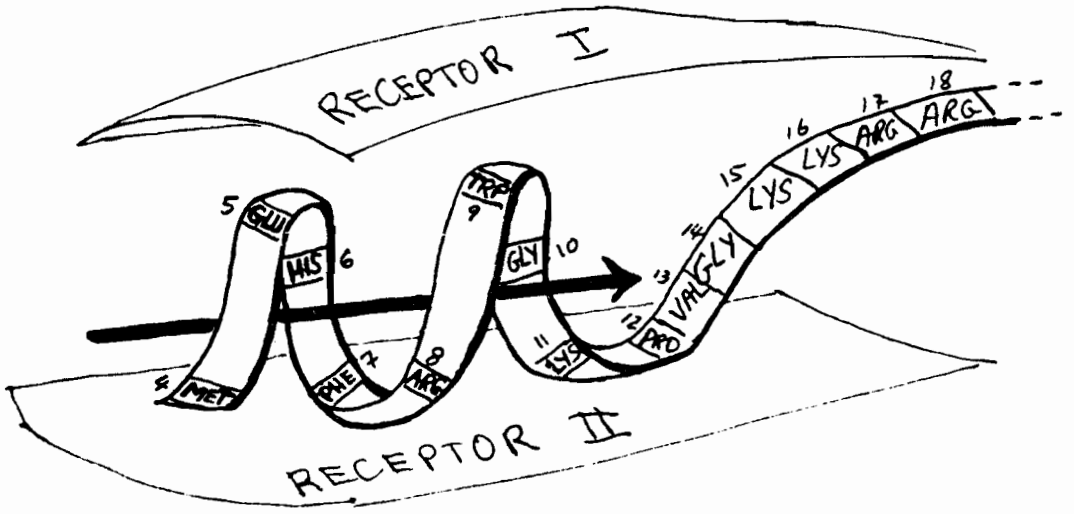
To account for the actions of peptides studied

It was Schwyzer's original idea that the 3-dimensional, α-helical arrangement of amino-acid residues in the 4-10 region of ACTH was responsible for the fact that different message sequences occur (139). Depending on the spatial orientation of a particular receptor,

FIG 34

a-Helical Arrangement of

message sequence 4-10



certain amino-acids on the α-helix would be "seen" by the receptor, while others would be out of reach. The idea is illustrated in Fig. 34. A model is hereby proposed that 3 different receptors can recognize certain combinations of residues in the 4-10 region of ACTH. If one imagines one receptor (I) facing the top of the α-helical segment, another (II) facing the under-side, while a third receptor (III) (not shown in Fig. 34) is located in front, facing the peptide from the direction of the observer's eye.

According the their respective orientations, receptor I would be in close contact with (message 1):

MET^{4}--- HE^{7}-ARG^{8}---LYS^{11}-PRO^{12}-VAL^{13} where PHE^{7}-ARG^{8} are "excitors" and MET^{4} or LYS^{11}-PRO^{12}-VAL^{13} are "binders". Receptor II would be close to (message 2):

GLU^{5}-HIS^{6} --- TRP^{9} with LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18} as address ("binder") and receptor III will be associated with (message 3):

GLU^{5}---ARG^{8}-TRP^{9}--LYS^{11} also with LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18} as address.

Explanation of findings with model (Fig. 35)

I. It is postulated that in the absence of the address segment (15-18) ACTH is recognized by receptor I, and hence fragments lacking the address segment should contain message 1 for activity, i.e., PHE-ARG plus either MET^{4} or LYS^{11}-PRO^{12}-VAL^{13}.

The following fragments would therefore be active:

ACTH (4-10) with MET^{4}, PHE^{7}, ARG^{8}

(5-14) with PHE^{7}, ARG^{8}, LYS^{11}, PRO^{12}, VAL^{13}

(1-13) with all

(7-13) with PHE^{7}, ARG^{8}, LYS^{11}, PRO^{12}, VAL^{13}

The following would be inactive:

ACTH (5-10) with PHE^{7}-ARG^{8} but without any "binders".

II. In the presence of LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}, ACTH is postulated to be recognized by either receptor II or receptor III and therefore ACTH fragments should carry GLU^{5}-HIS^{6}--TRP^{9} (for receptor II) or GLU^{5}---ARG^{8}-TRP^{9}-LYS^{11} (for receptor III) for activity.

Thus these fragments would be active:

ACTH (3-18) with GLU-HIS--TRP

ACTH (5-24) with GLU-HIS--TRP

and the following would be inactive:

ACTH (7-38) without GLU or HIS

ACTH (11-24) without GLU, HIS, PHE, ARG, TRP

STUDIES WITH ANALOGS

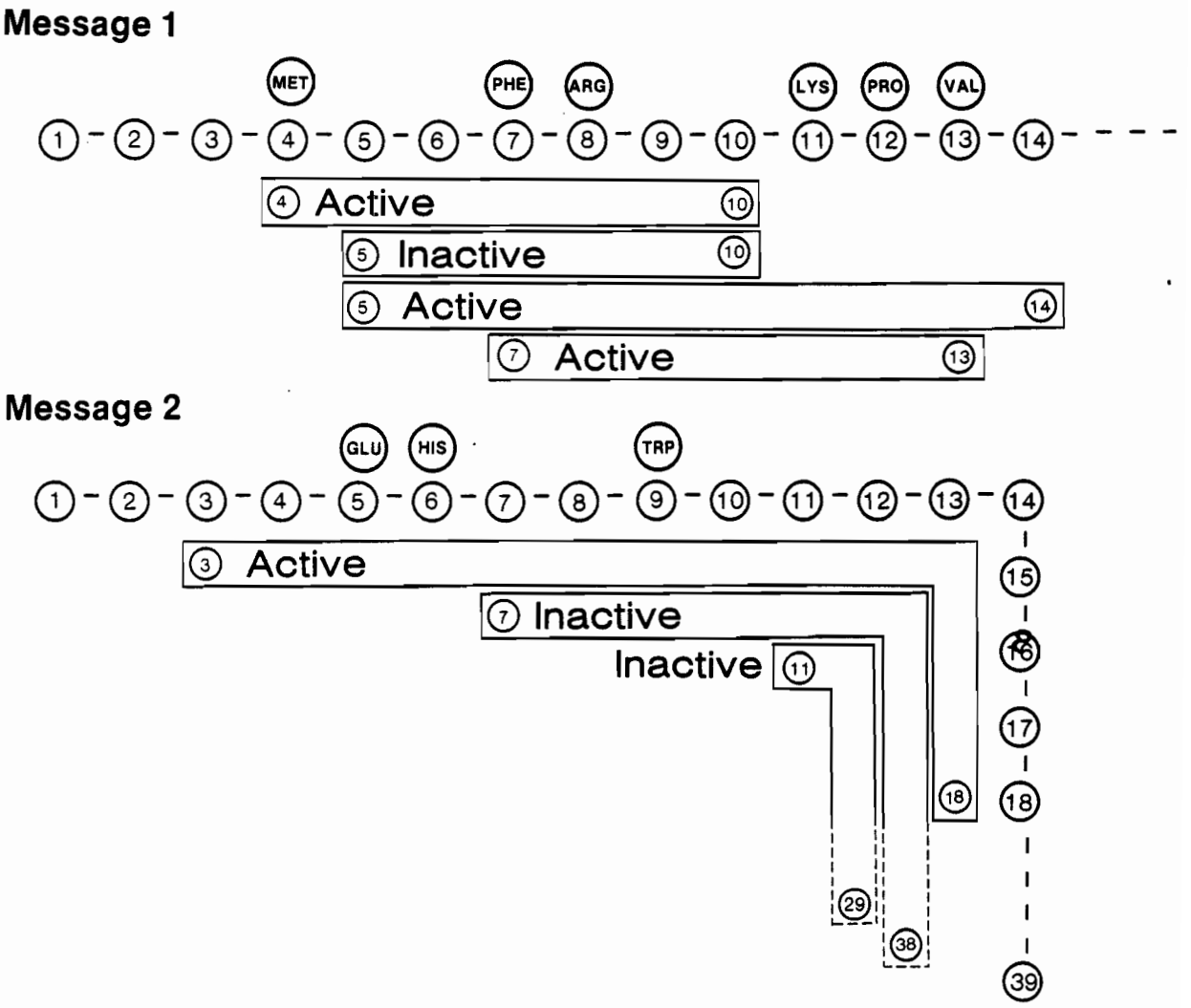
Cases of dissociation between glycolysis and steroidogenesis

Certain substitutions at position 8 (ARG) and position 9 (TRP) led to a clear dissociation between the glycolytic and the steroidogenic responses:

(1). With ($LYS^{8}PHE^{9}$) ACTH instead of ($ARG^{8}TRP^{9}$) ACTH, there was no steroidogenic activity whereas aerobic glycolysis was stimulated.

(2). With NPS-ACTH, there was maximal steroidogenic stimulation

FIG 35



Correlation of findings with model

but only 30% glycolytic stimulation. There was virtually no stimulation of CAMP production.

Consider the postulate that these two kinds of analogs are recognized by receptor III. They should therefore carry GLU^{5}-ARG^{8}-TRP^{9}--LYS^{11} for activity.

The data seem to indicate that ARG^{8} is required for triggering the steroidogenic receptor whereas TRP^{9} is the major trigger for the stimulation of aerobic glycolysis. Specifically, H-bonding and/or charge-donation would be the role of TRP for stimulation of the glycolytic response. In contrast, hydrophobic binding would be the TRP role in the activation of the steroidogenic response.

The results indicated that CAMP formation required the hydrogen-bond capability by the indolyl-NH of TRP and the presence of LYS^{11} as potentiator. Thus NPS-TRP could not stimulate CAMP formation.

The data from the analogs study seem to suggest that

(1) ARG^{8} is required for triggering the steroidogenic receptor. (Thus substitution of ARG^{8} by LYS^{8} led to loss of steroidogenic activity).

(2) ARG^{8} is not required for glycolysis (hence the LYS^{8} analog was capable of maximal glycolytic activity).

(3) TRP^{9} is required for stimulating glycolysis and inducing CAMP formation. Specifically, the ability of the indolyl-NH to form H-bonds is required for excitation of the receptor whereas aromaticity of the side-chain is essential for affinity.

Thus NPS-ACTH is not capable of maximal glycolytic stimulation. ALA^{9}-ACTH is inactive.

(4) The data also indicate that TRP only contributes to affinity for the steroidogenic receptor but not involved in its excitation.

Thus, NPS-ACTH has low steroidogenic potency but is fully capable of maximal steroidogenic action, and (ARG^{8},ALA^{9}) is steroidogenically almost inactive.

(5) LYS^{11}-PRO^{12}-VAL^{13} seem to potentiate stimulation of CAMP formation. PTH, lacking LYS^{11}-PRO^{12}-VAL^{13}, is only a weak agonist for CAMP formation. Further, PTH, with its MET^{18}-GLU^{19}-TRP^{23} is capable of maximally stimulating aerobic glycolysis, but not as effective at inducing steroidogenesis (lacking ARG at position 22).

CONCLUSIONS

The present data suggest the essential regions of ACTH for the stimulation of aerobic glycolysis are:

1. LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18} for affinity
2. LYS^{11}-PRO^{12}-VAL^{13} for potentiation
3. TRP^{9} for intrinsic activity
4. MET^{4}-GLU^{5}-HIS^{6} for potentiation
5. L-configuration of amino-acid residues
   - for correct bioactive conformation
6. GLY^{10} - for conformational freedom and affinity

MECHANISMS AND SIGNIFICANCE OF ACTH-INDUCED AEROBIC GLYCOLYSIS

As demonstrated in these structure-activity studies, stimulation of steroidogenesis by ACTH-peptides is not always accompanied by enhanced aerobic glycolysis. Similarly, glycolysis can be induced without any steroidogenic activity. Nevertheless, parallelism often existed and certain exogenously supplied adrenocortical steroids have been shown in this laboratory to stimulate aerobic glycolysis. It is thus possible that ACTH-induced glycolysis may be partially steroid-mediated. Steroids could elicit allosteric activation of glycolytic enzymes by reducing ATP/ADP, AMP ratios as a result of steroid metabolism requiring NADPH synthesis or by direct inhibition of the NADH-oxidase system.

Manner by which peptides directly enhance aerobic glycolysis could include the enhancement of glucose entry into the cell, an attractive concept in view of the evidence that exogenous glucose is required for the ACTH-induced glycolytic response (76) and the observation that ACTH

stimulates glucose transport (73).

SUMMARY OF PROJECT A.

The effects of the dibutyryl derivatives of cyclic GMP and cyclic AMP on lactic acid and steroid production was compared in intact mouse adrenal glands at concentrations of 0.5 to 1 mmol/l and in mouse adrenal cell suspensions at concentrations of 0.01 to 1 mmol/l. The dibutyryl derivative of cyclic GMP had little or no effect on lactic acid production in either tissue preparation. It caused a slight stimulation of corticosteroid output in intact glands at a concentration of 1 mmol/l, amounting to one-tenth of the response observed with 1 mM dibutyryl cyclic AMP. Dose-dependent increases in lactic acid and steroid production were obtained with dibutyryl cyclic AMP in cell suspensions. AMP and GMP increased lactic acid but not steroid production.

All the substrates tested (glucose, glucose-6-phosphate, fructose, fructose-6-phosphate, fructose-1,6-diphosphate, 10 mmol/l; pyruvate and glycerol, 20 mmol/l) stimulated basal glycolysis in intact glands and cell suspensions and none affected basal steroid production significantly. By far the greatest increase in lactic acid production was noted with fructose-1,6-diphosphate. However, only glucose and, in unsectioned glands, pyruvate exerted a potentiating effect on the glycolytic response to ACTH. Glucose potentiated the steroidogenic response to ACTH also, but only in intact glands.

The relative ineffectiveness of dibutyryl cyclic GMP is in accord with the species-dependent differing responses to the free form of the cyclic nucleotides noted in mouse and rat adrenal glands. The substrate requirements are in keeping with a rate-limiting role of

phosphofructokinase and an action of ACTH at some site between the entry of glucose into the cell and the formation of fructose-1,6-diphosphate.

ORIGINAL CONTRIBUTIONS TO SCIENTIFIC KNOWLEDGE

A.Nucleotide and substrate requirements of ACTH-induced glycolysis were established:

1. Dibutyryl cyclic GMP caused a slight increase in corticosterone production in unsectioned mouse adrenal glands in vitro but was found to be less effective in this respect than dbcAMP.

2. DbcGMP had either no effect on lactic acid production or caused an increase not exceeding that obtained with GMP in contrast to dbcAMP which was far more effective than AMP.

3. The same difference in responsiveness to dbcGMP and dbcAMP was also noted in mouse adrenal cell suspensions and contrasted greatly with the nearly equipotent in vitro response to dbcGMP and dbcAMP in the rat adrenal.

4. Basal glycolysis (but not steroid production) was increased by glucose, glucose-6-phosphate, fructose, fructose-6-phosphate, fructose 1,6-diphosphate, pyruvate and glycerol in intact glands and cell suspensions, fructose 1,6-diphosphate being by far the most effective substrate.

5. Only glucose potentiated the glycolytic response to dbcAMP and ACTH in cell suspensions. In intact glands a synergistic effect on lactic acid production was also obtained with pyruvate and fructose 6-P although glucose was far more effective.

6. The steroidogenic response to ACTH and dbcAMP in cell suspensions was not enhanced by any of the substrates indicating a

dissociation between the glycolytic and steroidogenic effects.

It was concluded that ACTH, via cAMP, stimulates glycolysis at some site between the entry of glucose into the cell and the elaboration of fructose 1,6-diphosphate. Possibly, fructose 2,6-biphosphate may mediate the hormone-induced glycolytic response in a manner similar to vasopressin-induced glycolysis in hepatocytes.

B. Glycolytically-active regions in the ACTH molecule were investigated:

1. Steroidogenesis was fully activated by (NPS-TRP^{9}) ACTH (1-24) whereas glycolytic activity was suppressed. (LYS^{8},PHE^{9}) ACTH (1-24) was a strong agonist for glycolysis but almost inactive in stimulating steroidogenesis.

2. The sequence LYS^{11}-PRO^{12}-VAL^{13} greatly potentiated glycolysis in the absence of the basic tetrapeptide (LYS^{15}-LYS^{16}-ARG^{17}-ARG^{18}). However, this tetrapeptide (also considered as the "address segment") was shown to greatly contribute to both the glycolytic and steroidogenic potencies of ACTH peptides.

3. The fragment ACTH (7-38) that completely inhibited steroidogenesis also inhibited glycolysis. The results showed that cAMP did not always play a major role in either glycolysis and steroidogenesis.

4. For the first time, it demonstrated that human parathyroid hormone can stimulate aerobic glycolysis in adenocortical cells whereas vasoactive intestinal peptide is inactive.

5. The first N-terminal residues, SER, TYR, potentiate both glycolysis and steroidogenesis induced by ACTH.

6. The studies suggest the existence of 3 message domains

with overlapping ability to stimulate glycolysis or steroidogenesis or cAMP formation:

I: (MET^{4}) PHE^{7}-ARG^{8}-- (LYS^{11}-PRO^{12}-VAL^{13})

II: GLU^{5}-HIS^{6}---TRP^{9}

III: GLU^{5}---ARG^{8}-TRP^{9}--(LYS^{11})

where II and III require LYS^{15} LYS^{16} ARG^{17} ARG^{18} for maximum affinity.

7. Evidence has been obtained, in the present study, for the role of TRP^{9} as the main trigger of a glycolysis-associated receptor, and ARG^{8} as the main executor for the steroidogenic receptor.

The findings suggest that more than one kind of ACTH receptor is associated with pathways stimulating aerobic glycolysis and steroidogenesis; that different regions of the molecule can trigger the same receptor; and that the same region can also trigger different receptors.

APPENDIX A

COMPUTER PROGRAM FOR CALCULATING Rmax, D_{50} AND POTENCIES

Computation of Rmax and D_{50} were by non-linear least squares fit of the data to the dose-response equation (rectangular hyperbola).

Non-linear least squares fit was by the Newton-Raphson method*: Initial estimates of D_{50} and Rmax were done by a linear transformation of the dose-response equation. The linear transformation was done by a Taylor's series expansion of the equation.

The estimates were then refined through minimized sum of squares of deviations of the observed from the predicted values.

The program (following page) was based on Cleland's* algorithm, and written for Radio Shack computers in Level II BASIC by the present candidate, J.W. Hinson.

* W.W. Cleland (1967). "The Statistical Analysis of Enzyme Kinetic Data". Advances in Enzymology, 29, 1-32.

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1 TROFF:DEFINTU:DIMV(20),A(20),W(20),S(3,4),Q(3),SM(3),SS(3):CLS
2 PRINT"<<<<NON-LINEAR FIT-DOSE RESPONSE CURVE>>>>":KC=0:JJ=0
3 W(12)=0.001:W(11)=0.01:W(10)=0.1:W(9)=1.0:W(8)=10.0:W(7)=100.0:W(6)=1000.0:W(5
)=10000.0:W(4)=1.0E+05
4 INPUT"ENTER CONTROL";CT
10 KC=KC+1:INPUT"ENTER A AND V";U,VC:V(KC)=VC-CT:A(KC)=W(U):INPUT"OVER(Y/N)";Z$:
IFZ$<>"Y"THEN10
12 TRON:NP=KC:M=1:N=2:P=NP-N:N1=N+1:N2=N+2:GOTO200
15 Q(1)=(V(I)[2)/A(I):Q(2)=V(I)[2:Q(3)=V(I):GOTO1300
16 CK=S(1,1)/S(2,1):NT=0:M=2:GOTO200
17 D=CK+A(I):Q(1)=A(I)/D:Q(2)=Q(1)/D:Q(3)=V(I):GOTO1300
18 CK=CK-S(2,1)/S(1,1):NT=N+1:IFNT=>3THEN1800ELSE200
200 FORJ=1TON2:FORK=1TON1:S(K,J)=0:NEXTK,J:FORI=1TONP:ONMGOTO15,17
1300 FORJ=1TON1:FORK=1TON:S(K,J)=S(K,J)+Q(K)*Q(J):NEXTK,J,I:FORK=1TON:SM(K)=1/SQ
R(S(K,K)):NEXTK:SM(N1)=1:FORJ=1TON1:FORK=1TON:S(K,J)=S(K,J)*SM(K)*SM(J):NEXTK,J:
SS(N1)=-1:S(1,N2)=1:FORL=1TON:FORK=1TON:SS(K)=S(K,1):NEXTK
1400 FORJ=1TON1:FORK=1TON:S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1):NEXTK,J,L:FOR
K=1TON:S(K,1)=S(K,1)*SM(K):NEXTK:ONMGOTO16,18
1800 S2=0:FORI=1TONP:S2=S2+(V(I)-S(1,1)*A(I)/(CK+A(I)))[2:NEXTI:S2=S2/P:S1=SQR(S
2):SL=CK/S(1,1):VI=1/S(1,1):VK=1/SL:FORJ=2TON1:FORK=1TON:S(K,J)=S(K,J)*SM(K)*SM(
J-1):NEXTK,J
1900 SV=S1*SQR(S(1,2)):SC=S1*SQR(S(2,3))/S(1,1):SI=SV/S(1,1)[2:S(1,3)=S1*SQR((CK
[2)*S(1,2)+S(2,3)+2*CK*S(1,3)):SP=S(1,3)/S(1,1)[2:SK=S(1,3)/CK[2:PT=(1/CK)*100
2000 TROFF:CLS:PRINT"A50=  ";CK;"10*-9M +/-";SC:PRINT"VMAX=  ";S(1,1)+CT;"+/-";S
V:PRINT"A50/VMAX=  ";SL;"+/-";SP:PRINT"1/VMAX=  ";VI;"+/-";SI:PRINT"VMAX/A50=  "
;VK;"+/-";SK:PRINT"POTENCY=  ";PT;" %"
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