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Studies of the Interaction of Adrenocorticotropic Hormone with Adipocyte Receptors

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ABSTRACT

STUDIES OF THE INTERACTION OF ADRENOCORTICOTROPIC HORMONE WITH ADIPOCYTE RECEPTORS

Catherine Marie Behrens

The interaction of adrenocorticotropin with its putative receptors on the isolated adipocyte has been investigated from several experimental vantage points. Structure-function studies with analogues of ACTH synthesized to contain diiodinated tyrosine selectively at position 2 or 23 (or both) indicated that the integrity of tyrosine 2 is essential for ACTH's full biological activity, as diiodinated tyrosine in this position yields a molecule with less than 5% of the potency of the native hormone. However, diiodination of the tyrosine at position 23 effected little or no loss of activity, and diiodination at both positions 2 and 23 caused no greater decrease in potency than the halogenation of the tyrosine 2 alone. The immunological reactivity of the iodinated ACTH analogues was also tested with a highly specific radioimmunoassay. The ACTH analogue containing diiodinated tyrosine at position 2 was found to compete as effectively as native ACTH in displacing tritiated ACTH from its antibody, while diiodination at tyrosine 23 caused a highly significant decrease in immunological reactivity of the analogue.

The structure-function studies indicated clearly that a radioiodinated analogue of ACTH would not be an acceptable

probe in studying the molecular basis of hormone-receptor interactions. Consequently, the preparation of a tritiated ACTH molecule with high specific activity and complete biological and chemical integrity was undertaken. Utilizing the method of reductive dehalogenation of synthetic or chemically iodinated ACTH analogues accomplished with pure tritium gas in the presence of a catalyst, tritiated ACTH of specific activities of 45ci/mmole (from the 3,5-I_2Tyr^{23}ACTH) and 90ci/mmole (from the 3,5-I_2Tyr^{2,23}ACTH) were prepared. The tritiated hormones were found to behave identically to native ACTH in all chemical characterizations, and to possess indistinguishable biological potencies relative to the unmodified hormone as well. The achievement of maximal theoretical specific activities was found to be highly dependent on conducting the catalytic dehalogenation in an aprotic solvent in which no protons would be available for exchange with the iodine atoms on the tyrosine ring. Therefore, to insure maximum specific activities for the tritiated ACTH the following conditions were adopted: the solvent system consisted of 0.1N acetic acid: hexamethylphosphoramide: dimethylformamide (1:10:90 v/v); the catalyst was palladium oxide on calcium carbonate.

The tritiated ACTH of 90ci/mmole has been employed to investigate the binding of the hormone with its putative rat adipocyte receptor. The binding reaction to isolated cells was observed to be rapid and reversible, and to occur over a concentration range which parallels quite closely that

which elicits a functional lipolytic response (K_D= $2x10^{-9}$M). These high affinity receptors were also found to be finite in number, with approximately 8500 sites calculated to be present per cell. The problem of non-functional binding and/or adsorption which was encountered in these measurements cannot be overemphasized; ACTH was found to bind with high affinity to a number of artificial materials with a magnitude that could often mask the receptor binding.

In order to facilitate the measurement of the interaction of ACTH with its receptor and ultimately to isolate this molecular entity, an attempt was made to regulate and modulate selectively the ACTH receptor population of the adipocyte ghost by the administration of dexamethasone to adrenalectomized rats, as had been reported in the literature (Braun and Hechter, 1970). It was observed that in the rat adipocyte ghost, adrenalectomy resulted in a loss of both ACTH and epinephrine-induced cAMP production, and that this deficiency was somewhat restored after dexamethasone administration; however, no selective loss/induction of the ACTH-induced cyclase stimulation could be confirmed. Both hormonally-induced cAMP production and lipolysis were also measured in isolated adipocytes from adrenalectomized rats. In the whole cells, adrenalectomy caused no apparent loss of response to either ACTH or epinephrine stimulation; furthermore, dexamethasone administration, although it caused both a super-normal production of cAMP and an extremely elevated lipolytic response, did not increase these responses to

levels higher than those seen in the steroid-deficient rats when the magnitude of the stimulation was expressed relative to the basal state. The binding of tritiated ACTH to isolated adipocytes from normal, adrenalectomized and adrenalectomized/dexamethasone treated rats was also measured and confirmed that no detectable changes occur in the receptor populations of adipocytes from these three metabolic states.

The final aspect of this study has involved the characterization of the protein components of the rat adipocyte membrane by one- and two-dimensional SDS polyacrylamide gel electrophoresis. This method has allowed the identification of over 30 membrane proteins ranging in molecular weight from 17,000-200,000; two carbohydrate containing moieties (with apparent MW 70,000 and 85,000) have also been identified. In addition, the adipocyte membrane from the normal versus the adrenalectomized rat has been analyzed on the one- and two-dimensional systems; however, no gross differences in the protein components of the membranes which could account for the observed differences in hormone responsiveness have as yet been found.

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PREFACE

The investigation of the mechanism of hormone action has occupied the attention of a great number of scientists for as long as the past one hundred years. The earliest attempts to elucidate hormone activity involved the removal of a particular gland and the subsequent observation of the changes that were effected in the organism; this approach was typified by A.A. Berthold in the castration experiments which he performed in 1849. During the remaining half of the nineteenth century investigators were able to demonstrate even more convincingly that hormones exert predictable effects in an organism, by such experiments as Murray's administration of sheep thyroid gland extracts to relieve the symptoms of hypothyroidism. Although the investigations of this era were largely empirical, they did serve to lay the basis for the more mechanistic approach to the study of hormonal action which was to follow.

Influenced perhaps by the recently proposed Mendelian view of inheritance or the relativistic theory of Einstein, endocrinologists in the early twentieth century intensified their interests in the chemical nature of hormones and the manner in which they effect responses from target tissue; for example, at this time insulin was shown by at least two groups to promote the uptake of glucose into cells, thereby allowing its phosphorylation to take place via catalysis by the hexokinase enzyme (Price et al., 1945;

Levine et al., 1949).

Following the application of biochemical techniques to endocrinology, it became possible to study the mechanism of hormone action on a much more molecular basis. The proposal in 1957 of cyclic 3',5'-adenosine monophosphate (cAMP) as the hormonal "second messenger" (Sutherland and Rall, 1957) contributed enormously to the understanding of polypeptide and protein hormone action, and at the same time challenged the imagination and expertise of investigators to determine the steps and details which preceded and followed the activation of the adenylyl cyclase. In the years that followed, particular attention was directed to cAMP-mediated enzyme phosphorylations within the cell, while hormone-receptor interaction, the dynamics of membrane structure and ion transport systems were studied as events occurring at the cell surface.

It was at this stage in hormone mechanism research that this particular study was conceived, with the intention of determining more precisely how the polypeptide and protein hormones of the anterior pituitary gland stimulate their target cells. Although the cell-specific response had been defined for nearly all of the anterior pituitary hormones, the steps prior to this response remained to be elucidated. Consequently, this study undertook an investigation of the events which occur at the cell membrane during hormonal stimulation; more precisely, this dissertation will concern itself

with the nature of the hormonal interaction with the receptor, the modulation of the receptor population and its association with the membrane-bound cyclase, and the structural components of the membrane.

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LIST OF ABBREVIATIONS

ACTH -- adrenocorticotropin

ATP -- adenosine triphosphate

BSA -- bovine serum albumin

cAMP -- cyclic 3',5'-adenosine monophosphate

CMC -- carboxymethyl cellulose

DMF -- dimethylformamide

DTT -- dithiothreitol

EDTA -- ethylenediaminetetraacetic acid

EGTA -- ethyleneglycol-bis(β-aminoethylether)N,N'-tetraacetic acid

GTP -- guanosine triphosphate

HMPA -- hexamethylphosphoramide

IEF -- isoelectric focusing

KRB -- Krebs-Ringer bicarbonate buffer

KRBG -- Krebs-Ringer bicarbonate buffer with glucose

LTI -- lima bean trypsin inhibitor

MW -- molecular weight

NPS -- o-nitrophenyl sulphenyl

PAGE -- polyacrylamide gel electrophoresis

TCA -- trichloroacetic acid

TEMED - N,N,N',N'-tetramethylethylene-diamine

SDS -- sodium dodecyl sulfate

CHAPTER 1

THE ADIPOCYTE AND ADRENOCORTICOTROPIN AS A MODEL SYSTEM FOR THE INVESTIGATION OF HORMONAL MECHANISMS

Introduction

In order to investigate the mechanism of action of the polypeptide and protein hormones of the anterior pituitary it seemed prudent to select a representative model system from which experimental information could be obtained and then extrapolated to other perhaps more complex systems. For this purpose, the interaction of adrenocorticotropic hormone (ACTH) with the isolated rat adipose cell was selected; the extensive characterization of the adipocyte response to hormone stimulation and the wealth of information on the structure and chemistry of ACTH made this particular system an attractive candidate for studying the mechanism of interaction.

As early as 1951 adipose tissue was recognized as being sensitive to hormonal stimulation when it was demonstrated that insulin directly promoted glucose uptake into the tissue *in vitro* (Krahl, 1951). By the late 1950's and early 1960's hormonally-induced lipolysis was observed for several hormones including epinephrine and norepinephrine (Gordon and Cherkes, 1958), glucagon (Steinberg *et al.*, 1959), melanotropin (Rabin *et al.*, 1961), vasopressin (White and Engel, 1958), and of course ACTH (White and Engel, 1958a), already establishing adipose tissue as an attractive system for mechanism studies. This attention was intensified by the discovery

that lipolysis was most likely mediated by cAMP when it was demonstrated that the activation of phosphorylase via cAMP could be effected in adipose tissue by ACTH as well as the other lipolytic hormones (Vaughan, 1960; Klainer et al., 1962). The ever increasing interest in the adipose tissue as a target for hormonal activation lead to the development of a method for isolating homogeneous populations of single adipose cells (Rodbell, 1964); this technique was executed by digestion of the tissue with collagenase and subsequent separation of fat cells from stromal and vascular tissue by simple centrifugation of the lighter adipocytes from all other inclusions. Rodbell was also responsible for introducing a protocol for preparing a membrane-enriched fraction, the so-called adipocyte "ghost" (Rodbell, 1967; Birnbaumer et al., 1969), which was to prove to be of great value in studying hormonal interaction with the adipose cell. This fraction was prepared by the induced swelling of the isolated adipocytes which were exposured to a hypotonic salt medium and their subsequent lysing by gentle agitation; this procedure yielded vesicles of intact delipidated membranes containing mitochondria, endoplasmic reticula, and occasionally a nucleus. When processed in the presence of adenosine triphosphate (ATP), adipocyte ghost adenylyl cyclase activity is preserved and found to be responsive to the lipolytic hormones (Rodbell, 1967; Birnbaumer and Rodbell, 1969), thereby making this

preparation another important tool for investigating the mechanism of hormone action. It must be noted, however, that a great deal of sensitivity to hormonal stimulation is lost in this fraction; for ACTH, for example, a concentration of three orders of magnitude greater is required to achieve half-maximal stimulation of the ghost as compared to the isolated adipocyte. This dramatic loss of sensitivity has been attributed to detrimental structural changes incurred during preparation (Birnbaumer and Rodbell, 1969).

It is not difficult to appreciate that within little more than a decade the adipose cell developed rapidly as a promising model system of hormonal activation. Investigators could confidently obtain a homogeneous population of cells which exhibited an easily quantitated cell-specific response - namely, lipolysis which could be detected by measuring either free fatty acid or glycerol release; moreover, they could also monitor adenylyl cyclase activity in membrane-enriched preparations by determining the production of cAMP, the first measurable biochemical event following cell stimulation.

The amenability of the adipocyte to investigation has been well confirmed in the literature during the last ten years. A review of all these studies would, however, certainly be beyond the scope of this discussion. Therefore, only a brief survey of those investigations in which the adipocyte has been employed for the elucidation of

the mechanism of action will be presented; the reader is referred to the Handbook of Physiology, Sec. 5 for further information. In order to complete the introduction of the model system, the known structure-function relationships of ACTH will also be discussed.

The Structure of the Adipocyte

Before approaching the biochemical characteristics of isolated adipocytes, a brief description of the cell structure would seem to be in order, so that the experimental geography will be appreciated. Under the light microscope, adipose cells present a fairly simple profile (Napolitano, 1965); they are characteristically spherical in shape with an average diameter of approximately 50 microns. The cell contains a plasma membrane, a large lipid inclusion and a flattened nucleus which has been pushed to the periphery. The electron microscope picture provides more detail, but again shows the adipocyte to have a rather uncomplex subcellular structure (Napolitano, 1965), dominated as expected by a large lipid droplet which is devoid of any membrane enclosure. Again, the nucleus appears to be flattened and a distinct nucleolar region can be identified. Mitochondria are present, ranging in shape from ovoid to filamentous; additionally, small dense granules are observed within these structures. The cytoplasm also contains small granules (which have been identified as ribonuclear particles),

an occasional endoplasmic reticulum and infrequently, a Golgi zone. In addition, pinocytotic granules are often evident at the cell surface. From this profile, it can be inferred that although the adipose cell is known primarily as a reservoir for fat storage, it is also a dynamic cell which is capable of protein synthesis, oxidation-phosphorylation, and of releasing cellular inclusions by pinocytosis.

Hormonal Activation of the Adipocyte

As indicated previously, the adipocyte of the rat is sensitive to stimulation by a number of hormones of disparate molecular structure, and responds with a single lipid mobilizing activity (Rizak, 1965). Moreover, these hormones all seem to exert this effect through the activation of the adenylyl cyclase enzyme (Sutherland and Robison, 1966; Butcher et al., 1968). In theory, the membrane-bound cyclase is thought to consist of both a regulatory and a catalytic subunit, where the regulatory unit faces the extracellular fluid, while the catalytic unit directs its active center to the interior of the cell. Presumably, the hormone receptor is associated in some particular way with the regulatory unit via a "coupling" or transducing structure. Experimental evidence from the work of Rodbell (Birnbaumer et al., 1969) has indicated that a Mg^{++}-ATP complex must bind to the catalytic unit to activate the enzyme, and also that there is a second Mg^{++}

binding site which is distinct from the catalytic site but which influences the catalytic center. It is this second Mg^{++} site which appears to be regulated by hormonal influence and this will be elaborated on below. The activated cyclase enzyme is then capable of cleaving adenosine triphosphate (ATP) to produce cAMP. Under the influence of the phosphodiesterase enzyme, the cAMP is degraded to 5'-AMP (Sutherland and Rall, 1958). Several known phosphodiesterase inhibitors exist and most belong to the general class of the methylxanthines (Butcher and Sutherland, 1962); experimentally, one of these phosphodiesterase inhibitors is usually included in the cyclase assay medium to facilitate the detection of the cAMP produced.

Because, as stated above, the adipose adenylyl cyclase is apparently capable of being activated by a number of hormones, as a model system, it presents particularly interesting prospects in terms of deciphering the mechanism by which the information contained in the hormone's structure is transmitted and translated into a specific cellular response. This potential was recognized by several investigators. By employing the adipocyte ghost and monitoring adenylyl cyclase activation Birnbaumer *et al.*,(1969) and Bar and Hechter (1969) independently were able to demonstrate that distinctive selectivity sites for ACTH, epinephrine and glucagon could be differentiated in this preparation. In these studies, it was demonstrated that calcium ion was obligatory only for ACTH-induced

cyclase activation, that a β-adrenergic blocking agent selectively abolished the stimulatory effect of epinephrine, and that neither of these conditions effected the glucagon activation. Having established recognition sites for the various hormones, investigators then questioned whether there existed a number of hormone specific membrane cyclases or whether a single cyclase was shared. Several studies have demonstrated subsequently that maximally stimulating concentrations of activating hormones when assayed in combination do not display activities greater than when assayed separately, that is, additive effects are not obtained when maximal doses are combined (Birnbaumer and Rodbell, 1969; Bar and Hechter, 1969; Rodbell et al., 1970). It would appear, therefore, that although separate recognition sites capable of distinguishing various hormones do exist in the adipocyte ghost preparation, all hormones share one and the same set of adenylyl cyclase molecules. The information obtained from these experiments has led to a generalized model system where a hormone, ACTH for example, reacts with a highly specific membrane-associated discriminator or receptor, and that this hormone-receptor complex acts through a coupling mechanism to stimulate the membrane-bound cyclase. The cAMP thus generated is considered to be the ubiquitous second messenger, allegedly responsible for initiating a sequential set of reactions; these reactions, which differ in various cell types, are then amplified to effect a specific cellular response that is characteristic of

hormonal stimulation. In the case of the adipocyte, for example, where the cell-specific event is lipolysis, cAMP has been shown to bind to the cAMP binding protein, an event which then triggers the phospohorylation of a kinase and the subsequent activation of the lipase enzyme (Vaughan, 1960).

Before completing the description of the adipocyte cyclase model it is worth noting that the ideas presented thus far might prove to be far more complex, and that consequently the events occurring during hormonal binding and cyclase activation should be scrutinized from a more molecular perspective. In one investigation of this nature, DeHaen (1974) has carefully analysed the kinetics of several published studies and has confirmed that in all probability a sharing of cyclase molecules does occur in a multireceptor adenylyl cyclase system, although there need not be a simple stoichiometric relationship between receptors and cyclase units; for example, there may exist systems with more receptors than cyclase molecules or vice versa. In addition, DeHaen proposes that the occupation of the receptor by the hormone greatly increases the affinity between receptor and cyclase; this initial interaction will therefore have the capability of directing the enzyme's activation by changing its energy state. Here we begin to appreciate the complexity of this model, particularly in reference to the nebulous coupling mechanism which was alluded to above and which will be discussed again in Chapter 4. The proposed mechanism of

hormonal cyclase activation has also been embellished by Rodbell who has recently investigated the effect of guanyl nucleotides on the adipose cell adenylyl cyclase (Rodbell, 1975). The results of his study, which suggest a modification of the DeHaen model, indicate that it is the guanine nucleotide and not the hormone which is capable of transforming the enzyme from its ground state E (low V_{max}) to an activated E' state (with a higher V_{max}) in which it displays a greater affinity for its protonated substrate, $ATPH^{3-}$. Rodbell proposes further that it is at this stage that the hormone enhances the rate of isomerization to a third level (E'') which in turn effects the cyclase stimulation. Rodbell's model therefore adds still another dimension to the regulation of the adenylyl cyclase, representing a so-called three-state system as compared to DeHaen's two-state model. Although, as discussed previously, it appears that the lipolytic hormones operate with distinct receptors but through a common cyclase, in view of the apparent role of the guanine nucleotides, it remains to be evaluated whether each hormone will display different nucleotide requirements. Should this be the case, a reassessment of the common cyclase theory would seem to be in order. In any event, a new aspect of information transfer has been proposed, and may be of importance in elucidating the way in which the activation of the adipocyte is regulated.

Hormonal Activation of the Rabbit Adipocyte

A great deal of the studies involving the adipocyte have been performed on the rat species; however, the rabbit adipose cell and ghost preparation have also been investigated (Tanaka et al., 1962; Rudman et al., 1964; Ramachandran and Lee, 1976), and found to exhibit some interesting species differences. To begin with, ACTH is a less potent lipolytic agent in the rabbit adipocyte, with a half-maximal concentration nearly two orders of magnitude greater than in the rat adipose cell. Furthermore, α-MSH, the melanocyte stimulating hormone, was found to be more effective than ACTH in promoting fat mobilization in rabbits, and is essentially devoid of activity in the rat adipocyte. These differences were amplified by the use of the o-nitrophenyl sulphenyl derivative of ACTH (NPS-ACTH) which contains a nitrophenylsulphenyl group on the single tryptophan moiety. This modification was found to selectively abolish the biological activity in rat adipocytes, while at the same time it clearly enhanced these activities in the rabbit system (Ramachandran and Lee, 1970). In addition, the dramatic loss of sensitivity to ACTH-induced cyclase activity which is observed in the rat ghost does not occur in the rabbit ghost, thereby making the latter more sensitive to ACTH stimulation (Ramachandran and Lee, 1976) than the rat preparation.

On the basis of these results it would appear that the rat and rabbit adipocytes exhibit different structural

requirements for productive hormonal interaction, presumably at the site of the receptor. Alternatively, as suggested by Rudman et al., (1964), perhaps different degradative enzyme systems are present in the two tissues which could account for the apparent disparate responses. In any event, this species specificity serves to increase the interest in the adipose cell as a model system to be used in investigating the molecular basis of hormone action.

The Structure and Function of ACTH

The remainder of this introductory chapter will be concerned with presenting a brief profile of ACTH which will be helpful in justifying its use in our model system and also in understanding the perspective from which this thesis work was undertaken.

ACTH or corticotropin was first isolated from porcine (White, 1953) and ovine (Li et al., 1954) pituitary glands. An extensive review on the isolation and purification of ACTH has been published (Li, 1956), as well as a revised isolation procedure (Canova-Davis and Ramachandran, 1976); the reader is referred to these articles for a detailed discussion. The amino acid sequences for several species including ovine (Li et al., 1955), porcine (Howard et al., 1955) and bovine (Li et al., 1958) were determined during the latter part of the 1950's and the human sequence followed in 1961 (Lee et al., 1961). The elucidation of the structure showed ACTH to be a polypeptide of 39 amino

acids, with the N-terminal 1-24 sequence precisely conserved from species to species; minor differences were found to occur between the 24^{th} to 33^{rd} amino acid. Fig.1 indicates the sequences of ACTH from various species.

The synthesis of a biologically active nonadecapeptide corresponding to the first 19 amino acids of ACTH was accomplished soon after the elucidation of the sequence (Li et al., 1960), and the total synthesis of porcine ACTH followed shortly (Schwyzer and Sieber, 1963). The success achieved in the synthesis of ACTH has resulted in the availability of a great many analogues of corticotropin; these synthetic variants of the native molecule have proved to be invaluable in defining the structural requirements for the expression of ACTH's biological activities. These will be summarized briefly below.

Chemically, ACTH is characterized as a basic peptide with a pI of 8.7 (Leonis and Li, 1959). The molecule contains three major chromophores, a single tryptophan in position 9 and two tyrosines in positions 2 and 23; collectively they contribute to an extinction of $\varepsilon \cdot {}^{1\%}_{280nm} = 1.72$ for ACTH. Physicochemical measurements would indicate that ACTH exists in solution as a flexible randomly coiled polypeptide (Squire and Bewley, 1965); Edelhoch and Lippoldt, 1969); however, there is some evidence that the hormone assumes a more compact conformation in alkaline pH than in acidic conditions (Squire and Li, 1961).

The biological function of ACTH was suspected as early

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Val-Lys-Val-Try-Pro
20 21 22 23 24

Ala-Phe-Pro-Leu-Glu-Phe
34 35 36 37 38 39

HUMAN	Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu 25 26 27 28 29 30 31 32 33	Riniker _et_ _al_., 1972
OVINE	Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Gln	Li, 1972
BOVINE	Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Gln	Li, 1972
PORCINE	Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu	Riniker _et_ _al_., 1972

Fig. 1. Amino Acid Sequences of ACTH from Various Species.

as 1926 when Smith observed that a secretion from the anterior pituitary demonstrated selective activation of the adrenal gland cortex. It is now well established that ACTH exerts both an acute adrenal effect which results in the production and secretion of steroids, as well as a chronic influence which serves to maintain the structure and function of the target gland. Moreover, ACTH possesses an extra-adrenal activity in its ability to stimulate fat mobilization from the adipocyte as described in the first section of this chapter. Additionally, ACTH has been found to cause the pigmentation of skin by its action on melanocytes (Li et al., 1960), and this activity has subsequently been attributed to the structural similarity between ACTH and the melanotropins (Ramachandran, 1973).

Having sketched the structural and functional properties of ACTH we are now in a position to discuss their relationship and how it will dictate the biological activity of the molecule. For the sake of brevity, only those aspects of ACTH's structure-function which are relevant to studies which will be described in this thesis shall be discussed at this time; the reader is referred to more extensive reviews (Ramachandran and Li, 1967; Ramachandran, 1973).

Perhaps the simplest way to summarize the structure-function of ACTH is to envision the manner in which the hormone interacts with its receptor site. As described by Ramachandran (1973), the binding of the sequence 6-13 appears to trigger those cellular events which are ultimately responsible for the biological response. This has

been demonstrated by the fact that any substitution in this part of the sequence results in a very dramatic loss of activity (Blake and Li, 1972; Chung and Li, 1967; Tesser and Rittel, 1969). The probability that this critical interaction of residues 6-13 will occur is enhanced by an "anchoring" of the molecule to the cell surface, thereby increasing its local concentration; this is effected through an attachment of the basic core (residues 15-18), as well as the amino terminal (1-5). The function of these additional attachment sites had been proposed after the demonstration that substitution in these areas resulted in varying degrees of depressed activity, attributable to an apparent decreased affinity of the hormone for its receptor. It has also been shown that the amino acid terminal region and the basic core are not essential for ACTH to exert its biological activity, since when substituted, the maximum response elicited remains unchanged, although the concentration required for half-maximal stimulation is increased. The importance of the basic core as an auxilliary attachment site is illustrated by Ramachandran (Ramachandran and Li, 1967; Ramachandran et al., 1965; Ramachandran, 1973) who has demonstrated that a systematic deletion of basicity in residues 15-18 is accompanied by a need for higher concentrations of the analogue to achieve a half-maximal response. Some illustrations of the effect of substitutions in the amino terminal region (residues 1-5) are observed in the acetylation of

the amino terminal serine which results in a 90% loss of activity (Lebowitz and Engel, 1963), or in the oxidation of methionine in position 4, resulting in approximately the same degree of diminishment. In contrast, substitution of tyrosine 2 for phenylalanine results only in a minor decrease in potency, as the analogue exhibits 60% of the activity of the native molecule (Geiger et al., 1964).

Before concluding this discussion it should be noted that the structural requirements for the receptor site on the rat adipocyte have thus far been shown to be the same as those for the rat adrenal (Ramachandran, 1973). In addition, it is apparent that only residues 1-19 of ACTH are needed to effect complete biological activity in in vitro systems; the C-terminal 20-39 residues appear to stabilize and protect the hormone in the circulation and are necessary for full potency to be expressed in vivo.

It can be concluded from this brief review of ACTH'S structure-function relationship that the region 6-13 residues appears to contain the message portion of the molecule, while the basic core (15-18) and probably the amino terminal 1-5 residues interact with binding sites on the surface of the cell, thereby effecting the concentration of 6-13 in the vicinity of the receptor. It is not difficult to appreciate that a cooperative effort which depends on the structural integrity of several regions of ACTH enable this molecule to exert its full biological activity.

CHAPTER 2

THE PREPARATION OF TRITIATED ACTH BY CATALYTIC DEHALOGENATION OF IODINATED ANALOGUES

Introduction

The search for a convenient parameter which could be used to monitor directly some of the events involved in the mechanism of hormone action has posed an ongoing challenge to investigators. As stated previously, biochemical events such as the generation of cAMP or the transport of ions have been measured in an attempt to gain some insight into the nature of hormonal-cell interactions, but uncertainty persists as to whether these events are truly relevant or merely coincidental. In attempting to obtain a more accurate measure of hormonal activation, it seems almost imperative to approach the problem with a radioactively labeled hormone preparation which can be used to probe cellular interactions directly. This chapter shall therefore be concerned with the description of the preparation and characterizaton of radiolabeled ACTH of high specific activity and full biologic potency.

The Effect of Iodination on the Biological Activity of ACTH

In the preparation of radiolabeled molecules for biochemical and physiological research, the isotope of choice has most often been ^{125}I or ^{131}I due to the high specific activities which can be achieved with their incorporation (2,200 and 16,000 ci/milliatom respectively). Currently, there exist three generally accepted methods which make possible either the chemical or enzymatic radioiodination

of peptides and proteins. The chloramine-T procedure (Hunter and Greenwood, 1962; Greenwood, 1971) is based on the oxidation of carrier-free $Na^{125}I$ or $Na^{131}I$ by chloramine-T, with the subsequent spontaneous reaction of I_2 at the tyrosine, histidine or cysteine residues of the protein. (The mechanism of peptide and protein iodination will be considered in greater detail below). Alternatively, peptides and proteins may be enzymatically iodinated with carrier-free ^{125}I or ^{131}I in the presence of lactoperoxidase and a small amount of hydrogen peroxide (Morrison and Bayse, 1970; Thorell and Johansson, 1971). In this reaction, the enzyme specifically catalyses the direct iodination of tyrosine, and is quite sensitive to enviromental conditions such as pH or solvent. The third method, described by Bolton and Hunter (1973) involves the iodination, by chloramine-T, of an N-hydroxysuccinimide ester containing a phenolic group, and the subsequent attachment of the ester to the ε-amino groups of the peptide or protein to be radiolabeled.

Although each of these three methods is capable of introducing ^{125}I or ^{131}I into proteins and peptides of biological interest to high specific activities, there are severe limitations. In both the chloramine-T and the lactoperoxidase methods, oxidative damage to the chemical structure of the molecule with concomitant loss of biological activity is known to occur. In the case of ACTH, for example, iodination by chloramine-T has been observed

to result in almost complete loss of potency (Greenwood et al., 1963; Landon, 1967; Ress et al., 1971). More recently, other investigators have reported that although iodinated ACTH prepared under more mild oxidative conditions retains some biological activity, the retention of complete potency has not been achieved. For example, McIlhinney and Schulster (1974), using the lactoperoxidase method, have described the preparation of ^{125}I-ACTH which retained 50% of its steroidogenic activity; iodination was predominantly at the tyrosine in position 2 and the extent of iodination was estimated at less than one atom per molecule of ACTH. Lefkowitz et al., (1970), using a modified chloramine-T method, have also prepared an ^{125}I analogue which was reported to have retained at best 50% activity. It should be stated here that some ambiguity exists in the interpretation of these data in that it has been reported that iodinated analogues (as well as those of insulin (Garrat, 1964; Massaglia et al., 1969) and glucagon (Rodbell et al., 1964)) must contain less than one iodine atom per molecule in order to retain full biological activity. Since iodine content is reported only as an average, it stands to reason that some molecules remain uniodinated and could be responsible at least in part for the activity observed.

Although the oxidative damage which can occur during the radioiodination of peptides and proteins presents a serious problem, a recent study by Rae and Schimmer (1974)

has indicated that this oxidation may be reversed with reduction with cysteine, at least in the case of monoiodinated ACTH. This work suggests that perhaps peptides can be iodinated without structural damage provided that extremely careful chemical characterizations and separations are executed throughout the modification. Before leaving the subject of iodination-induced oxidations, it should be pointed out that the Bolton and Hunter method could perhaps offer a viable alternative in the sense that this procedure does not involve the direct exposure of the peptide or protein to either the oxidizing radioiodine solution or any of the iodinating reagents. However, this modification does result in the introduction of a bulky iodine and phenolic group onto potentially important lysine and N-terminal residues; as discussed in Chapter 1, this method would therefore not be acceptable for iodinating ACTH, although it could be useful for introducing radioactive halogen into other molecules of interest.

Assuming that the chemical damage which may be incurred during the iodination can be circumvented or at least controlled, the effect which an alien, bulky, and electrophilic iodine atom might have on biologically important peptides and proteins, particularly when they will be used in mechanism studies,must next be considered. The presence of the large iodine atom, the very significant change in pK value of iodinated tyrosines versus native tyrosine (monoiodo:pK 8.2; diiodo:6.4; tyrosine:10.0), and the resulting lipophilic character of the iodinated aromatic

amino acid must all at least be suspected of altering the physical and biological properties of the iodinated analogue as compared to the native molecule. Moreover, as discussed in Chapter 1, it has been well established that the integrity of the tyrosine in position 2 is important for the peptide to exert full biological activity.

In order to either confirm or disprove whether the presence of iodinated tyrosine does effect the potency of ACTH, a systematic investigation was undertaken to determine the biological activities of the iodinated analogues of ACTH. The use of synthetic derivatives, prepared by Drs. S. Lemaire and D. Yamashiro (1977) using the solid-phase method of Merrifield with the incorporation of 3,5-diiodotyrosine, eliminated the possibility of oxidative damage occurring during iodination procedures; these experiments should indicate, therefore, whether suppressed activity of the iodinated analogues is attributable to oxidation or to the halogen itself. Moreover, since ACTH contains two tyrosines with equal potential to be iodinated (to be discussed below), and since at present there exists no method to selectively halogenate, synthesis of the analogues provided the only means of obtaining ACTH which is iodinated at tyrosine 2 only, tyrosine 23 only, and at both tyrosines 2 and 23; with these analogues, it was therefore possible to ascertain the effect of iodination of a specific tyrosine residue on the biolgical activity.

The biological potencies of the iodinated analogues, relative to the unmodified parent ACTH, were determined in

three assay systems: 1)stimulation of corticosterone and cAMP in isolated rat adrenal cells; 2)stimulation of lipolytic activity in isolated rat adipose cells; 3)dispersion of melanin in frog melanophores. In all assay systems, the presence of the iodine atoms on tyrosine 2 was observed to cause a marked decrease in the ability of the hormone to exert its biological activity. Diiodination of the tyrosine in position 23, however, caused little or no suppression of hormonal stimulation, and the combination of halogenation of both tyrosines 2 and 23 effected no further decrease than iodination at tyrosine 2 alone. A detailed description of experimental procedures and results, followed by a discussion of the implication of these data will be presented at this time. In addition to the biological activity data, the results of a highly specific radioimmunoassay for ACTH in which the iodinated analogues were allowed to compete with the unmodified ACTH for binding to the antibody will also be discussed.

Methodology

Materials:

Bovine serum albumin (BSA), Fraction V, was purchased from Reheis or Armour and was purified as follows: albumin (10% w/v) was dissolved in deionized water, the pH was lowered to pH 3 with cold dilute HCl, and the protein was dialysed at 4°C against Norit charcoal (approximately 0.2% w/v in deionized water) for 18 hours. Following the

charcoal dialysis, the solution was dialysed against several changes of deionized water with stirring for 24 hours at 4°C. The pH of the solution was then adjusted to 7.4 with 1N NaOH; the protein was lyophilised and stored at 4°C until use.

All other chemicals and reagents were used without further purification. Collagenase and Lima Bean Trypsin Inhibitor were products of Worthington; DNase was purchased from Sigma. Chromotropic acid (4,5-dihydroxy-2,7-naphthalene disulfenic acid, disodium salt) was obtained from J.T. Baker. For the radioimmunoassays, tritiated corticosterone (specific activity 40-60ci/mmole) was from New England Nuclear; tritiated α_h-ACTH (specific activity 46ci/mmole) was prepared in this laboratory as described in this chapter; Norit A charcoal was purchased from Pfanstiehl Chemical Company (Waukegan, Ill.); Dextran T70 was a product of Pharmacia Fine Chemicals; PCS was from Amersham/Searle. All synthetic diiodotyrosine human ACTH analogues were prepared by Drs. S. Lemaire and D. Yamashiro and were kindly supplied by Dr. C.H. Li.

For the steroidogenic assays, adrenals were obtained from Nembutal-sedated (0.1ml/100g body weight) male Sprague-Dawley rats (350-400g) purchased from Simonsen (Gilroy, CA); for lipolysis, epididymal fat from male Sprague-Dawley rats (140-180g) was used. In the frog skin assay, Rana pipiens of both sexes were obtained from Dahl Biological Supplies (Emeryville, CA), and were sacrificed

by decapitation.

Methods:

Steroidogenic Assay:

Steroidogenesis was measured according to the method of Moyle et al., (1973). Adrenals were removed from rats and de-fatted with forceps. Decapsulation was achieved by cutting each adrenal in half and gently expressing the tissue from the capsule with forceps. The adrenals were chopped with a razor blade and placed in a 50ml polypropylene conical tube with a small volume of Krebs Ringer Glucose Buffer (KRGB) in ice. Digestion was accomplished by re-suspending the adrenals in fresh KRGB (approximately 1ml/rat) containing 0.5% BSA, 0.4% collagenase, 0.01% DNase, and incubating at 37° C with gentle shaking for one hour. At the conclusion of the incubation the cells were allowed to settle and the supernate was removed. Cells were mechanically dispersed by adding a few milliliters of fresh KRGB-BSA with 0.01% trypsin inhibitor (defined as the incubation buffer), drawing the cell suspension in and out of a siliconized glass Pasteur pipette, allowing the suspension to settle, and then filtering the cloudy supernate through cheesecloth. This process was repeated until the supernate was clear and no undispersed cells remained at the bottom of the tube. The filtered cells were then centrifuged for ten minutes at 200rpm, the supernate was removed, and the cell pellet was washed two times with the incubation buffer.

For the assay, the cells were suspended in fresh incubation buffer and distributed in 0.5ml aliquots to the assay tubes. Hormones (dissolved in 0.001N HCl) were added in 0.1ml aliquots and the tubes were incubated at 37°C for one hour under an atmosphere of 95% O_2/5% CO_2. Steroid production was terminated by boiling the tubes for 10 minutes. Corticosterone was estimated by specific radioimmunoassay using antibodies raised by Dr. A.J. Rao against corticosterone-21-hemisuccinate coupled to BSA (Erlanger et al., 1957). Briefly, an aliquot of the incubation medium was incubated overnight at 4°C with tritiated corticosterone and the antibody in 50mM phosphate buffer pH 7.4 with 0.5% BSA. The bound and free corticosterone were separated by adsorption with charcoal which was accomplished by the addition of 0.5ml charcoal suspension (4mg/ml with 0.025% Dextran in 50mM phosphate buffer pH 7.4), and subsequent centrifugation. An aliquot (0.5ml) of the supernate was added to 4.5ml PCS Solubilizer and counted in a Packard Tricarb Liquid Scintillation Spectrometer.

Lipolytic Assay:

Isolated adipose cells were obtained essentially according to the method of Rodbell (1967) as follows. Epididymal fat pads were removed from rats and placed in normal saline until processed. The tissue was then blotted on filter paper, minced with scissors into pieces 2-3mm square, and placed in a 50ml conical tube containing collagenase (6mg/rat) in Krebs Ringer Bicarbonate Buffer

(KRB) with 4% BSA (3ml/rat). Digestion was carried out with moderate shaking at 37°C until a slurry of cells could be detected when the suspension was swirled, and no large pieces of tissue were evident (this generally took between 30 and 45 minutes). The digested tissue was then expressed through cheesecloth into polystyrene tubes and then centrifuged a 200rpm for 1-2 minutes. The infranate was discarded and replaced with fresh KRB-BSA warmed to 37°C, inverted to mix, and re-centrifuged; this washing was carried out at least twice. The washed cells were re-suspended in fresh warmed KRB-BSA with 0.01% trypsin inhibitor and were then distributed to the assay tubes (10-75mm polystyrene) in 0.5ml aliquots. Hormones were diluted in either 0.001N HCl or KRB-BSA and were added to the cells in 0.5ml aliquots. Incubation was performed at 37°C with gentle shaking for two hours, at which time the lipolysis was terminated by the addition of 1ml ice cold 10% trichloroacetic acid (TCA). Glycerol levels were determined by the chromotropic acid method of Korn (1955), adapted briefly as follows. The TCA-treated incubation mixtures were vortexed and centrifuged at 2000rpm for 20 minutes. An aliquot (0.5ml) of the infranate was removed and acidified with 0.1N H_2SO_4 (0.1ml). The glycerol was oxidized to formaldehyde with 50mM sodium periodate (0.1ml), and after 5 minutes the excess periodate was reduced by the addition of 0.1ml sodium bisulfite (10%w/v). Chromotropic acid reagent (1g dissolved in 100ml water and diluted with 400ml

of 12.5N H_2SO_4) was added (5ml), the tubes were vortexed, covered and boiled for 30 minutes. After vortexing the tubes, the color which developed was read at 570nm, using Glycerol (Merck) as standards.

Frog Skin Assay:

The frog skin assay was performed as described by Ramachandran (1970), briefly as follows. The skins of the sacrificed animals were prepared according to the method of Schizume et al.(1954). The reflectance of the skins was measured with a Photovolt photoelectric reflection meter(model 610) and all readings were taken while the skin was immersed in 20ml of solution in a 50ml beaker. The original reading of the skin after soaking in Ringer's solution for one hour was taken as the base line, and a decrease in reflectance was measured from this value. Readings were taken one hour after the addition of the hormone.

ACTH Radioimmunoassay:

All incubations were carried out in 10-75mm siliconized glass tubes. The hormone, diluted in 50mM phosphate buffer pH 7.4 with 0.5% BSA (assay buffer), was added in a 0.1ml aliquot, followed by the antibody (0.05ml) and the tritiated ACTH (about 10,000 cpm in 0.5ml, diluted in assay buffer). The tubes were incubated at 4°C overnight, and the bound hormone was separated from the free by addition of 0.5ml charcoal suspended in assay buffer (20mg/ml with 1% Dextran), and centrifuged at 3000rpm for 20 minutes. An aliquot

(0.25ml) was removed, added to 4.5ml PCS and then counted in the liquid scintillation counter for tritium content.

Results and Discussion

Biologic Activity of Iodinated ACTH Analogues

As could be anticipated from the structure-function studies discussed previously, both the dose reponse curve in Fig. 2 and the calculated potencies in Table 1 confirm that the integrity of the tyrosine in position 2 is essential for the complete steroidogenic activity of ACTH, while modification of tyrosine 23 depresses the activity only slightly. From the dose response curve, it can be seen that the concentration required for half maximal stimulation has shifted from only $5x10^{-11}M$ for native ACTH to approximately $6.5x10^{-11}M$ for $(3,5\text{-}I_2Tyr^{23})$-ACTH, but to $2x10^{-9}M$ for $(3,5\text{-}I_2Tyr^{2})$ and $(3,5\text{-}I_2Tyr^{2,23})$-ACTH (assuming that stimulation would have reached the same maximum as the native ACTH had a higher dose been tested). Calculation of potencies (Borth, 1960) relative to native ACTH demonstrated that $(3,5\text{-}I_2Tyr^{2})$ and $(3,5\text{-}I_2Tyr^{2,23})$-ACTH are only about 2% as active as the parent molecule, while $(3,5\text{-}I_2Tyr^{23})$-ACTH retains 64% of its activity. It is interesting to note that in the analogue iodinated at both tyrosines 2 and 23 there is no additional suppression of activity relative to $(3,5\text{-}I_2Tyr^{2})$-ACTH. Furthermore, these results are in almost quantitative agreement with Lowry et al., (1973) who assayed iodinated synthetic analogues of $ACTH_{1-24}$ for steroidogenic activity and found potencies

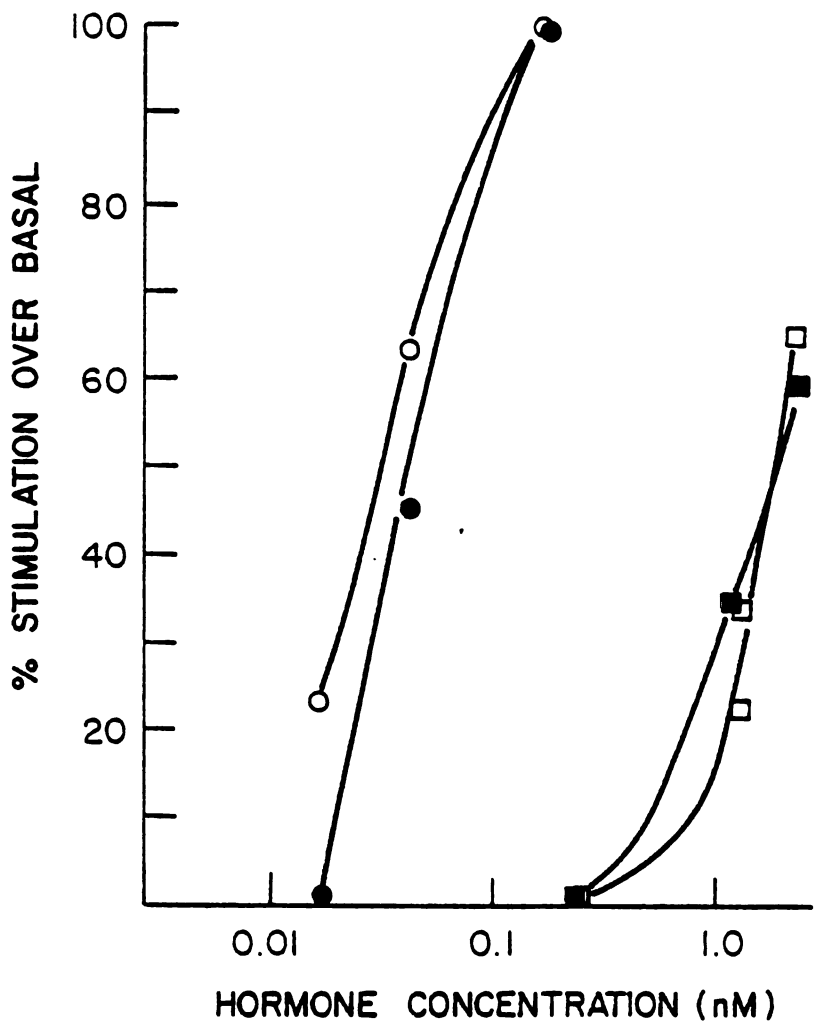


Fig. 2. Stimulation of corticosterone production in isolated adrenal cells by native human ACTH (○), (3,5-I_2Tyr^2) α_h-ACTH (□), (3,5-I_2TYR^{23}) α_h-ACTH (●), and (3,5-I_2Tyr^{2,23}) α_h-ACTH (■).

TABLE 1

Biological Potencies of Iodinated ACTH Analogues

Hormone	Dose, ng/ml	Response	Relative Potency[a] %	95% Confidence	λ
STEROIDOGENESIS					
Natural α_h ACTH	0.1	144.0±4.4[b]	100		
	0.33	165.0±3.6			
$(3,5\text{-}I_2Tyr^2)\text{-}\alpha_h\text{-}ACTH$	5.0	150.4±1.9	2.4	2.0-2.8	0.13
	15.0	164.2±3.0			
$(3,5\text{-}I_2Tyr^{23})\text{-}\alpha_h\text{-}ACTH$	0.1	130.6±3.6	64	42-89	0.13
	0.33	156.0±5.1			
$(3,5\text{-}I_2Tyr^{2,23})\text{-}\alpha_h\text{-}ACTH$	5.0	140.4±3.7	2.2	1.7-2.5	0.17
	15.0	158.4±5.0			
LIPOLYSIS					
Natural α_h ACTH	0.8	1.88±0.021[c]	100		
	4.0	7.50±0.05			
$(3,5\text{-}I_2Tyr^2)\text{-}\alpha_h\text{-}ACTH$	20.0	1.29±0.010	4.1	3.9-4.4	0.02
	100.0	6.4±0.051			
$(3,5\text{-}I_2Tyr^{23})\text{-}\alpha_h\text{-}ACTH$	0.8	2.05±0.051	104	101-109	0.03
	4.0	5.86±0.072			
$(3,5\text{-}I_2Tyr^{2,23})\text{-}\alpha_h\text{-}ACTH$	20.0	1.24±0.051	4	3.6-3.9	0.02
	100.0	6.40±0.051			
MELANOTROPIC ACTIVITY					
Natural α_h ACTH	30	36.5±4.0[d]	100		
	90	53.8±7.8			
$(3,5\text{-}I_2Tyr^2)\text{-}\alpha_h\text{-}ACTH$	30	10.8±4.6	33.4	6.4-64.5	0.24
	90	36.5±2.8			
$(3,5\text{-}I_2Tyr^{23})\text{-}\alpha_h\text{-}ACTH$	30	33.5±3.7	116	64.1-232	0.22
	90	45.5±5.3			

[a]Compared with the potency of natural human ACTH.
[b]Nanograms of corticosterone/10^6cells/h; values in mean ± S.E.
[c]Micromoles of glycerol/g fat/2h.
[d]Percent decrease in reflectance.

of 57% for (3,5-I_2Tyr^{23}) and 3% for (3,5-I_2Tyr^{2})-ACTH. As anticipated, cAMP production in these adrenal cells paralleled precisely steroid production for all of the analogues.

Figure 3 and Table 1 illustrate the results obtained in the lipolytic assay; again a dramatic decrease in biological activity is observed for (3,5-I_2Tyr^{2})-ACTH (half-maximal stimulation 4.4×10^{-10}M for the native ACTH versus 2.0×10^{-8}M for the analogue, or a potency of 4% relative to the native), while the modification on tyrosine 23 causes no apparent suppression of potency. The melanocyte stimulating activity shows the same trends, with a diminishment of activity seen for the (3,5-I_2Tyr^{2}) analogue and no appreciable loss of potency for (3,5-I_2Tyr^{23})-ACTH, as illustrated in Table 1. It is interesting to note that the impairment of the activity of the (3,5-I_2Tyr^{2}) analogue appears not to be as extensive as it is in steroidogenesis and lipolysis.

It is tempting to speculate on how these results might be interpreted in terms of ACTH's mechanism of action. Because the amount of hormone required to attain half-maximal stimulation for the iodinated analogues is shifted to higher concentrations while the magnitude of the response is equivalent to that of native ACTH, it is unlikely that the message portion of the hormone has been altered; rather, it would appear that the affinity of the analogues has been decreased, and that a higher concentration of the modified ACTH is now required for complete activation to be achieved.

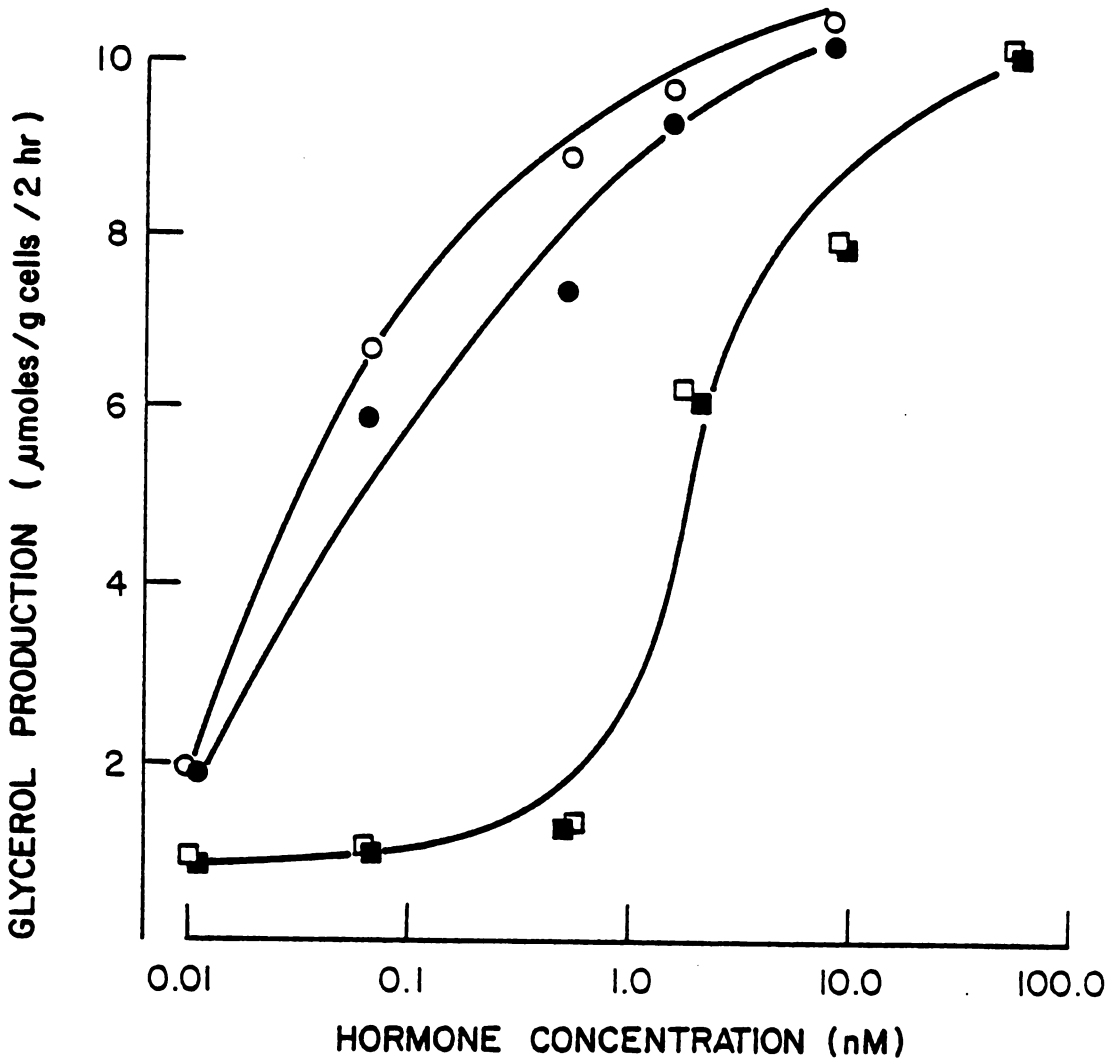


Fig. 3. Stimulation of glycerol production in isolated adipocytes by native human ACTH (○), (3,5-I_2Tyr^2) α_h-ACTH (□), (3,5-I_2Tyr^{23}) α_h-ACTH (●), and (3,5-$I_2Tyr^{2,23}$) α_h-ACTH (■).

In light of this, the dramatic decrease in activity of ($3,5\text{-}I_2Tyr^2$)-ACTH could easily result from a steric hindrance imposed by the bulky iodine atoms which would prevent the proper anchoring of the molecule near its receptor site. Moreover, the change in pK of the tyrosine from 10.0 to 6.4 for diiodotyrosine could mean that since the diiodotyrosine is largely ionized at physiological pH's, there may also be a charge interference which again makes it difficult for the analogue to approach the cellular binding site. Alternatively, the presence of the iodine atoms could also alter the rate of degradation or turnover of the hormone in *in vitro* systems. Saez *et al.*, (1975) have reported on this phenomenon in isolated adrenal membranes and postulate at least two enzyme systems capabale of degrading $ACTH_{1-24}$ and $ACTH_{11-24}$ differentially. Perhaps even more importantly, however, is the fact that the iodination of the tyrosines increases dramatically the hydrophobicity of the analogues (Lemaire *et al.*, 1977), thereby making them much less soluble in aqueous systems; it is possible that this decrease in solubility reduces the concentration of the iodinated hormones in the vicinity of the receptor.

The relative insensitivity of the biological response to iodination at tyrosine 23 agrees well with the accepted concept that only residues 1-19 are necessary for ACTH to exert its full biological activity in *in vitro* systems (Ramachandran, 1973). It is interesting to note, however, that the data presented here would indicate that the

steroidogenic activity is more sensitive to modification at the C-terminal tyrosine than are the lipolytic or melanocyte activities. In its broadest interpretation this observation could imply that there is a difference in the topography around the receptor, and that modification of tyrosine 23 somehow interferes with adrenal but not adipocyte or melanocyte response. This situation would seem unlikely, however, in view of the fact that despite the many analogues tested, adrenal and adipocyte receptors have always exhibited the same structural requirements for activation (Ramachandran, 1973). Perhaps the presence of the iodine atoms on tyrosine 23 alters slightly the solubility and/or the rate of degradation of the molecule (as discussed above), and this subtle decrease in adrenal-stimulating activity is more readily detected by a radioimmunoassay for corticosterone than by a chemical analysis for glycerol concentrations. The final interpretation of the discrepancy between the steroidogenic and lipolytic activities of the ($3,5\text{-}I_2Tyr^{23}$) analogue must however await a more intensive analysis.

Immunologic Activity of Iodinated ACTH Analogues

In addition to the biological activity, the immunreactivity of the iodinated ACTH analogues was also investigated, using a highly specific radioimmunoassay for ACTH (Rao, A.J., Behrens, C. M., Ramachandran, J., manuscript in preparation). From Fig. 4 it can be seen that for the antibody used in this study, the immunological activity of ACTH is virtually insensitive to iodination of the tyrosine in position 2, while a significant decrease in the ability

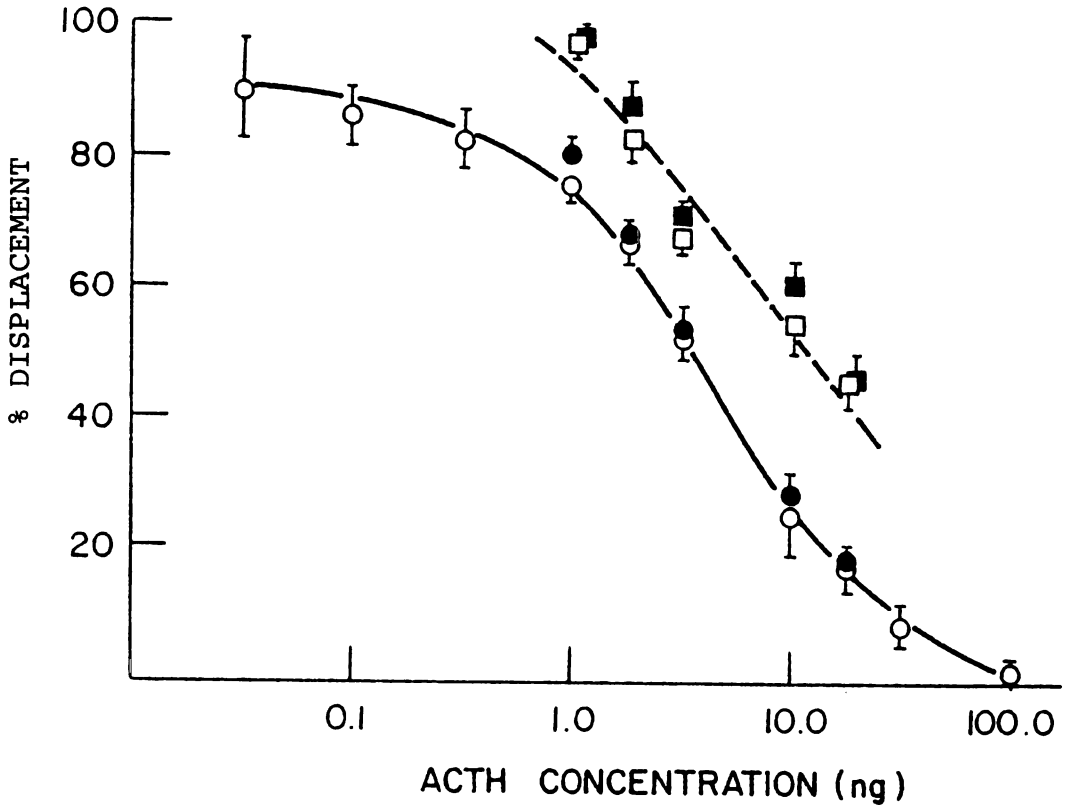


Fig. 4. Displacement of $^{3}H\ \alpha_h$-ACTH from its specific antibody by native human ACTH (○), (3,5-I_2Tyr^2) α_h-ACTH (●), (3,5-I_2Tyr^{23}) α_h-ACTH (□), and (3,5-I_2Tyr^{2,23}) α_h-ACTH (■).

to displace native ACTH from its antibody is observed for ($3,5\text{-}I_2Tyr^{23}$) and ($3,5\text{-}I_2Tyr^{2,23}$). In view of the fact that the sequence of the peptide near the site responsible for biological activity is generally conserved from species to species, it is not surprising that antigenic activity is directed against a portion of the molecule not involved in receptor interaction, and which evolutionarily might be more susceptible to change. The use of iodinated analogues in both biological and immunological systems illustrates perfectly the dichotomy between these two molecular properties and the fact that one activity need not (and indeed probably will not) parallel the other. The widespread use of ^{125}I or ^{131}I hormones in radioimmunoassay must be interpreted cautiously, particularly when immunological activity is used as an index of biological function. As has been illustrated for ACTH, the actual presence of iodinated tyrosines may very well alter the biological and immunological properties of the molecule, while the process of iodination itself may cause structural change and/or damage. Each iodinated hormone must be carefully tested to assess these problems; although the iodinated analogues will be used in biological systems, the chemical integrity of the molecules cannot be compromised.

To return to the problem at hand, the case with ACTH is clear. If an ^{125}I or ^{131}I analogue were to be used in mechanism studies, the methionines, cysteines and tryptophan must be protected from oxidation. In addition, the ^{125}I

or ^{131}I must be directed exclusively to tyrosine 23 if full biological activity is to be preserved; at present, however, no method exists to selectively iodinate one tyrosine over another. Perhaps it would be feasible to synthesize an ACTH analogue with phenylalanine in position 2 (a small loss of biological activity occurs (Geiger et al., 1964)), and then iodinate via lactoperoxidase with ^{125}I or ^{131}I so that tyrosine 23 would be labeled; nevertheless, some immunological activity would be compromised. For the present mechanism studies, it was decided that a tritiated ACTH of maximum specific activity possessing full biological and immunological potency would be the optimal molecule to use, and this next section will discuss its preparation and characterization.

Tritiation of $\alpha ACTH_{1-39}$

The problem of introducing tritium to a high specific activity into a peptide or a protein molecule has been approached by a number of investigators who have explored a variety of methods. One of the earliest attempts involved the incorporation of tritiated leucine in the synthesis of oxytocin by du Vigneaud et al., (1962); although chemically successful, the synthetic method yielded a tritiated hormone of extremely low specific activity (0.117ci/mmole). This technique was largely abandoned perhaps because of the expense which would be involved in introducing a concentration of tritiated amino acid high enough to increase the specific activity. However, Halban and Offord (1975) have recently adapted the semi-synthetic

preparation of insulin to a micro scale, and were able to introduce tritiated phenylalanine in sufficient quantity to obtain a specific activity of 20ci/mmole.

Several methods which introduce tritium through the chemical modification of specific amino acids have been applied to peptides and proteins with limited success. Acetylation with tritiated acetic anhydride results in the labeling of primary amino acids and tyrosines (Cuatrecasas et al., 1971), and although it can be accomplished under relatively mild conditions with no oxidation or denaturation involved, the specific activity of the product is severely limited by the specific activity of the anhydride, and the number of reactive groups as well. Furthermore, for many molecules modification of primary amino groups will result in a loss of biological potency; with ACTH, for example, both the N-terminal ε-amino group and the lysines are essential for the full expression of activity, as discussed in Chapter 1. A similar method, involving the tritiated acetamidination of the free amino groups has also been employed (Hunter and Ludwig, 1962), but this procedure suffers from the same disadvantages as the acetylation, particularly where ACTH is concerned.

Other chemical modifications involving the reaction of ε-amino groups with Schiff's base followed by reduction with sodium borotritide have also been employed (Rice and Means, 1971; Churchich, 1965); however, the peptides and proteins tritiated in this manner are again of low specific activity

and reduced biological potency.

A somewhat unique method for introducing tritium into biologically relevant molecules has generated a great deal of interest during the last few years; this procedure, a modification of the Wilzbach method, involves the bombardment of the substrate with tritium atoms, which have been generated by the microwave activation of tritium gas (Hembree, 1975). The material which is tritiated with this method will, however, be randomly labeled, as tritium atoms will be placed in any position occupied by an exchangeable hydrogen atom. There is also some question about the destructiveness of this technique, although Hembree et al., (1973) have reported the tritiation of $ACTH_{1-24}$ to a specific activity of 15ci/mmole and full biological potency.

It would appear from the literature that the most consistently successful method for preparing tritiated peptides is by the reduction of their halogenated analogues with tritium gas in the presence of a catalyst (Morgat et al., 1970; Morgat et al., 1970a; Pradelles et al., 1972; Marche et al., 1972; Brundish and Wade, 1973). In this technique, as in those used for radioiodination, the susceptibility of the tyrosine residue to halogenation is again a key aspect; however, in catalytic dehalogenation it is only an intermediate step which serves to introduce a non-radioactive iodine atom which can then be replaced through reduction with a tritum atom. With this procedure several small biologically active peptides have been tritiated to near-theoretical specific activities, for example, angiotensin II

(Morgat et al., 1970), gonadotropin releasing factor (Marche et al., 1972), and oxytocin (Morgat et al., 1970a). However, lower specific activities and poorer yields were generally obtained when this method was applied to more complex peptides such as lysine-vasopressin (Pradelles et al., 1972), although Brundish and Wade (1973) have succeeded in tritiating protected $ACTH_{1-24}$ to a specific activity of 45ci/mmole. A slight modification of this catalytic dehalogenation procedure, in which dehydro-amino acid double bond rather than a halogen is reduced by tritium gas in the presence of palladium/charcoal, has also been used (Schwyzer and Karlaganis, 1973).

In view of the apparent success of employing catalytic dehalogenation to incorporate tritium into biologically active peptides, this procedure was adopted for the preparation of the tritiated $ACTH_{1-39}$ to be used in this study. It was therefore necessary to chemically iodinate the ACTH with non-radioactive iodine (that is, before the synthetic iodinated analogues became available), and then reduce this halogenated peptide in the presence of tritium gas; these procedures will be described in detail at this time.

Iodination of $ACTH_{1-39}$

Materials:

Synthetic 1-39 porcine ACTH was obtained from Ciba Pharmaceuticals; for chromatography, microgranular carbomethyl cellulose (CMC) was purchased from Calbiochem. Trypsin (TPCK) and leucineaminopeptidase were products of Worthington; acid protease was obtained from Miles.

Methods:

Iodination: Synthetic porcine ACTH (18mg) was dissolved in 3ml 0.02M bicarbonate buffer, pH 9.0 and was then reacted with 0.175ml 0.1M I_2 in KI (a 4-molar excess over the peptide) at 0°C; the KI/I_2 was added stepwise at 0, 15, and 60 seconds. Disappearance of the iodine color was almost instantaneous; however, the reaction was allowed to proceed for 5 minutes at which time it was quenched with 10 µmoles DTT. The reaction mixture was lyophilized and then desalted on Sephadex G-25 equilibrated with 0.1N acetic acid. The desalted peptide was chromatographed on a column of microgranular CMC (1x25cm) equilibrated with 0.01M ammonium acetate, pH 4.5; the gradient was established by introducing 100ml 0.1M ammonium acetate, pH 4.8 through a 125ml mixing chamber containing the starting buffer, then substituting 0.2M ammonium acetate, pH 4.8 for the solution flowing into the mixing chamber, followed by 0.4M ammonium acetate, pH 4.8. The flow rate was approximately 60ml/hour and fractions of 2ml were collected; the absorbance was read on a Beckman DB-GT grating spectro-

photometer at 280nm.

Characterization:

Ultraviolet absorbance spectra were scanned in a Beckman DK-2 recording spectrophotometer in 1cm path length matched silica cuvettes. Peptides were dissolved in 0.1N acetic acid, and the same solvent was used as the reference solution. The absorbance was measured from 360 to 250nm; light scattering was corrected according to the method of Beaven and Holiday (1952).

The difference spectra of the peptides were obtained under similar conditions, with the exception that both sample and reference cuvettes contained the peptide (at the same concentration) in 0.1N acetic acid, and the pH of the solution in sample cuvette was raised to 12 with the addition of a small volume (10µl) of concentrated NaOH(10N). The difference in the absorbance of the ionized versus the unionized peptide was then measured from 360 to 260nm.

Enzymic hydrolysis of the peptide was accomplished as follows. The peptide (0.5mg) was dissolved in 0.01N HCl and incubated with acid protease (0.05mg) at 37° overnight, lyophilized, reconstituted in 0.05M Tris buffer pH 8.5 with 0.01M $mgCl_2$. Leucineaminopeptidase (0.015mg) was added, and the mixture was re-incubated at 37° overnight. Amino acid analyses were performed on a Beckman Model 120B Analyzer.

Results and Discussion:

The iodination of peptides and proteins was investigated as early as 1945 by Li who studied the reactivity of serum albumin and pepsin. Under mild reaction conditions the iodination of peptides and proteins will affect only the tyrosyl, histidyl and cysteinyl residues, while with more rigorous conditions, tryptophan and methionine may also be reacted (Means and Feeney, 1971). The mechanism of iodination is believed to be a classic electrophilic substitution as illustrated in Fig. 5.

The strategy adopted for the halogenation of ACTH was to design conditions where both tyrosines would be diiodinated in positions 3 and 5, thereby establishing the potential for achieving maximum specific activity in the tritiation. However, to insure that the tritiated molecule would still possess full biological activity, it was also important to minimize any oxidative side reactions (since ACTH contains both methionine and tryptophan), to make certain that only tyrosines were halogenated, and to avoid any deamidation of the molecule. Consequently, relatively mild reaction conditions of low temperature (0°), high pH (9.0), the generation of I_3 from KI/I_2, no additional iodine above the required 4-molar excess over peptide, and short duration of reaction (5 minutes) were selected.

In order to isolate the reaction products the mixture was applied to a CMC column; theoretically, the iodine atoms undergo hydrophobic interactions with the cellulose,

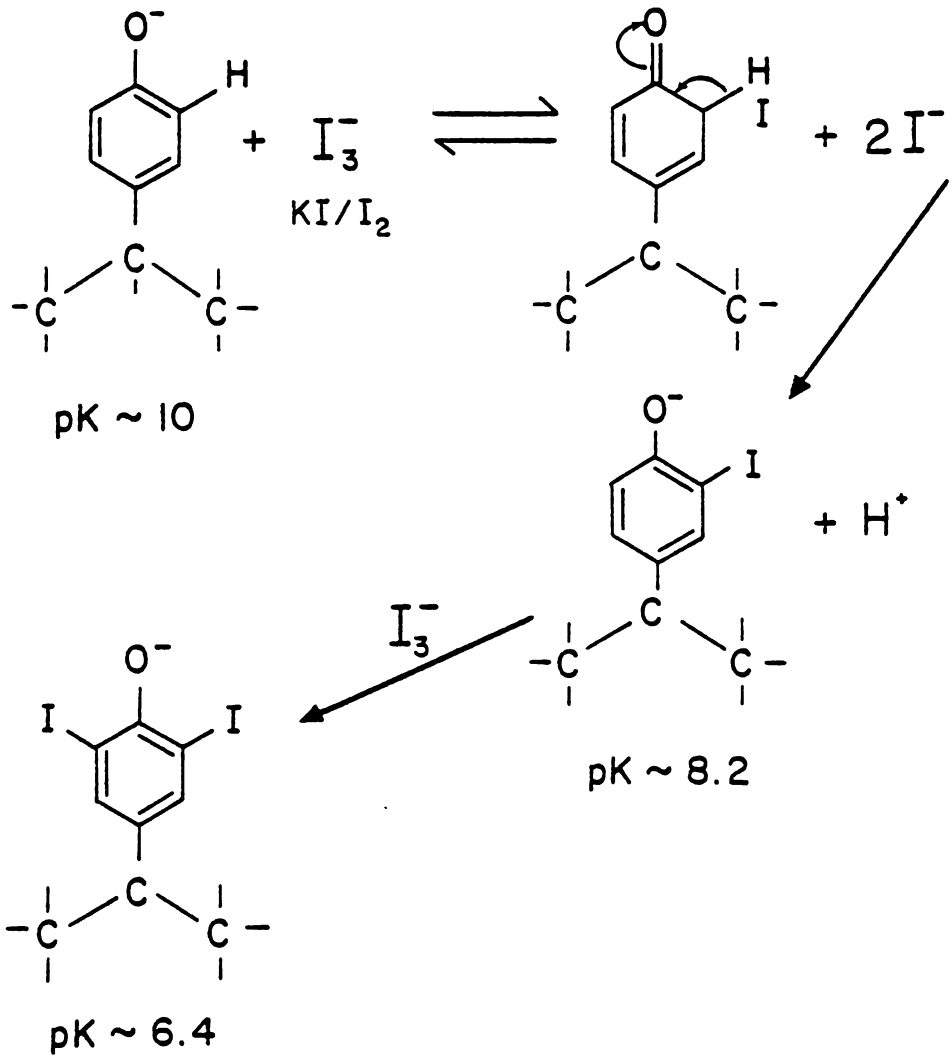


Fig. 5. The mechanism of tyrosine iodination is assumed to proceed via a classic electrophilic substitution. In this mechanism, I^+ attacks the ring; the intermediate structure is stabilized by the ionized oxygen atom which can share 2 pairs of electrons with the benzyl ring and thereby accommodate the positive charge. The reaction is completed by the loss of H^+; the second iodine atom can also react to form diiodotyrosine.

and consequently, iodinated species are retarded on the column. However the presence of the iodine on the tyrosine will easily effect its ionization at neutral pH and thereby cause the modified ACTH to elute from the column prematurely. These two opposing physical tendencies would result in the elution of iodinated ACTH in an extremely broad peak; therefore, in order to suppress the ionization of the iodinated tyrosines, the pH of the gradient must remain acidic throughout the fractionation. Under these conditions, the separation will depend only on the hydrophobic interaction of the iodine atoms; the elution pattern of the iodination reaction mixture is illustrated in Fig. 6. Lefkowitz et al. have reported a more complete separation of iodinated ACTH on CMC using a linear rather than a convex gradient (1970).

Fractions were pooled as indicated, and although the extent of iodination could be anticipated from the elution position, the exact degree of halogenation was determined by the technique of difference spectroscopy (Woody et al., 1966; Edelhoch, 1962). Since reference will be made to this technique several times, and since it also plays an essential role in monitoring the tritium reduction, the principles involved in difference spectroscopy will be described briefly below. The difference spectra of the CMC fractions resolved the iodination reaction mixture into three major species: Fraction 1, (3-ITyr)-ACTH and unreacted ACTH (Fraction 1 was further resolved on partition chromatography (Yamashiro, 1964) in butanol: acetic acid:

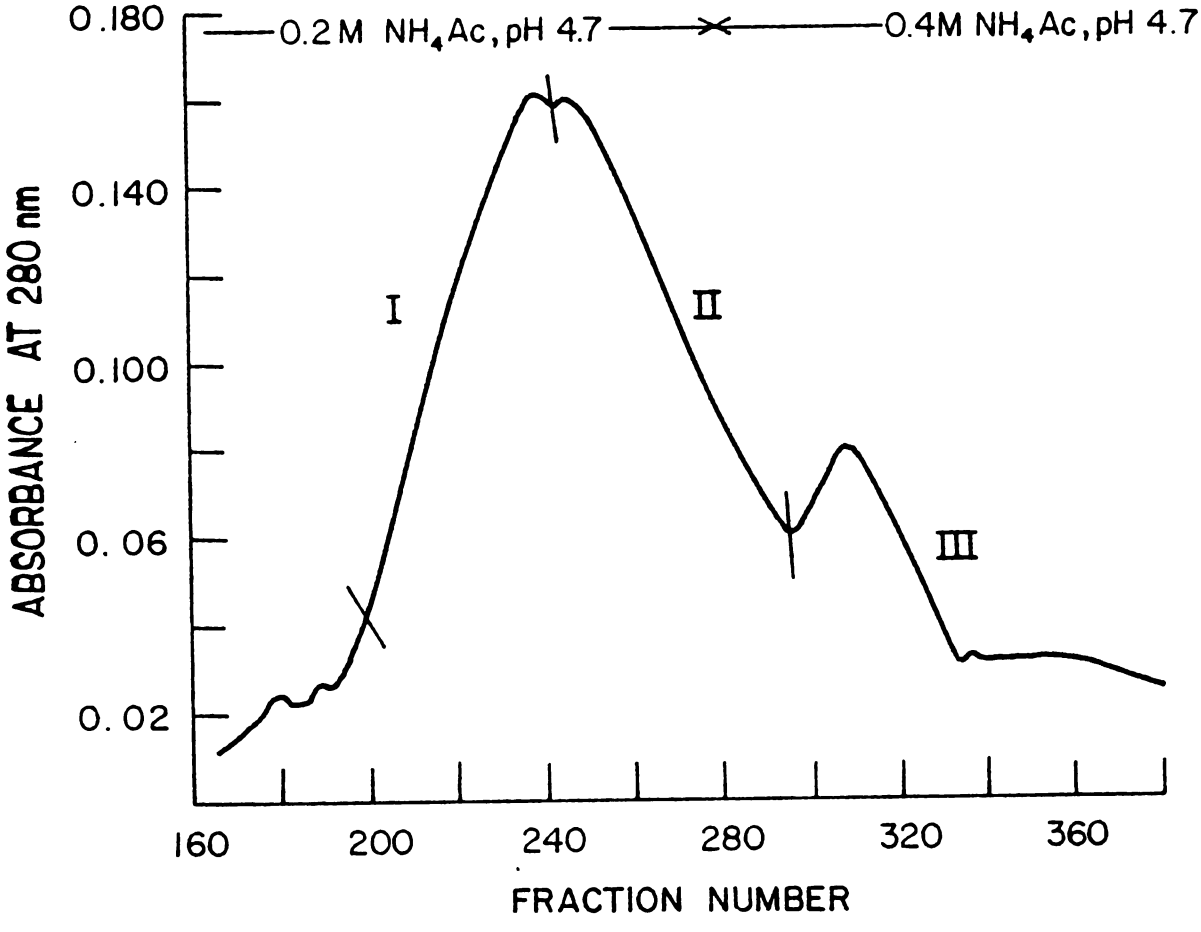


Fig. 6. Chromatography on carboxymethyl cellulose of reaction mixture from the chemical iodination of porcine ACTH. The product of iodination obtained after de-salting on Sephadex G—25 was applied to a column of Whatman microgranular cellulose (1x25cm) equilibrated with 0.01 M ammonium acetate, pH 4.5. The gradient was established by introducing 0.1 M ammonium acetate (pH 4.8) through a 125ml mixing chamber containing the starting buffer. After 60 fractions were collected the gradient was increased by substituting 0.2 M ammonium acetate as the solution flowing into the mixing chamber; after 120 fractions, completion of the gradient was accomplished by introducing 0.4 M ammonium acetate, pH 4.8. The flow rate was 60ml/hour and 2ml fractions were collected; the absorbance was read at 280nm.

water, 4:1:1 to obtain these results); Fraction 2, (3-ITyr^{2,23})-ACTH; Fraction 3, (3-ITyr^{2,23})-ACTH (the extremely low yield made analysis difficult, but it appears to be the same species as Fraction 2, which had been eluted abruptly when the gradient was changed).

Because sufficient iodine was reacted to diiodinate each of the two tyrosines (hence, the 4-molar excess over peptide), it was at first surprising that the molecule was not halogenated to a greater extent. Analysis by difference spectroscopy indicated that approximately 2/3 of the ACTH was monoiodinated at both tyrosines, while approximately 1/3 remained unmodified; it is interesting to note that no diiodotyrosine could be detected. There are, however, several explanations for the limited halogenations. To begin with, iodinations carried out above pH 8 are known to favor the formation of the monoiodinated species (Means and Feeney, 1971). Secondly, at alkaline pH's several reactions of iodine with water have been reported to occur (Harrington and Neuberger, 1936), and this phenomenon could certainly reduce the effective iodine concentration. Finally, the short reaction time, the low temperature, and the overall mild reaction conditions which were selected to insure the integrity of the other residues, have no doubt suppressed the extent of iodination.

Because it had been iodinated more extensively, Fraction 2 was selected for reduction with tritium. Amino acid analysis following leucineaminopeptidase digestion was

performed on the peptide and confirmed that half the tyrosine content had been modified (as it no longer eluted with tyrosine), and also that the iodination did not promote the oxidation of any of the residues.

As mentioned previously, before proceeding to the tritiation, a discussion of the technique of difference spectroscopy and how it has been used to determine the extent of tyrosine iodination, will be presented at this time. To begin with, the direct ultraviolet spectrum of a peptide or protein represents the absorption bands of a chromophore as it undergoes a transition from the energy level of the ground state to an excited state. The energy for this transition is provided by the incident light which is passed through the chromophore, and the spectrum will show the absorbance as a function of the wavelength of this light.

The characteristic absorbance of proteins and peptides in the 240 to 300nm region is attributed to the presence of tyrosine and tryptophan, with some minor contribution from phenylalanine. As early as 1905 it had been known from the work of Baly and Ewbank (Baly and Ewbank, 1905) that the spectrum of tyrosine is extremely sensitive to pH changes, while the absorbance of tryptophan will remain virtually unaltered whether measured at acid or alkaline pH's. It is now well established that the tyrosine absorbance shifts to longer wavelengths at alkaline pH's, and this phenomenon has been ascribed to the ability of the phenolic ring to undergo ionization. It should be recalled that the presence of

iodine reduces dramatically the pK_a value of tyrosine, or in other words, the iodine enhances the ionization potential of the tyrosine. It would seem that this iodine atom, by providing another nucleus, increases the probability that the phenoxide electron will be found away from the oxygen atom; in a sense therefore, this electron becomes more loosely bound and as a consequence the energy required for its promotion to an excited state is reduced. Thus, the absorbance of an ionized iodinated tyrosine will be shifted to a higher wavelength, which reflects the lower amount of energy required for the transition.

In order to focus on the absorbance of the tyrosine chromophore and to make this transition more apparent, the technique of difference spectroscopy is applied. In this method, both the sample and reference cuvettes contain the same peptide at identical concentrations, the difference being that one peptide is exposed to pH 12 where all the tyrosines will be ionized, while the other is kept at pH 3, where no tyrosyl residues have undergone ionization. Since tryptophan residues will be unaffected by this pH change, the resultant spectrum will indicate only the difference in the absorbance of the ionized and unionized tyrosines; the magnitude of the wavelength shift will reflect the extent of iodination.

In order to quantitate this technique, the difference spectra of various model solutions containing relative amounts of all chromophores which would be present in native ACTH and all of its mono- and diiodinated analogues were

determined. Table 2 indicates the maximum wavelengths which were characteristic of the varying degree of iodination. The wavelength of maximum absorbance was also determined for the synthetic iodinated ACTH analogues, and as can be seen in Table 2, these values agree precisely with their corresponding model peptides. Fraction 2, which was the most retarded major fraction of the CMC chromatograph of the iodination reaction mixture and which was selected for tritiation, displayed a λ_{max} of 305nm; this wavelength maximum strongly suggested the presence of two monoiodotyrosines, and this was later confirmed in the tryptic digest of the tritiated peptide.

Although the difference spectrum is the optimal method for determining the extent of tyrosyl iodination, an approximation can be made from the direct ultraviolet spectrum, particularly in those instances when the amount of peptide required for the difference spectrum is not available (for example, in monitoring column eluate). Using the synthetic iodinated analogues prepared by Lemaire and Yamashiro, the effect of diiodotyrosine on the extinction of the peptide has been investigated as indicated in Fig. 7. It can readily be seen that whereas the ratio of $\text{extinction}_{275}/\text{extinction}_{285}$ in the unmodified ACTH is approximately 1.5, the substitution of one diiodinated tyrosine for unmodified tyrosine results in a ratio of about 1.2 while the replacement of both tyrosines with the diiodo analogue yields a ratio of approximately 1.0. This increase in the

TABLE 2

λ_{max} From Difference Spectra of Iodinated Analogues

	λ_{max}, nm
MODEL PEPTIDES	
Tyr, Tyr	293
ITyr, Tyr	297
ITyr, ITyr	305
I_2Tyr, Tyr	306
ITyr, I_2Tyr	309
I_2Tyr, I_2Tyr	311
SYNTHETIC ACTH ANALOGUES	
(3,5-I_2Tyr^2, Tyr^{23})-α_h-ACTH	306
(Tyr^2, 3,5-I_2Tyr^{23})-α_h-ACTH	306
(3,5-$I_2Tyr^{2,23}$)-α_h-ACTH	311
CHEMICALLY IODINATED ACTH	
Fraction II	305

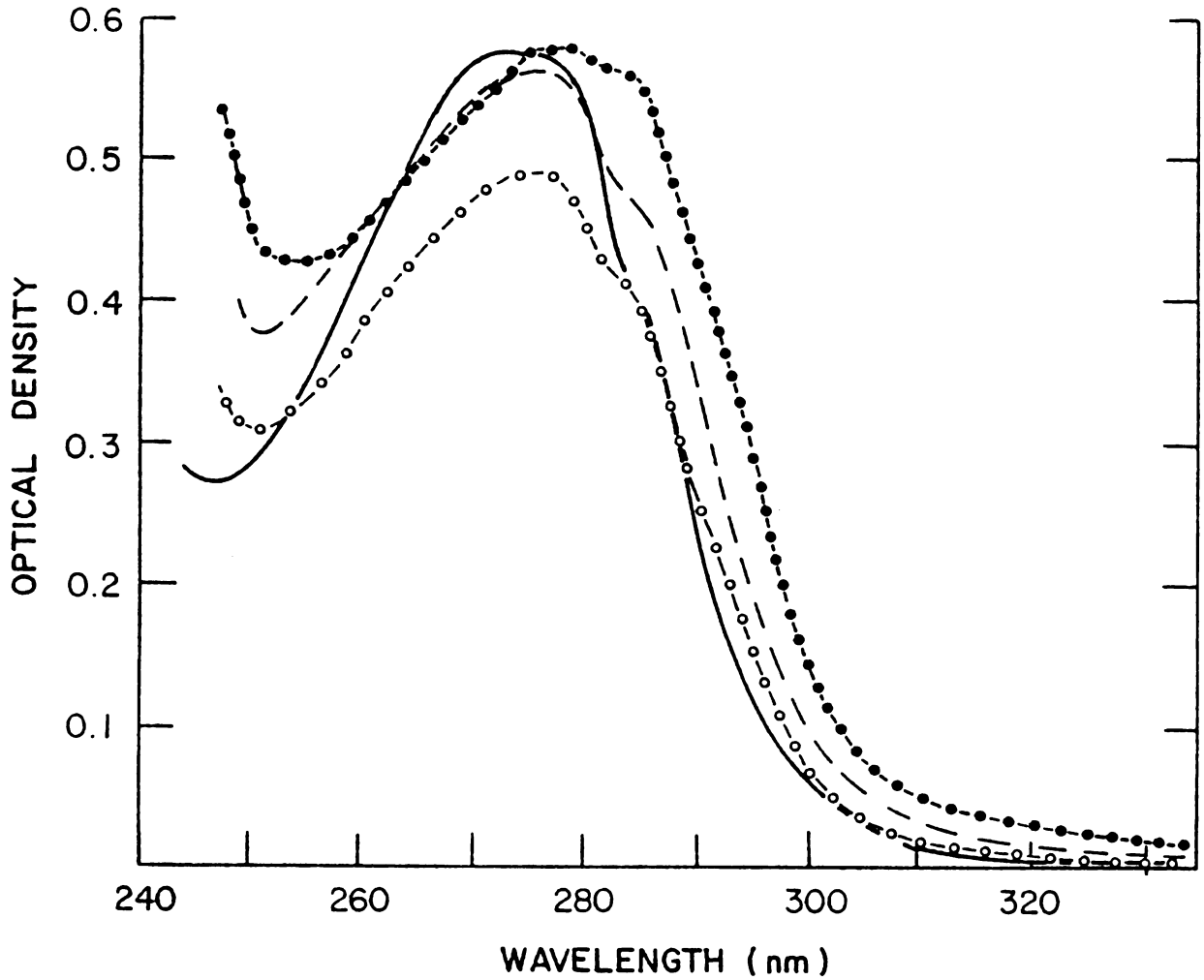


Fig. 7. The U.V. absorption spectra of the iodinated ACTH analogues. (——) native human ACTH; (- - -) (3,5-I_2Tyr^2) α_h-ACTH; (-o-o-) (3,5-I_2Tyr^{23}) α_h-ACTH; (-o-o-), (3,5-I_2Tyr^{2,23}) α_h-ACTH. All spectra were taken as described in the text. The following extinction coefficients were determined for the analogues: (3,5-I_2Tyr^2) α_h-ACTH, $\varepsilon^{1\%}_{280nm}$ = 1.79; (3,5-I_2Tyr^{23}) α_h-ACTH, $\varepsilon^{1\%}_{280nm}$ = 1.79; (3,5-I_2Tyr^{2,23}) α_h-ACTH, $\varepsilon^{1\%}_{280nm}$ = 1.86.

absorbance of the iodinated peptides at 285nm reflects the shift to higher wavelengths which is characteristic of iodinated tyrosines (Gemmill, 1955), and monitoring this shift can provide at least a qualitative assessment of the extent of tyrosine iodination.

Reduction and Tritiation of Iodinated $ACTH_{1-39}$

Materials:

Synthetic porcine ACTH was monoiodinated at tyrosines 2 and 23 as described above. Synthetic ($3,5\text{-}I_2Tyr^2$), ($3,5\text{-}I_2Tyr^{23}$) and ($3,5\text{-}I_2Tyr^{2,23}$)-α-human $ACTH_{1-39}$ were prepared by Drs S. Lemaire and D. Yamashiro and were kindly provided by Professor C.H. Li, as was the synthetic α-human $ACTH_{1-39}$. Palladium black was freshly prepared from palladium chloride (Matheson) as follows (Willstatter and Waldschmidt-Leitz, 1921). Palladium chloride (100mg) was dissolved with heating in 2N HCl (5ml). The pH of the solution was brought to 7.0 with 2N KOH. Formic acid (0.2ml) was added dropwise until the palladium precipitated out. This reaction was allowed to proceed until the evolution of CO_2 ceased; the palladium was filtered and washed thoroughly with water. Palladium oxide was obtained from Matheson. Dimethyformamide and hexamethylphosphoramide were products of Aldrich. Fluorescamine was obtained from Hoffman-LaRoche and PCS scintillation cocktail was from Amersham/Searle.

Methods:

This method has already been described (Ramachandran and Behrens, 1977), but will be reviewed here in greater detail. Catalytic dehalogenation in the presence of pure tritium gas was performed at the Lawrence Berkeley Labs in the tritium system described by Eterovic et al. (1975). Briefly, this system contains a uranium trap where uranium is converted to the hydride as follows: $2U + 3H_2 \rightleftharpoons 2UH_3$. The trap is heated and begins to release pure $^3H_2$ at approximately 400°C.

Tritiation 1:

The chemically iodinated α-porcine (3-1Tyr^{2,23})-ACTH (6mg) was dissolved in the reaction vessel in 2ml 0.1N acetic acid to which was added aproximately 10mg freshly prepared palladium black. The peptide and catalyst were exposed to the tritium gas at a pressure of 650-700mm of Hg at room temperature with stirring for two hours. At the conclusion of the reaction period, the catalyst was removed by centrifugation and washed several times with 0.1N acetic acid; the combined liquors were pooled and lyophilized to remove most of the exchangeable tritium atoms. All of the above procedures were performed in the glove box at the Lawrence Berkeley Labs. The lyophilized peptide was then re-lyophilized from a large volume of water (200ml) to remove additional labile tritium. The twice-lyophilized peptide was reconstituted in 0.01M ammonium acetate pH 4.8 and was applied to a microgranular CMC column (1x

25cm) using the same gradient as described previously, with the exception that the gradient was not started until at least two hold-up volumes were run through the column to remove the remaining exchangeable tritium ions. Periodic aliquots of column eluant were checked for radioactivity, and the gradient was not begun until these counts reached a low, stable background - approximately 25,000 cpm/0.1ml. It is interesting to note that despite two lyophilizations, a large amount of exchangeable tritium remained associated with the ACTH and this could apparently be removed only by extensive washing of the CMC-bound peptide.

Tritiation 2:

Synthetic α-human (3,5-I_2Tyr^{23})-ACTH (6mg) was dissolved in the reaction vessel in 2ml 0.1N acetic acid to which was added 10mg pulverized palladium oxide (it was observed that the reduction proceeded more quickly if the catalyst was first ground to a fine powder in a glass mortar and pestle). The rest of the tritiation was carried out exactly as in Tritiation 1.

Tritiation 3:

Synthetic α-human (3,5-I_2Tyr^{23})-ACTH (6mg) was dissolved in 0.025ml 0.1N acetic acid, to which was added 0.25ml haxamethylphosphoramide (HMPA) and 2.5ml dimethylformamide (DMF). The catalyst was prepared as follows. Palladium oxide (20mg) and calcium carbonate (200mg) were pulverized and mixed well. A portion of the mixture (50mg) was deposited in the reaction vessel with the dissolved peptide and

an additional 50mg of catalyst mixture were placed in the "boat". The reduction was carried out as described previously for 1 hour (the palladium oxide was observed to turn black after 15 minutes, indicating that it had been reduced to palladium); at the conclusion of the first hour, the catalyst in the boat was added to the solution and the reduction was continued for an additional 1.5 hours. At the end of the reduction period, 1ml of 1M solution of DTT/0.1N acetic acid was added to the reaction mixture; the catalyst was then easily centrifuged out and washed twice with 0.1N acetic acid. The pooled liquors were lyophilized overnight to remove the most labile tritium and this was followed by a second lyophilization from a large volume. The peptide was then reconstituted in 0.1N acetic acid (1ml) and de-salted on Sephadex G-25 equilibrated in 0.1N acetic acid. CMC chromatography was carried out exactly as described previously.

Determination of Specific Activity:

Whenever possible, peptide concentrations were determined by ultraviolet absorption at 280nm. Peptides were dissolved in 0.1N acetic acid and scanned against the same solvent on a Beckman Model DK-2 recording spectrophotometer from 360 to 250nm; matched silica cuvettes of 1cm path length were used. Light scattering, when present, was corrected by extrapolating the absorbance from 360 through 340nm to 280nm and subtracting this value, as described by Beaven and Holiday (1952). A molar absorptivity of 1.72

was assumed for ACTH.

Where concentrations were too low to be determined spectrally, fluorescamine analysis was performed essentially according to the method of Nakamura and Pisano (1976). Briefly, peptides were diluted in 1ml of 0.1M borate buffer pH 9.0; to this was added 0.5ml of 0.02% fluorescamine (w/v in spectral grade acetone) while vortexing vigorously. After 30 minutes, the fluorescence at 390nm excitation and 510 emission was determined on a G.K. Turner Model 111 fluorometer. Synthetic α-human ACTH was used as a standard, and this method could reliably detect levels as low as 1μg/ml of ACTH.

The specific activity was determined by removing a small aliquot of peptide from a solution of known concentration and pipetting it directly into a scintillation vial containing 4.5ml of PCS. At least two concentrations of the peptide were used in determining the specific activity. Siliconized glass pipettes were used for all transfers and they were rinsed out several times in the PCS to remove any adsorbed counts. Radioactivity was counted in a Packard Tricarb liquid scintillation spectrometer Model 3320; a counting efficiency of 32% was calculated, using tritiated water as the standard.

Results and Discussion:

The procedure of catalytic dehalogenation of iodinated peptides in the presence of tritium has been well documented and applied successfully to obtain a number of tritiated

peptides, as described in the introduction to this chapter. However, some difficulty was encountered in obtaining an ACTH of high specific activity in the early phases of this study; theoretically, a specific activity of 29ci/mmole can be expected for each ^{3}H atom incorporated. Schwyzer and Karlaganis (1973), reducing the dehydro-norvaline residue of Phe^{2}, 4,5-dehydronorvaline^{4}-$ACTH_{1-24}$, also obtained a tritiated ACTH of low specific activity (7.42ci/mmole) with a 10% biological potency; additionally, Pradelles et al. had little success with lysine vasopressin (1972). A systematic study of this problem has been conducted and has resulted in the preparation of tritiated ACTH of 46ci/mmole (from the $(3,5\text{-}I_2Tyr^{23})$ analogue) and 90ci/mmole (from the $(3,5\text{-}I_2Tyr^{2,23})$ analogue) or approximately 80% of the obtainable theoretical values. In scrutinizing the problem of reduced specific activities, some insights into the mechanism of the catalytic dehalogenation have been garnered, and these shall be discussed here in some detail.

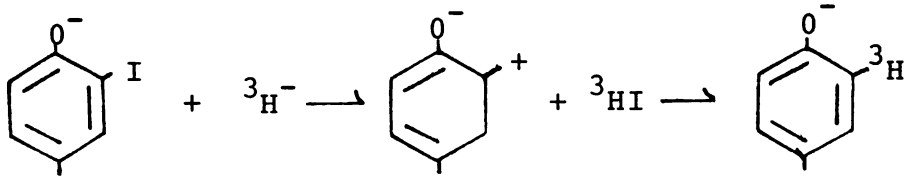
In the first attempt at tritiation, the chemically iodinated preparation of porcine ACTH containing two iodine atoms per molecule was dissolved in 0.1N acetic acid and reduced with pure tritium gas in the presence of freshly prepared palladium black. The product isolated by CMC chromatography (as described above) revealed a single major peak migrating in the same position as unmodified ACTH, with radioactivity associated only with the peptide elution. Furthermore, both the ultraviolet and difference spectra

(λ_{max}293) indicated that the product was completely dehalogenated (or reduced). The radioactive peptide comigrated with native ACTH on paper electrophoresis at pH 3.7; a tryptic digest of the peptide was also subjected to paper electrophoresis at pH 6.7 and under these conditions the C-terminal peptide was observed to migrate to the anode while another radioactive fragment migrated toward the cathode. These results indicated that the product contained approximately equal amounts of tritium associated with each of the tyrosines at positions 2 and 23, and confirmed our earlier evidence from the difference spectroscopy. Moreover, the tritiated ACTH exhibited both full steroidogenic and lipolytic activities as compared to the unmodified hormone. Despite the apparent success of the tritiation, the specific activity of the radioactive ACTH was determined to be approximately 8ci/mmole, far short of the theoretically obtainable 58ci/mmole.

In an attempt to isolate the source of dilution of specific activity, it was learned that diiodotyrosine (0.5mg/ml) dissolved in 0.1N acetic acid could be reduced spontaneously to tyrosine in the presence of freshly prepared palladium black (10mg) - without the introduction of any H_2gas; the reduction was already evident within 15 minutes (λ_{max}297) and was complete within 30 (λ_{max}293). It was believed at first that the catalyst might contain large amounts of hydrogen which had become dissolved in its surface during preparation. In order to circumvent this possibility, the fresh palladium black was replaced with

palladium oxide, a compound which would have no catalytic activity until it itself was reduced to palladium by the tritium gas; under these conditions, the catalyst could contain no extraneous hydrogen. This catalyst system was used in Tritiation 2 in which the synthetic ($3,5\text{-}I_2Tyr^{23}$) α_h-ACTH was reduced; again, although the reduction was successful, the specific activity of the peptide remained in the range of 5 to 8ci/mmole.

Having eliminated the suspicion about the catalyst, it was concluded that the only additional source of cold hydrogen which could be competing with the tritium would have to be provided from the solvent. Since the mechanism of reduction had been thought to occur via the tritide (as illustrated), we were reluctant to implicate the protons from the solvent in diluting out the specific activity.



However, Morgat et al. (1970) have mentioned that palladium is known to catalyse an exchange between the hydrogen of water and molecular hydrogen, and that in an aqueous solution this exchange will take place together with the dehalogenation process. Perhaps this alternate dehalogenation is more easily viewed as a reversal of the iodination of tyrosine, where H^+ is capable of displacing the iodide in the presence of the catalyst (see Fig.5). From the vantage

point of this new mechanism, it is not difficult to see that the relative rates of both dehalogenation processes will be an important factor in determining the final specific activity of the labeled product. Obviously the rate of deiodination of a peptide the size of ACTH is sufficiently slow to allow a great deal of exchange of iodine with solvent protons to occur (some insights into the kinetics of the reduction will be discussed below).

This problem was approached by attempting the reduction in an aprotic solvent; however, in the case of ACTH, and indeed many peptides and proteins, a small amount of aqueous solvent is needed to effect solubility. Consequently, the following solvent system was devised: 0.1N acetic acid: hexamethylphosphoramide: dimethylformamide (1:10:90,v/v). This solvent mixture was employed in Tritiation 3; again, palladium oxide was used as the catalyst and calcium carbonate was included as a solid support to increase the catalyst surface area and also possibly to trap iodide and stabilize the pH (Brundish and Wade, 1973). In addition, DTT was added to the reaction mixture at the end of the reduction to poison the catalyst and thereby prevent any proton exchange which could occur during the workup in aqueous solvents. These procedural modifications resulted in the preparation of tritiated ACTH with a specific activity of 46ci/mmole (80% theoretical). Characterization of the tritiated hormone revealed that the radioactivity co-migrated with the peptide on both CMC (Fig.8) and paper

electrophoresis (Fig.9), and that the tritiated peptide behaved in both systems as would native ACTH. Furthermore, tryptic digest followed by paper electrophoresis at pH 6.7 indicated that the peptide was labeled specifically at tyrosine 23 (Fig. 10); finally, the tritiated hormone exhibited full steroidogenic and lipolytic activities, as demonstrated in Figures 11 and 12. It should also be stated that the yield of the tritiation was quite acceptable, calculated to be approximatley 30%. This same procedure has also been employed to tritiate $(3,5\text{-}I_2Tyr^{2,23})\alpha_h$-ACTH and has resulted in a final product with a specific activity of 90ci/mmole (78% theoretical); this tritiated peptide has been assessed by the above criteria and found to be chemically homogeneous with complete biological activity. Brundish and Wade (1973) have also reported the successful tritiation of the protected $ACTH_{1-24}$ in dimethylformamide to a specific activity of 45ci/mmole; however, they were unable to increase this activity significantly when the tritiation of tyrosine in position 2 was attempted (Brundish et al., 1976).

During the determination of the specific activity the problem of loss of ACTH through adsorption was first appreciated, and was found to be particularly prevalent in dilute solutions of peptide (especially those below 150µg/ml). Table 3 illustrates that bovine serum albumin at a concentration of 1% alleviates this problem to a large extent. Therefore, in all assays performed after the

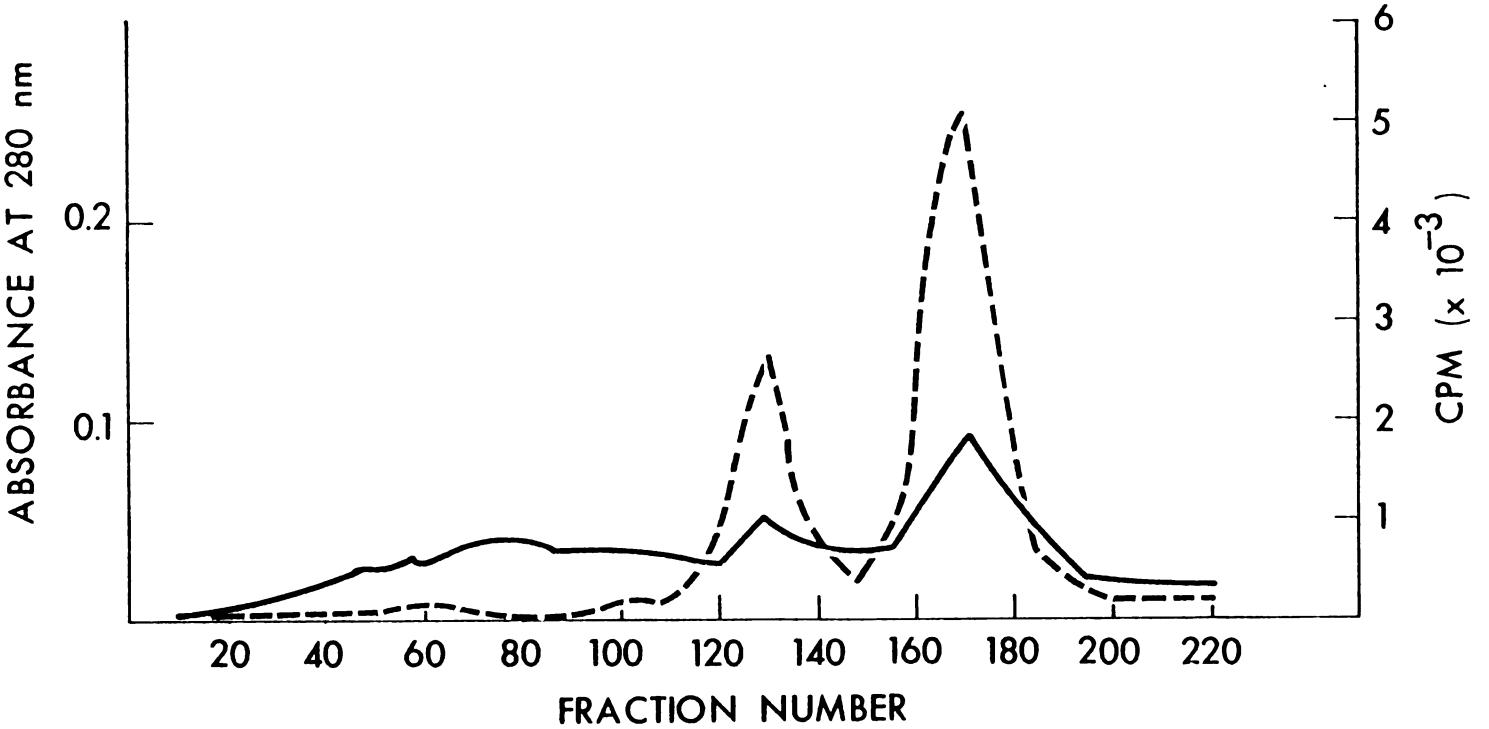


Fig. 8. Chromatography of tritiated ACTH on carboxymethyl cellulose. The product of catalytic dehalogenation obtained after gel filtration on Sephadex G-25 was applied on a column of Whatman microgranular cellulose as described in Fig. 6. For radioactivity ( ) determination, 5µl aliquots were mixed with 5ml of PCS and counted. The minor peak preceding the ACTH was considered to be deamidated ACTH.

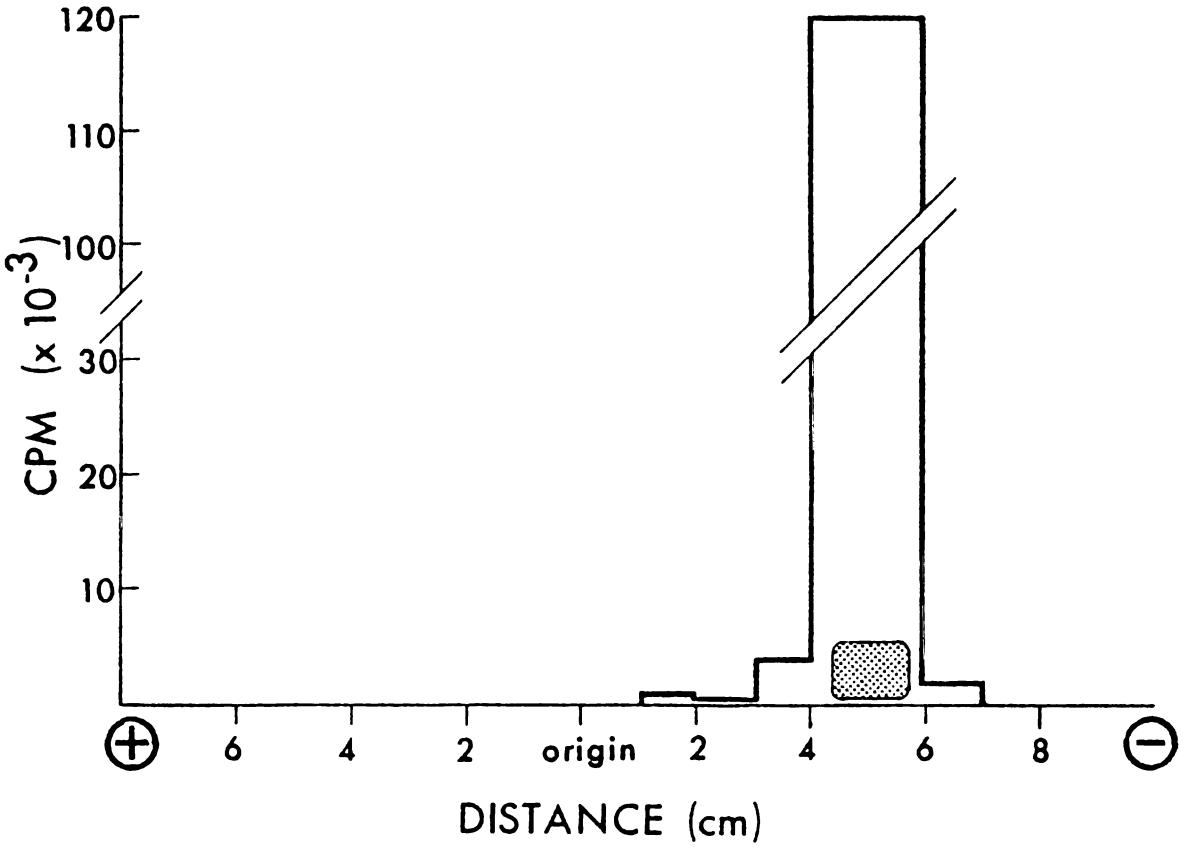


Fig. 9. Electrophoresis of ^{3}H α_h-ACTH Synthetic (0.1 mg) was mixed with tritiated ACTH isolated after CMC chromatography and subjected to electrophoresis on Whatman No. 3 paper in pyridine-acetate buffer, pH 3.7 for 4 hr at 400 V. The peptide was visualized by staining with ninhydrin. The paper was cut into 1 cm strips, mixed with the scintillant and counted.

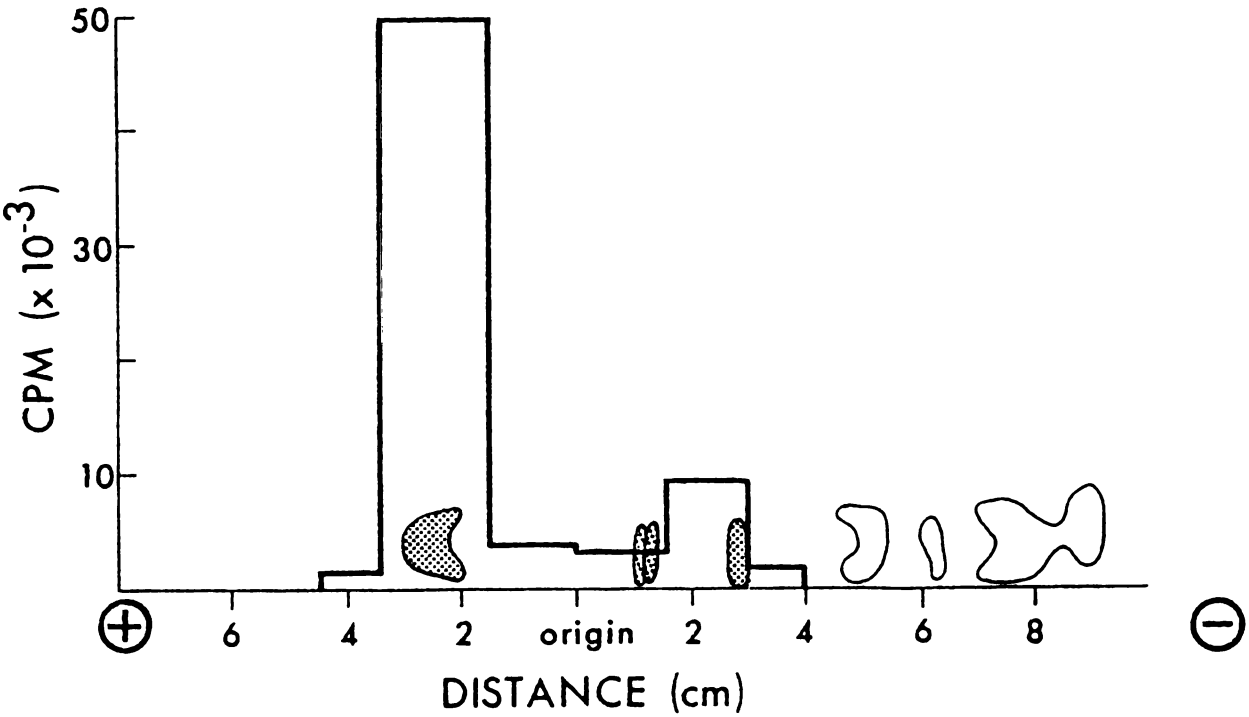


Fig. 10. Electrophoresis of tryptic digest of ^{3}H α_h-ACTH. Synthetic α_h-ACTH (0.1 mg) mixed with tritiated ACTH was dissolved in 0.1 ml of 0.01 M Tris -HCl buffer, ph 8 containing 10 mM Mg^{++} and incubated with trypsin (2µg) for 20 hr at 37 . The reaction mixture was lyophilized and redissolved in 0.1 N acetic acid. The digest was then subjected to electrophoresis on Whatman No. 3 paper in collidine-acetate buffer, pH 6.7 for 4 hr at 400 V. The paper was stained with ninhydrin followed by the Pauly reagent. Peptides which stained only with ninhydrin are shown as open spots and those stained by both ninhydrin and Pauly reagents are shown as hatched spots. The radioactivity associated with the peptides was determined by cutting the paper into strips and counting in the presence of the scintillant.

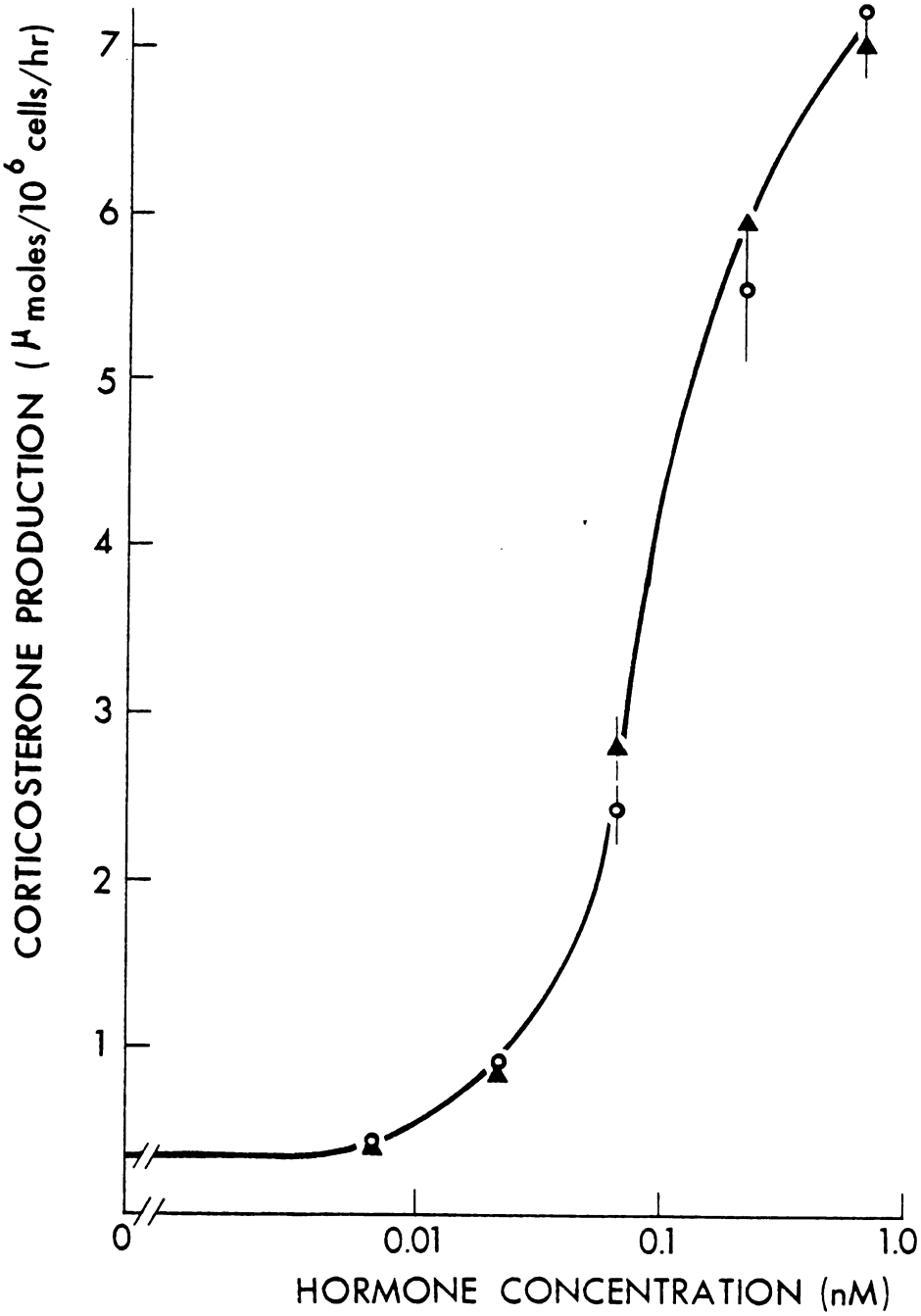


Fig. 11. Stimulation of corticosterone (A) production in isolated adrenal cells by synthetic α_h-ACTH (▲) and tritiated α_h-ACTH (O).

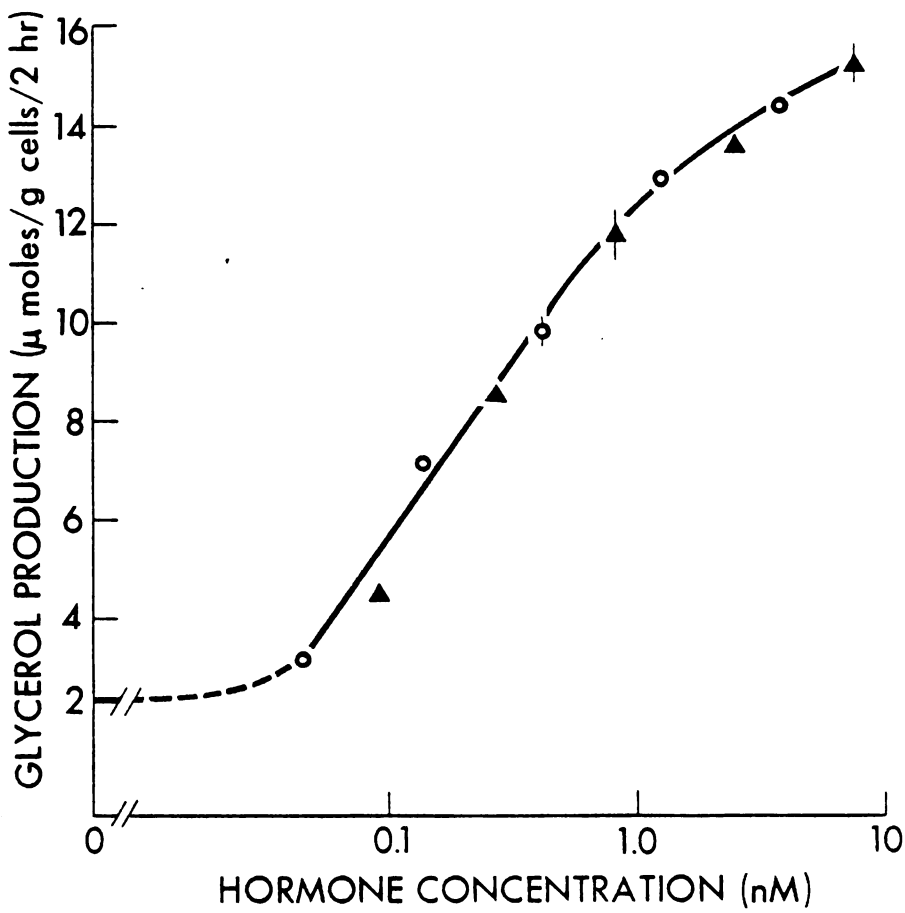


Fig. 12. Glycerol release in isolated adipocytes by synthetic α_h-ACTH (▲) and tritiated α_h-ACTH (O).

TABLE 3

Effect of Bovine Serum Albumin in Alleviating the Adsorption of ^{3}H-ACTH

Bovine serum Albumin concentration (%)	Radioactivity (CPM)
0	87,000
0.11	215,000
0.33	234,000
1.00	275,000
3.00	270,000

Tritiated ACTH (5λ) from a stock solution in ethanol: 0.001NHCl (1:1) was added to 1 ml of Krebs-Ringer bicarbonate buffer containing varying amounts of bovine serum albumin taking care to thoroughly rinse the micropipette with the diluent. An aliquot (100 λ) was mixed with 4.5 ml of the scintillant and counted.

adsorption problem was appreciated, ACTH dilutions were made in the presence of BSA (at least 0.5%), or when this was not practical, siliconized glass tubes and pipettes were used to minimize the adsorption. This property of ACTH became even more apparent during the binding studies, and will be discussed again in that context in the following chapter.

Kinetics of Reduction

Reference was made above to the kinetics of the reduction of the iodinated ACTH analogues. The use of palladium oxide as a catalyst makes this difficult to determine since the reduction of the peptide does not begin until the palladium oxide is converted to palladium. However, by observing that the oxide turns from brown to black in color upon reduction, it has been determined that the reduction of the catalyst usually occurs during the first 15 or 30 minutes after the hydrogen or tritium gas has been introduced into the system. By monitoring the difference spectra of the peptide undergoing reduction, the following data have been obtained. For the porcine (3-ITyr^{2,23})-ACTH in 0.1N acetic acid with palladium oxide:

	λ_{max}
0 minutes	298
120 minutes	295
150 minutes	293

For (3,5-I_2Tyr^{23})-ACTH in the organic solvent system described above, it was noticed that the absorbance of the diiodinated tyrosine was shifted well upfield to approximately 320nm and that the disappearance of this peak during the reaction could be followed instead of the

difference spectrum. The reduction in this solvent system in the presence of palladium oxide appeared to require 120 minutes for completion, as indicated by the disappearance of the absorbance at 320nm. From these kinetics, and from those observed in the aqueous solvent as well, it can be concluded that the reduction of halogenated ACTH in the presence of palladium oxide is a slow process and that the exchange of the tyrosyl iodine with solvent protons can indeed have been responsible for the low specific activities obtained when the reduction was carried out in aqueous solvents. Furthermore, the failure to achieve 100% theoretical specific activity for tritiations in the aprotic solvent system can probably be attributed to the exchange which takes place with the small amount of water used to dissolve the peptide. It should be emphasized that this exchange of tyrosyl iodine with solvent protons occurs only in the presence of a catalyst; this has been ascertained by the demonstration that diiodotyrosine is not at all reduced in 1N acetic acid alone, even when left at room temperature for 24 hours.

Stability of Tritiated ACTH

Before concluding this chapter on the tritiation of ACTH, mention must be made of the stability of the radioactive hormone, particularly since this represents another distinct advantage to labeling with tritium rather than ^{125}I or ^{131}I. The tritiated hormone was normally stored at $-20^{\circ}C$ in 50% ethanol. After six months the integrity of the peptide was tested by subjecting it to paper electro-

phoresis at pH 6.7 as described previously. With the preparation tritiated at both tyrosines 2 and 23, it could be seen that a negatively-charged C-terminal radioactive fragment was present (Fig. 13), indicating that a peptide bond had been cleaved in the vicinity of the $^{3}H_{2}$-Tyr^{23}. Furthermore, some loss of biological activity, which could be due to methionine oxidation, was also suspected. In order to restore the chemical and biological integrity of the tritiated ACTH, the hormone was incubated with 10% DTT (w/v in 0.1N acetic acid) at 37° for 72 hours to reduce any oxidized methionine (Dedman et al, 1961); the peptide was then subjected to chromatography on Sephadex G-25 (SF) to remove any fragments. Fig. 14 illustrates this elution pattern. The tritiated ACTH recovered from this purification was found in the paper electrophoresis system to co-migrate with native ACTH as a single radioactive spot, with no radioactivity migrating towards the anode. The biological activity was found to be at least 80%. The stored tritiated ACTH was checked periodically for degradation, and it appears that after the initial damage which occurs during the first six months, further degradation is much slower.

The study of the tritiation of ACTH has resulted in the development of a reliable method of radiolabeling biologically active peptides and perhaps proteins as well. The procedure has the advantage of being applicable to either natural or synthetic peptides, and produces chemically intact products in a high yield. Furthermore,

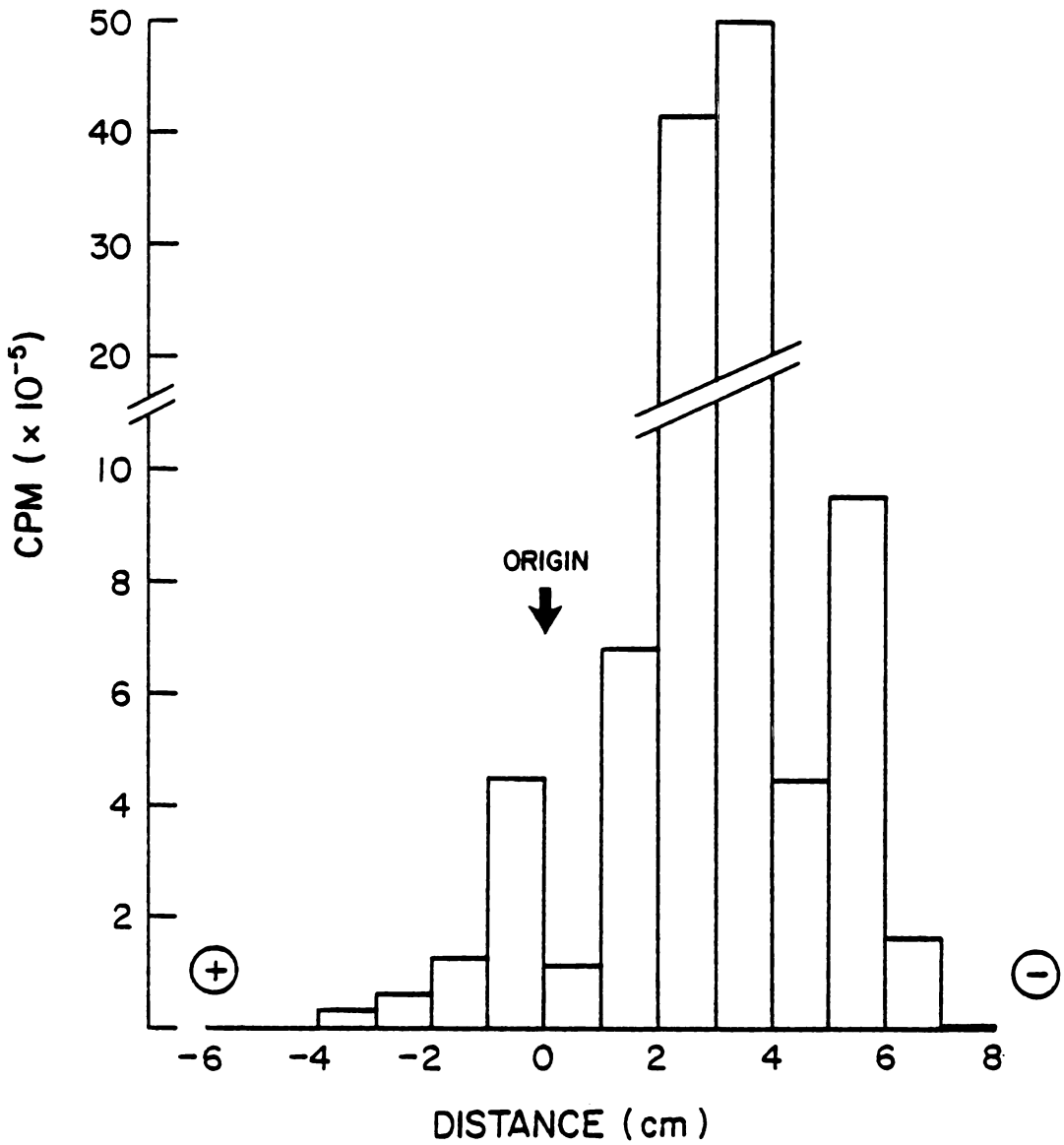


Fig. 13. Electrophoresis of tritiated ACTH after 6 months storage at -20°C. The electrophoresis was carried out at pH 6.7 and the peptide was visualized as described in Fig. 9.

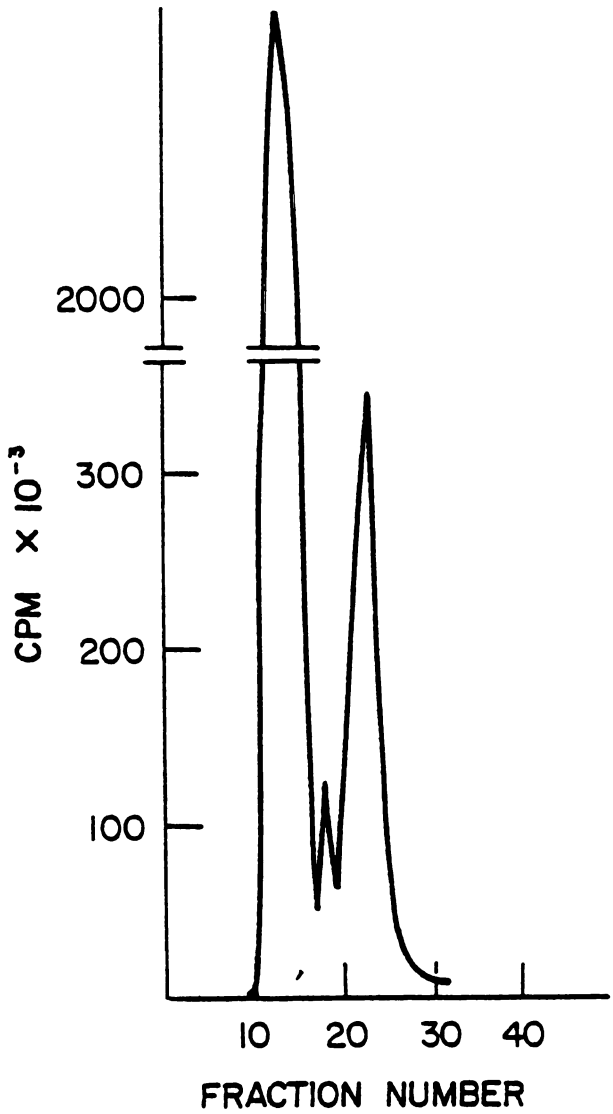


Fig. 14. Purification of tritiated ACTH. The stored tritiated peptide was reduced with DTT (as described in the text) and applied to a Sephadex G-25 super fine column (5x250mm) equilibrated in 0.1 N acetic acid. Fractions of 5 drops were collected, and radioactivity was determined by counting a 5λ aliquot in 5ml PCS. The major peak was cut conservatively as indicated; a recovery of at least 50% was achieved.

we are now in a better position to make intellegent predictions of how modifications of the method, which might be necessary in applying this procedure to other peptides and proteins, will affect the outcome of tritiation. This more complete understanding of the reductive dehalogenation process which this study has provided, therefore renders this method even more flexible and more valuable.

CHAPTER 3

THE BINDING OF TRITIATED ACTH TO ISOLATED ADIPOCYTES AND ADIPOCYTE MEMBRANES

Introduction

Within the field of hormonal mechanism of action, the 1970's ushered in a period of scientific curiosity concerning the initial interaction of the hormone at the cell surface. The target cells and biological activity of many of the hormones had already been established, as had the concept that these hormones most likely transmitted their chemically encoded messages to specific recognition sites located on the plasma membrane (Hechter and Halkerston, 1964). Moreover, the recent progress which had been made in the preparation of radiolabeled hormones during these years (as discussed in the previous chapter) challenged many investigators to locate, measure and define these putative sites. An incredible amount of collective energy began to be channeled into so-called binding studies and the search for the elusive hormone receptor.

The investigation into the mechanism of action of ACTH which was undertaken in this thesis work progressed quite naturally to a study of this nature. This chapter will therefore present the insights into the binding of ACTH to its adipocyte receptor which have been obtained; however, a good portion of this discussion must also be concerned with the difficulties, misuse, and misconceptions which have become an integral part of any binding study.

Binding studies have been conducted with virtually every available hormone and every accessible cell system. Review articles on polypeptide binding studies are numerous (Cuatrecasas, 1974; Cuatrecasas and Hollenberg, 1976; Freychet, 1976; Roth, 1973); representative reviews on receptors for steroids (Gorski et al., 1968) proteins (Dufau and Catt, 1975), and prostaglandins (Humes et al., 1976) are also cited.

The binding phenomenon has been visualised as the first step in the action of a polypeptide hormone, and is believed to result in the formation of a hormone-receptor complex that is responsible for setting in motion the events which will ultimately effect the cellular response. Perhaps the most infamous attempt to demonstrate that polypeptide hormones do indeed exert their activity by binding to cell surface recognition sites was Cuatrecasas' insulin-bound Sepharose study (1969). Although these experiments have largely been discredited (Katzen and Vlahakes, 1973; Butcher et al., 1973) as has the wisdom of using Sepharose-bound hormones in mechanism studies (Kolb et al., 1975), no definitive experimental evidence has since been proposed to confirm this theory. Indirect evidence comes from investigations in which highly purified membranes are shown to retain some hormonal sensitivity, for example, in the form of a membrane bound cyclase activity (Harwood and Rodbell, 1973). More recently, reports have appeared which suggest the polypeptide and protein hormones actually enter the cell to exert their

activity (Goldfine and Smith, 1976; Szego, 1974). The fact that these investigations have been conducted largely with iodinated hormone analogues discounts their credibility (for reasons discussed in Chapter 1 and this chapter as well); however, even if it can be demonstrated that peptide and protein hormones do enter the cell to express their function, some membrane recognition sites must still exist. At least from an academic perspective, therefore, it must be possible and profitable to measure the interaction of hormones with cell surface binding sites.

From a practical perspective, binding studies are executed by incubating a radiolabeled hormone with either intact cells or membrane preparations, separating out the unbound hormone from the system, and then detecting the hormone which remains bound by scintillation counting. In any binding study an appreciation of the experimental techniques which are used to isolate the receptor binding is so essential to the interpretation of the results; therefore, a detailed discussion concerning the nature of the labeled hormone, the receptor preparation, and the method employed to separate bound from free hormone (or distinguishing specific from nonspecific binding) will be presented at this time. Before proceeding, however, it is necessary to comment on the terminology associated with binding studies. The word "receptor", for example, has unfortunately come to be used in describing any site to which the hormone may bind. In its original sense,

"receptor" was defined to designate only that component of the target cell membrane which: 1) selectively recognizes and demonstrates a rapid and reversible high affinity for a particular hormone; 2) exists in finite numbers; and 3) when occupied by the hormone is capable of transmitting this recognition to effect the biological response. It is only when these three minimum criteria are fulfilled that the term "receptor" will be used; all other components to which the hormone demonstrates an affinity, will be referred to simply as "binding sites".

Some confusion exists also in the use of the terms "specific" and"nonspecific" binding. For the sake of simplicity this discussion will adopt the most generally accepted definitions introduced by Cuatrecasas (1971); "specific" binding will refer to those counts which are displaced by high concentrations of unlabeled ligand, while "nonspecific" will indicate radioactivity that cannot be displaced in the presence of the unlabeled hormone. This terminology differs from that employed by DeMeyts and Roth (1975) who refer to "high affinity" binding (presumably specific) as that which dissociates slowly, while low affinity binding (presumably nonspecific) exhibits a rapid dissociation.

Radiolabeled Hormones:

Some discussion about the widespread practice and the disadvantages associated with the use of radioiodinated hormones in biological studies has already been presented.

However, in the context of binding, some additional comments must be made about the use of these analogues. As discussed in Chapter 2, iodinated derivatives often display diminished biological activities which must reflect some chemical damage which occurred during iodination or a modification-induced change in the molecule's physical properties. It should also be recalled that these iodinated analogues are prepared to contain "less than one iodine atom per molecule". Again, this must indicate that some molecules may contain two atoms of iodine while some remain unmodified; or even more importantly, it has been reported (Rodbell et al., 1971) that iodination-induced damage to unreacted molecules causes them to co-migrate with the iodinated species. In the context of binding studies, the necessity to carefully characterize the radiolabeled ligand cannot be overestimated, particularly if these investigations are to contribute to an understanding of the mechanism of hormone action.

Some criticism must also be directed at the practice of using the native hormone to displace the iodinated analogue in determining the specific binding. In view of the possible alteration in the affinity of the halogenated hormone for the receptor, displacement by the unmodified hormone is an imperfect control, and it is essential to recognize the limitations of data obtained under these conditions. Such displacement experiments are perhaps useful in determining and correcting for the amount of damaged hormone which has been bound inappropriately;

it would however, be unwise to use this data, for example, in measuring the kinetics of dissociation or to propose models for receptor cooperativity (this latter phenomenon will be discussed in greater detail below). Despite the compromise of a lower specific activity, there is no question that a tritiated hormone which is prepared to insure maximum tritium incorporation and which is characterized carefully, represents the most appropriate radioligand for use in mechanism studies.

Receptor Preparation:

The advent of techniques which make possible not only the isolation of cells, but the fractionation of their component structures as well, has given the investigator a variety of preparations to which the binding may be determined. However, isolated cell preparations offer an attractive advantage over subcellular fractions since they permit simultaneous measurement of both binding and cell response. Once the intact system has been characterized in terms of the specifity, affinity and number of binding sites, it is then possible to disrupt the cell and ultimately measure the binding to a purified membrane preparation. This is of particular interest in those systems in which purified membranes retain some measurable ligand-responsive activity which can be correlated to the binding; adipose cell "ghosts" and purified membranes present such a system (Harwood and Rodbell, 1973). Although these fractionated preparations can result in a higher concentration of

receptors, they can however introduce some problems in binding studies. For example, these preparations may at once lose sensitivity to some hormones while their response to others may be intensified (Jarett et al., 1971; Allen and Beck, 1972; Rosselin and Freychet, 1973). Furthermore, fractionation may induce the exposure of nonspecific binding sites, or even result in a concentration of degradative enzymes which can rapidly digest hormones, as has been reported for ACTH (H.J.P. Bennett et al., 1974; Saez et al., 1975), as well as insulin (Le Cam et al., 1975).

Separation Techniques and Distinguishing Between Specific and Nonspecific Binding:

The separation of free labeled hormone from the receptor- hormone complex is perhaps the most critical aspect of the binding assay. At the present, two techniques are generally employed, namely, centrifugation to isolate a receptor-containing pellet, or the filtration of cells or membranes through a filter to eliminate the free ligand. It is in this separation step that nonspecific adsorption is usually introduced, and it can and often does result in the obliteration of the binding to the receptor; this important problem therefore deserves particular attention.

Historically, the early binding studies which were performed might best be described as "idealized". The investigators, armed with often poorly-characterized iodinated hormones with reduced potencies, were able to observe a displaceable or "specific" binding, and therefore assumed

that this represented a biologically significant event. It was not until about 1975 that the severity of the problem of nonspecific binding which is introduced into these artificial *in vitro* systems began to be generally acknowledged in the literature (Cuatrecasas and Hollenberg, 1975; Phillips, 1976). It is difficult, if not impossible, to evaluate the extent of nonspecific adsorption in the early published binding studies. However, some insight is offered (albeit unintentionally) by Cuatrecasas *et al*. in an extensive review article which deals specifically with nonspecific "specific" binding (1975). From the data presented in this article it can be calculated that in the absence of cells or tissue, insulin (at concentrations 10^{-11} to 10^{-10}M) can be bound"specifically" to the extent of 10,000cpm. In his earlier study of 1971 (Cuatrecasas, 1971), Cuatrecasas performed this same experiment with isolated adipocytes under very similar experimental conditions, and demonstrated a specific (i.e. displaceable) binding of insulin in this same concentration range of 2-4,000 cpm. It is possible to suspect from this information that despite Cuatrecasas' ability to show displacement with native hormone and a dose-dependent response which paralelled a biological function, in his 1971 study he might very well have been measuring a nonspecific adsorption of insulin to his filtration membrane, rather than a receptor binding. The problem of nonspecific adsorption was fully appreciated in the

early phases of this study and the measures adopted to circumvent its intrusion will be described in detail in the Methodology discussion below.

Methodology

In light of those points discussed in the introductory remarks, considerable care was exercised in selecting those conditions which would insure a low nonspecific adsorption of the peptide, and thereby provide the best means of avoiding artifacts so that the interaction of ACTH with its putative receptor could be selectively measured. To begin with, the rat adipocytes which were used as the receptor preparation were incubated under identical conditions as for the lipolytic assay, with the exception that the binding was measured at room temperature (rather than 37°C) to minimize any enzymatic degradation of the hormone. Furthermore, it had shown previously that our tritiated ACTH preparation behaved identically to native ACTH in biological and chemical aspects; an achievement of a specific activity of 90ci/mmole further justified the use of this radioligand to detect receptor binding. In this study however, some difficulty was experienced in separating the free from the bound hormone, and in distinguishing the binding to the putative receptor from any nonspecific adsorption introduced in this step. The propensity for ACTH to adsorb to many surfaces was first fully appreciated during the determination of the specific activity as

described in Chapter 2. As a result, several experiments were conducted to evaluate the extent of ACTH's non-specific adsorption and ultimately to devise the optimal method for separating the bound from the free hormone. Table 4 illustrates the adsorption of ACTH to a number of different materials under a number of different conditions. From these data, it was determined that a centrifugation step to pellet the hormone-receptor complex could be carried out in polypropylene or polystyrene test tubes with controllable adsorption, while glass displayed too great an affinity for ACTH. In addition, cellulose acetate, polyvinylchloride (Millipore) and glass filter membranes were found to be unacceptable materials for a separation of bound from free by filtration, although DEAE and polycarbonate (Bio-Rad) presented viable alternatives.

The low specific gravity of adipose cells makes it impossible to pellet out the hormone-receptor complex by centrifugation, although this method can easily be used for ghosts or purified adipose membranes. In order to measure the binding of ACTH to whole adipocytes, therefore, it was necessary to devise a filtration procedure which would minimize nonspecific adsorption. The filtration process was investigated and observed to involve a number of complex interactions. Table 5 illustrates a comparison between the behaviour of Millipore cellulose acetate (which exhibits a high affinity for ACTH) and DEAE (which has somewhat lower adsorptive properties); it is interesting

TABLE 4

Adsorption of ACTH to Various Materials

Material	Conditions	% Binding of ^{3}H-ACTH-Attributable to Adsorption
Cellulose Acetate (Millipore)	Filtration[a]	0.4
Deae	Filtration[a]	0.2
Glass Fiber	Filtration[a]	0.68
Polycarbonate	Filtration[b]	0.06
Polyvinylchloride (Millipore)	Filtration[b]	3.3
Cellulose Acetate (Millipore)	Filtration[b]	0.15
Polypropylene	Centrifugation[c]	0.05
Polystyrene		0.25

[a]Filtration was performed as described in the text; no cells were present.
[b]Filtration was performed as described; cells were present. Counts bound are those not displaced in the presence of excess cold ACTH.
[c]Centrifugation was performed as described; no ghosts or membranes were present.

TABLE 5

Extent of Non-Specific Adsorption as a Function of Cell Number

Dry Weight of Cells, mg	Cellulose Acetate (Millipore) CPM Bound	DEAE CPM Bound
0	970±143	540± 20
15	463± 29	757±172
50	867±167	1570±290
150	1160± 80	1433±170

to note that this adsorptive behavior appears to be altered as a function of cell number. It would seem that the Millipore filters as well as the cells exhibit a particular affinity for the tritiated ACTH, and that the affinity for the filters is greater than for the cells, as illustrated by the fact that fewer counts are bound to the Millipore when 15mg of cells are present than in the absence of cells. This is in contrast to DEAE, a positively-charged ion exchanger which has little affinity for the basic ACTH. Obviously, the cells cover a certain area on the filters, thereby rendering a number of Millipore binding sites inaccessible to the tritiated ACTH. This observation makes the use of a "cell-free" control difficult to apply and interpret, as an absence of cells leaves more surface area exposed to potential adsorption.

While investigating the filtration process it was also determined that the largest pore size filter possible must be used in order to minimize the sites available for adsorption, as well as to promote a quick filtration and insure the briefest time of contact between the tritiated ACTH and the filter. Despite an average diameter of some 50 microns for adipocytes, it was found that under filtration pressure, the cells escaped through filters of 3 or more microns. Therefore, filters of 1 micron were found to provide the best compromise of lowest surface area and fastest filtration, while still insuring the retention of all cells. It should also be mentioned at this time that the DEAE, although exhibiting a particularly low adsorption

was found not to retain the adipocytes entirely; its use was therefore abandoned in favor of the polycarbonate filters.

An examination of the filtration process also revealed that a constant maximum filtration pressure had to be maintained throughout the filtration in order to insure a consistent low background adsorption. The use of a filtration manifold which could accommodate several filtrations simultaneously greatly enhanced reproductibility.

Despite the careful selection of membrane filter material and the control of filtration conditions, non-specific adsorption could still obliterate cell binding, particularly at the lower ACTH concentrations. The greatest improvement introduced to circumvent this problem was the removal of the cell infranate after incubation with the tritiated hormone, and resuspending the cells in fresh incubation medium before filtration. This step removed approximately 80% of the unbound tritiated ACTH, and thereby greatly reduced the amount of labeled hormone which could potentially adsorb to the filter. Using this improved method with polycarbonate filters, it was possible to measure the binding of tritiated ACTH to isolated adipose cells up to a concentration of 50ng ACTH, beyond which the adsorption becomes highly significant.

Although a considerable amount of thought was given to choosing a control for non-receptor binding, no method proved to be entirely satisfactory. A cell free control should theorectically provide a correction for non-tissue binding; however, as discussed above, in the filtration method this leaves all the surface area of the filter exposed to potential adsorption, and consequently provides a high estimate of non-tissue binding. Boiling the cell preparation to destroy the receptor's capacity for binding was also attempted, but this procedure can cause alterations where either new nonspecific sites are exposed or existing ones are destroyed; in addition, the denaturation of the cell's proteins will most likely increase the possibility of occluding the labeled peptide in the interstitial spaces. The use of an analogous cell preparation containing no ACTH receptors was also considered but was dismissed because it would be difficult to find a tissue that has no affinity for the very basic ACTH; furthermore, the problem of normalizing the surface area and density of another cell to the adipocyte is extremely difficult.

Since no satisfactory alternative could be found, nondisplacement of bound hormone with excess unlabeled ACTH was taken to indicate nonspecific binding, while displacement suggested a rapidly reversible receptor binding. Despite this apparent concession, it must be emphasized that displaceability was considered necessary but certainly not sufficient evidence for specificity. Furthermore, the

displacement was accomplished with non-radioactive hormone which was identical to the labeled ligand and therefore did not, as in the case with many radioiodinated analogues, exhibit an affinity which differed from the bound hormone.

Materials:

For the "ghost" and membrane isolation, adenosine triphose (ATP) was obtained from either Schwarz/Mann or Sigma; all salts were Analytical Grade. For the binding assay, polycarbonate membranes were purchased from Bio-Rad (Richmond, CA); PCS and NCS were products of Amersham/Searle. All scintillation counting was done in a Packard Tricarb liquid scintillation spectrometer Model 3320 and an efficiency of 32% was determined using tritiated water as standard.

Methods:

Isolated adipocytes were prepared by the method of Rodbell (1964) as described previously. Adipocyte ghosts were also isolated according to the Rodbell protocol (Birnbaumer et al., 1969) as follows. All operations were carried out at 4°C in polystyrene test tubes. Stock Lysing Medium was prepared in the following manner: 2.5mM $MgCl_2$, 0.1mM $CaCl_2$, 1mM $KHCO_3$ were dissolved in 2mM Tris-HCL, pH 7.4 and stored at 4°C. On the day of ghost preparation, 2.5mM ATP was added to the Stock Medium and, when necessary, the pH was adjusted to 7.4 with a few grains of Tris.

Isolated adipocytes from 6-8 rats were washed twice

with lysing medium (5ml). Cell lysis was accomplished by inverting the swollen cells suspended in the lysing medium 20 times/minute for 1 minute, followed by centrifugation at 200xg for 1 minute and removal of the turbid infranate containing the released ghosts. This procedure was repeated at least 4 times to insure complete cell lysis. The pooled infranates were then centrifuged at 900xg for 30 minutes to pellet the ghosts; the supernate was discarded. The pellet was resuspended in 5ml 1mM $KHCO_3$ and centrifuged again at 900xg. Approximately 5mg ghost material were obtained from 8 rats, although this yield descreased sharply when tissue from less than 4 animals was used.

Purified membranes were isolated from the adipocyte ghosts on a sucrose gradient by a modification of the Harwood and Rodbell (1973) method. All procedures were carried out at 4°C. The Stock Gradient Medium was prepared to contain the following: 10mM Tris-HCl pH 7.4 with 1mM Ethylenediaminetetraacetic acid (EDTA). The sucrose gradient (27.6 - 54.1%w/v) was mixed in an LKB gradient maker in the following way. The appropriate amounts of sucrose were dissolved in the Stock Gradient Medium (containing 1mM ATP) and approximately 17ml of each of these sucrose solutions were poured into the gradient chambers; approximately 34ml were then allowed to flow slowly (0.5ml/min) into a 1" - 3.5" cellulose test tube. The ghosts were washed with and then suspended in Stock Gradient Medium (7ml) to which had been added 0.25M

sucrose and 1mM ATP. The ghost suspension was then gently layered on the gradient and the tubes were centrifuged in an SW 27 rotor at 24,000rpm for 60 minutes at 4°C. The gradient resolved two major fractions: a distinct band just below the gradient surface which contained the membranes, and a "mitochondrial" band at the bottom of the tube. The upper few milliliters of gradient were discarded, and approximately 7ml was taken to include all of the membrane fraction. This fraction was then diluted 4-fold in 1mM Tris-HCl pH 7.4 with 1mM EDTA, and centrifuged at 15,000rpm for 30 minutes; the resultant pellet was taken to be the purified membrane fraction.

Binding Procedure for Whole Cells:

Adipocytes (50-70mg per ml dry weight) were suspended in Krebs-Ringer bicarbonate buffer with 4% BSA and 0.01% trypsin inhibitor (incubation medium), and 2ml aliquots were distributed to polystyrene test tubes (17x100mm). Unlabeled ACTH (0.05 ml aliquot of 20mg/ml in 0.1N acetic acid) was added to half the tubes, while 0.05ml 0.1N acetic acid was added to the remainder. The appropriate concentration of tritiated ACTH (in 0.1ml) was then aliquoted to each tube, care being taken to swirl the cell suspension completely. (Note: The addition of the cell/BSA suspension to the test tubes before the tritiated ACTH greatly reduced the adsorption of the latter.) The tubes were incubated at room temperature with gentle shaking for 1.5 hours. Bound and free hormone were separated first by spinning the cells for 30 seconds and then using a Pasteur pipette to remove

the infranate, which contains at least 80% of the total counts added. The separation of the bound and free hormone was completed by resuspending the cells in 1.75ml ice cold incubation medium and then filtering 0.5ml aliquots through polycarbonate membranes (presoaked in incubation medium) on a Hoeffer filtration manifold. Three samples were filtered simultaneously. House vacuum was used during the filtration, and a pressure of 22-25 on the manifold gauge was always maintained. The filtered cells were washed once with 1ml ice cold incubation medium. The filter membranes were then placed in 5ml of PCS, incubated for 30 minutes at room temperature, and then counted.

Binding Procedure for Ghosts or Purified Membranes:

Ghosts (150-200µg) or membranes (30-50µg) were suspended in Krebs Ringer bicarbonate buffer with 0.5% BSA and 0.01% trypsin inhibitor and pipetted into either polypropylene microcentrifuge tubes (1.5ml capacity) or polyethylene test tubes (10-74mm). Tritiated ACTH was distributed in 0.05ml aliquots; cold ACTH, when designated, was added before the radiolabel. Cells were incubated with hormone for the presecribed time period, and the bound hormone was separated from the free by centrifugation; in the early experiments this was accomplished in a Sorvall Model RC-2 with a GSA Rotor at 5000 rpm, and in the later work in a Beckman Microfuge at machine speed (10,000rpm). The supernate containing most of the unbound hormone was

apirated off and the pellet was washed with 0.5ml ice cold incubation medium and recentrifuged twice. The tip of the centrifuge tube was cut off and placed in a scintillation vial containing 0.5ml NCS. The pellet was incubated with the NCS for at least two hours, at which time it was neutralized with 0.05ml glacial acetic acid and then counted in 5ml PCS. Alternately, after centrifugation and washing, the pellet was suspended in 0.5ml 0.1N acetic acid to dissociate the bound ACTH; after 20 minutes this wash was transferred to a scintillation vial and counted with 5ml PCS.

Protein Determinations:

All ghost and membrane protein content was determined according to the method of Lowry (Lowry et al., 1951) as follows. The reagent was prepared in the following manner: 1ml NaK tartrate (4% w/v in water) was added to 1ml $CuSO_4$ (2% w/v in water) and this was diluted to 100ml with 3% Na_2CO_3 (w/v in 0.1N NaOH). Proteins (0.2ml) were added to 2ml reagent and were allowed to stand at room temperature for 10 minutes. Phenol (0.2ml of a 1N solution) was added to each tube while vortexing vigorously. The color was allowed to develop for 30 minutes and was then read at 660nm. Bovine serum albumin was used as a standard.

Results

For all the experiments reported here, tritiated ACTH of 90ci/mmole and complete biological activity was employed. Fig. 15 demonstrates a typical dose-response curve for the binding of tritiated ACTH to isolated rat adipocytes. In another experiment the amount of labeled hormone bound was also found to be linearly proportional to the number of cells. From this curve it can be seen that the binding saturates at approximately 15ng/ml and that the K_D is estimated to be $2x10^{-9}M$. It should also be noted that the binding curve parallels almost precisely the dose-response of the lipolytic activity for the labeled hormone under the same conditions. It should be stated at this time that the lipolytic activity of the tritiated preparation was checked periodically and after the routine monthly purification of the labeled peptide (as described in Chapter 2), it was found to possess about 70% biological activity. This small loss of potency is due either to partial oxidation of the methionine or to inaccuracy in the concentrations which were determined from the specific activity of the original tritiated preparation.

Preliminary experiments on the kinetics of the binding of ACTH to adipocytes indicates that binding is apparently maximum after 5 minutes; more detailed experiments must be performed to obtain reliable values for both the association and dissociation rate constants.

Scatchard analysis of the binding will not be

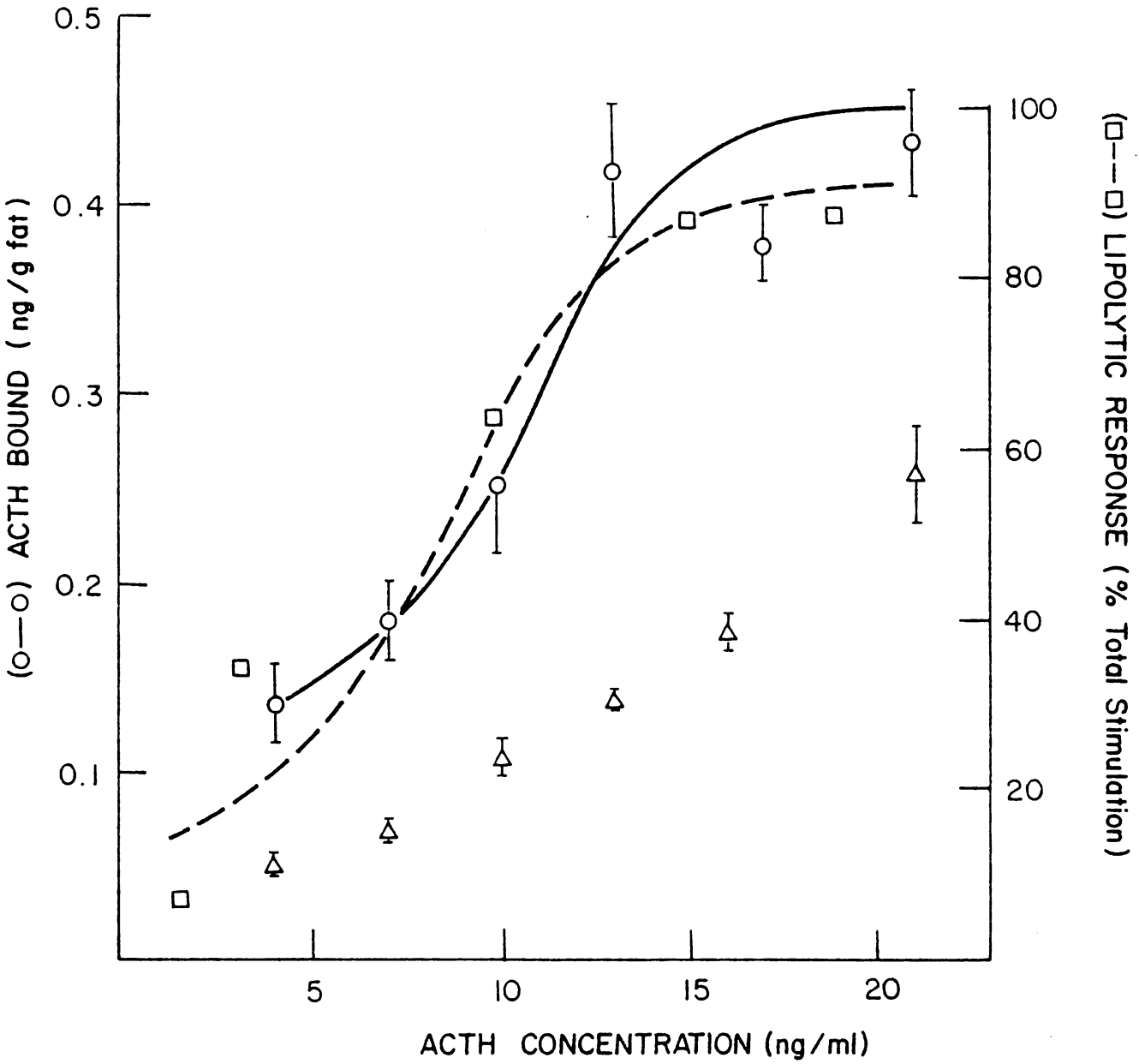


Fig. 15. The binding of tritiated ACTH (90ci/mmole) to isolated adipocytes. Binding was determined as described in the text. Counts not displaceable in the presence of 500µg/ml cold ACTH are represented (Δ). These non-displaceable counts were subtracted from the total radioactivity bound to obtain the specific binding (O). The lipolytic response obtained under similar experimental conditions with the tritiated ACTH is included for comparison (□).

performed at this time because of insufficient experimental points. However, the information which can be obtained from such a plot is readily available from the Michaelis-Menton dose-response as indicated in Fig. 15; the presence of multiple affinity sites and/or receptor cooperativity which is conventionally detectable only from Scatchard analysis will be discussed below. As has already been shown, the K_D value is easily determined from the half-maximal concentration of the dose-response curve. In addition, the number of binding sites may also be calculated from the number of counts bound at the point of saturation, assuming that all the receptors are occupied at this time. From the work of Lang and Schwyzer (Lang et al., 1974), it is estimated that 1mg dry weight of adipocytes is equivalent to 7800 cells. Therefore, using this estimate and the amount of hormone bound at saturation, a value of aproximately 8500 sites/cell is calculated. In terms of receptor density, this would indicate that there are approximately 2 high affinity ACTH receptor sites/micron^2 of the adipocyte surface area.

The binding of tritiated ACTH to adipocyte ghosts and purified membranes is also illustrated in Figures 16 and 17.

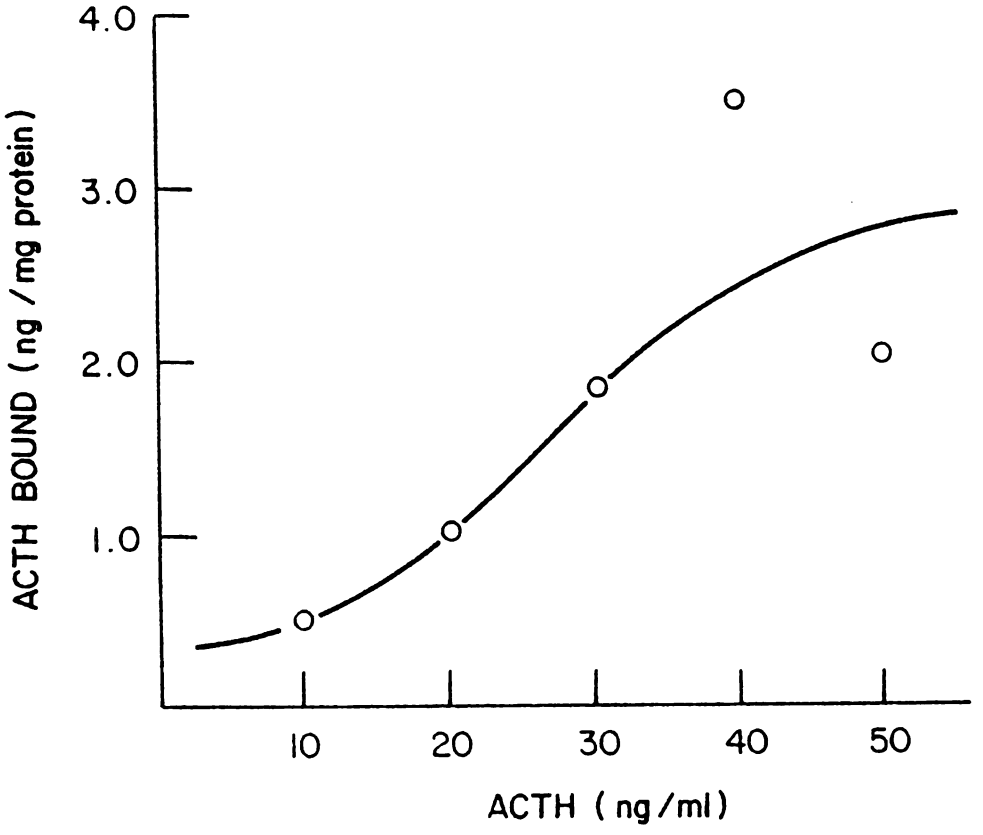


Fig. 17. The binding of tritiated ACTH (90ci/mmole) to purified adipocyte membranes. Hormone and membranes were incubated at 4° overnight. Counts not displaceable in the presence of 250µg/ml cold ACTH have been subtracted out to obtain the specific binding (O). 60µg of membrane preparation was present in each tube.

Discussion

In the introductory remarks of this chapter it should be recalled that several criteria were established and declared to be essential before experimentally-determined binding could be considered to represent true receptor binding. The results presented here do indeed thus far fulfill these requirements. To begin with, tritiated ACTH does demonstrate a rapid and reversible binding to its putative receptor on the adipocyte. The observation that binding appears to be complete within 5 minutes attests to the rapidity of this interaction; moreover, this agrees well with the concept of ACTH's mechanism of action which has been obtained through measurements of its biological activities. It is well established that the adipocyte cyclase is stimulated to produce cAMP within 3 minutes of incubation with hormone (Birnbaumer *et al.*, 1969). In addition, studies employing the nitrophenylsulphenyl analogue of ACTH (NPS-ACTH), a partial ACTH antagonist, indicate that dissociation of the native hormone is also rapid (Moyle *et al.*, 1973).

The reversibility of tritiated ACTH's binding, another criterion which must be fulfilled, is confirmed by the ability to displace the radioligand with excess amounts of unlabeled hormone. Again, it must be emphasized that this is an imperfect control and that under some conditions nonreceptor binding may be displaced as well, as discussed above for the Cuatrecasas study. Some comment should be

made here concerning the significant amount of tritiated ACTH which is not displaceable, even at high concentrations. The most logical explanation is that this represents at least in part irreversable (or nonsaturable) adsorption to the polycarbonate filters or test tube surface; however, other possibilities should also be considered. Since the chemical integrity of the tritiated ACTH was established at the outset of this study and was re-affirmed at monthly intervals, it is unlikely that these non-displaceable counts represent damaged label (as they perhaps do with short lived radioiodinated ligands). The non-displaceable counts could also be due to interstitially-occluded pools, although occlusion does not appear to present as much of a problem in filtration separation as it could in centrifugation. Another explanation for the non-displaceable counts might be that there occurs some exchange of label with cell components or that perhaps even an internalization of label takes place during the incubation period; this latter suggestion does not however appear to be too likely as there is yet no physiological evidence for such a phenomenon. It might be more straightforward to question the effects of excessive pharmacological doses of hormones in this cell system. Indeed, the mechanism of action might be altered under these circumstances; or as mentioned above, these counts probably represent the very significant nonspecific unsaturable adsorption which is induced at these high levels of hormone. Several orders of magnitude of non-labeled

hormone should therefore be tested for displaceability, in the hope that the receptor binding can be displaced specifically by a low concentration of cold ACTH, without incurring any pharmacological effects or upsetting the delicate balance which exists between true binding and adsorption.

To return to the receptor binding criteria, the affinity of the binding interaction must next be considered. In this present study, binding was measured only at physiologically significant doses and as discussed above, the hormone appears to be bound rapidly and reversibly at the same low concentrations which will trigger lipolysis. This affirms that the observed binding demonstrates high affinity, but perhaps even more importantly, it fulfills another essential receptor criterion - namely, that the binding can be correlated directly to a well defined biological event, for example lipolysis. It is only this biological function that can justify the existence of a so-called receptor.

The last criterion for true receptor binding, that there be a finite number of sites and that a binding saturation be observed, is the source of a great deal of controversy in this era of the "spare" receptor; this point shall be discussed in greater detail below. In the context of the binding experiment itself, however, achievement of saturation is an excellent way of differentiating between adsorption sites and what could be receptor sites. As

is indicated in Fig. 15 nonspecific binding is linearly related to the concentration of labeled hormone and within reasonable limits (less than 10^{-5}M) will not display saturation. Therefore, the apparent saturation at physiological concentration is a favorable indication that the observed binding is not due to nonspecific adsorption; it should be emphasized, however, that saturation is not a sufficient condition for receptor binding.

In summarizing the interpretation of the data presented here, it can be stated that the interaction of tritiated ACTH to isolated adipocytes has been determined to be a rapid, reversible, saturable binding of high affinity. A K_D of $2x10^{-9}$M has been measured and approximately 8500 sites/cell were calculated to be present. The effective binding concentration parallels the effective lipolytic concentration as measured under the same conditions. Because the binding was determined only at physiologically relevant concentrations and because it was measured with a radiolabeled but isoelectronic analogue of native ACTH (which exhibited complete chemical and biological integrity), there is every reason to believe that true interaction with the receptors has been observed.

The binding experiments conducted with subcellular fractions of the adipocyte are admittedly less straightforward than those in which intact cells were used. As discussed in Chapter 1, the preparation of the rat adipose cell ghosts or membranes results in a loss of sensitivity in response to ACTH stimulation; the concentration

required for half-maximal stimulation of cAMP production is approximately three orders of magnitude greater in the subcellular fractions than in the isolated cell (Ramachandran and Lee, 1976). To account for this depressed sensitivity, it has been proposed that a loss or damage of receptors might occur during fractionation or that the cyclase might become uncoupled from some receptors (Birnbaumer and Rodbell, 1969). In any event, measurement of the binding characterisitics of the ghost and membrane could lend some insight into this loss of sensitivity.

According to the cAMP dose-response curve, ACTH stimulates rat adipocyte ghosts at concentrations from 10^{-8} to 10^{-5}M. In view of the lengthy discussions and illustrations of the nonspecific adsorption problems which occur at these concentrations, it is not surprising that it is difficult and perhaps impossible to measure the binding interaction within this range. Typical experiments will show a total binding and a "displaceable" binding, but at these concentrations the difference (or specific binding) becomes the small difference between two large numbers. Mathematically it is impossible to obtain a statistically significant displacement under these conditions, and experimentally it becomes apparent that it is not feasible to "see" receptor binding in the presence of such high nonspecific adsorption.

Although the binding interaction could not be observed at the levels of hormone necessary to stimulate cAMP in the ghosts, it was possible to measure the binding at those

concentrations which would effect lipolysis in whole cells. Fig. 16 illustrates this binding. Achieving a saturation point was extremely difficult in these experiments despite the fact that a range of four orders of magnitude was attempted. A small but reproductible inflection point occurred at approximately 30-40ng/ml of tritiated ACTH. Again, although displacement of bound counts does occur when high concentrations of unlabeled ACTH are added, a good deal of radioactivity remains associated with the ghost preparation. Centrifugation was used to separate the bound and free tritiated ACTH and it is suspected that some counts remain occluded despite the thorough washing of the pellet. This hypothesis is supported somewhat by the fact that fractionated adipocyte preparations do not form fine suspensions and are difficult to disperse after centrifugation; furthermore, centrifugation at very high speeds (90,000xg) resulted in even more non-displaceable counts.

The binding of tritiated ACTH to adipocyte membranes which have been purified on sucrose gradients as described, follows a concentration dependency similar to that of the ghosts (Fig. 17), although much less protein was required to bind the same amount of ligand as would be needed with the ghosts.

The results obtained with the adipocyte ghosts and membranes would seem to imply that the loss of sensitivity to ACTH which occurs in their preparation does not appear to be at the level of the receptor. However, in view

of the pitfalls and difficulties of measuring binding in subcellular fractions, as described previously, conclusions should be withheld until additional experimental evidence is obtained. At the present, it cannot be stated unequivocally that this binding which has been measured represents true receptor binding since the correlation with a biological function for ghosts or membranes at these low tritiated ACTH concentrations cannot be demonstrated. We must therefore refer to them as high affinity ACTH binding sites at this time.

Comparison of Binding Results with Relevant Literature

A comparison of the results of this study with others in which the binding of ACTH has been measured will be introduced at this time. The only other investigation in which the ACTH binding to isolated adipocytes has been attempted is that of Lang and Schwyzer (Lang et al., 1974). The tritiated ACTH analogue Phe^{2}, 4,5-dehydro(4,5-^{3}H) $norvaline^{4}$-$ACTH_{1-24}$ of specific activity 7.42ci/mmole and biological activity 10% of the native ACTH was used in their study; separation of receptor-bound from the free hormone was accomplished on cellulose acetate (Millipore) filters and specific (or rapidly reversible) binding was determined by displacement with cold ACTH contained in the washing medium. This investigation reports that the binding equilibrium is reached within 1-2 minutes at 37°C and agrees with the observation of this present study that binding appears to be maximal within 5 minutes. Lang and Schwyzer report further the presence of at least 3 different orders of binding sites: a high affinity region

(10^{-11} to 10^{-10}M) which could not be measured with their ACTH preparation; the major region occurring between $5x10^{-10}$ to $3x10^{-8}$M; and a low affinity region (above $5x10^{-8}$M). For the major region, a receptor density of approximately 265 site/micron^2 or 1,325,000 sites/cell was also reported. These results indicate a much greater number of receptors with a lower affinity than has been found in our study. In addition, Lang and Schwyzer do not seem to achieve a saturation point in their binding. It is felt that the low specific activity and reduced biological potency of their tritiated ACTH preparation has made it difficult for them to measure exclusively receptor binding. However, they were able to demonstrate some parallelism beween binding and lipolysis, and do observe an increase in both these activities upon the addition of phenoxazone to their incubation; in light of these results, therefore, this work must be given some thought and consideration.

H.J.P. Bennett et al. (1974) have also published a brief reference to the binding of (3,5-$^3H_2$Tyr^{23})-ACTH$_{1-24}$ of 46ci/mmole to isolated rat adrenal cells and have calculated that only 120 sites/cell are occupied when approximately 80% of the steroid response has been induced. The paucity of binding data in this paper makes it difficult to evaluate the veracity of the study; however, their observation about receptor occupancy is quite interesting and should be pursued further.

A more extensive study has been conducted by McIlhinney and Schulster (1974) using lactoperoxidase-

prepared ^{125}I-ACTH$_{1-24}$ of specific activity 150-500μci/ug and 50% biological activity, to study the binding to isolated rat adrenal cells. Separation of bound from free hormone was accomplished by pelleting the cells in a test tube and cutting the tip off to count the receptor-bound ACTH. Their kinetic analysis shows that the binding reached equilibrium within 6 minutes at 37° which again concurs with the observations reported in this dissertation. McIlhinney and Schulster demonstrate that an increase in binding parallels an increase in steroidogenisis, although they failed to achieve saturation of binding at concentrations which were maximum for the biological response. They also propose two receptors of differing affinity: a high affinity with K_D 2.5×10^{-10}M present in some 3,000 sites/cell and a low affinity with K_D 1×10^{-8}M and greater than 30,000 sites/cell, the so-called "spare" receptors. In view of the apparent dissociation between cAMP production and steroidogenisis in the adrenal (Moyle et al., 1973), there may very well exist two separate receptors. However, McIlhinney and Schulster's proposal of the existence of spare receptors because they could not achieve binding saturation at maximal steroidogenisis should be investigated extensively to eliminate the possible occurrence of non-receptor interactions.

Lefkowitz et al. (1970) have reported on the binding of ^{125}I-ACTH to mouse adrenal tumor homogenates, using a filtration on Sephadex G-75 to separate bound from free hormone. They were able to correlate this binding to the stimulation of cAMP for ACTH and a number of analogues. In another study

using the same adrenal preparation (Lefkowitz et al., 1970a) they were able to demonstrate that Ca^{++} was not necessary for the initial binding of ACTH to its putative receptor, but that it must be present to stimulate the cyclase.

Models for Hormone Receptor Interactions

Before concluding this chapter on the binding interactions of ACTH some comments must be made on the interpretations and repercussions of binding studies in general, and of this one in particular. A question which often arises from binding studies is whether a one-to-one relationship of the binding to a biological response should exist. In many studies this does seem to be the case, for example with insulin (Cuatrecasas, 1971) or epinephrine (Lefkowitz et al., 1972). In this present investigation, the binding to whole adipocytes also seems to conform to this model, while in the ghosts and membranes this does not appear to be the case. Where the dose-response for the binding and function do not coincide there is a temptation to suggest that the binding is not representative of interactions with the receptor. However, it is perhaps reasonable to consider that a cell specific response is often far removed from the initial interaction of the hormone and receptor, and that many cascading or diminishing effects could intervene and cause dissociation between binding and biological function. In view of this, several models have been proposed to explain this apparent discrepancy without implicating nonspecific binding sites, and these ideas deserve some consideration in the context of a binding study.

A rather popular model which has evolved from binding studies is that of the "spare" receptor which contends that

the maximum biological response is attained when only a fraction of equivalent receptors are occupied (Stephenson, R. P., 1954; H.P.J. Bennett et al., 1974; McIlhinney and Schulster, 1974; V. Bennett et al., 1975). Although such a mechanism may exist in the cell, it must be asked if this hypothesis was proposed merely to rationalize the difficulty in achieving saturation which is often encountered in binding experiments. There appears to be at this stage no valid physiological role for these spare receptors, although one can invoke explanations involving receptor turnover or hormone degradation.

Another model which has been proposed and which is based on the opposite occupancy trend as spare receptors is that of the "threshold effect" (Rodbard, 1973). In this particular system a biological response may not be seen until a certain level of hormone occupancy is reached; in a dose-response curve, therefore, the binding will be observed at concentrations before the function is expressed. The binding results which we have obtained with the adipocyte ghost and membrane could be interpreted in terms of this model.

Perhaps the most popular model which has been proposed from binding data is that of the presence on a particular cell of receptors with varying affinities, as has been determined from nonlinear Scatchard plots. Some of the practical causes for Scatchard nonlinearity which can be introduced inadvertently into the binding assay have been

alluded to several times throughout this chapter- for example, radiolabeled ligands of differing affinity, the presence of nonspecific binding sites; an excellent discussion of several other causes can be found in the Rodbard article. Again, it must be considered that this multi-affinity receptor model has been offered to explain erratic binding data. Of course, certain situations do exist in cell response where two biochemical events may occur at differing hormone concentrations, as in the adrenal cell where steroidogenesis may be detected at ACTH concentrations at which little or no cAMP is produced, (Moyle et al., 1973). In such instances, two receptors of varying affinity could very well exist, as implied by McIlhinney and Schulster (1974). The presence of multi-affinity receptors is an interesting proposal; conceptualizing this idea in terms of a network of low affinity binding sites which saturate a cell surface and serve to concentrate the hormone around the limited number of high affinity receptors is certainly feasible. It cannot be overemphasized, however, that such models should not be proposed outside the context of biological function, simply on the basis of binding data.

The information obtained from binding experiments has also been used to propogate the idea of cooperativity among receptors - in particular, the so-called negative cooperativity (DeMeyts et al., 1976; Cuatrecasas and Hollenberg, 1975). Although most investigators have come to suspect some type of cooperativity among receptors

almost instinctively - indeed the sigmoidal dose-response curve suggests this strongly, it would seem that binding studies of themselves cannot provide sufficient information to either confirm or disprove this phenomenon. The detection of cooperativity relies on being able to measure precisely the rate constants of association and dissociation; again, the use of radioiodinated analogues which are either not well characterized or have a reduced affinity relative to the native hormone cannot be used to give the precise information which is necessary to confirm cooperativity. In addition, the use in these studies of pharmacological doses of hormones to promote dissociation most likely cannot mimic the situation as it occurs in vivo. The theory of receptor cooperativity is both interesting and important, and as such deserves to be investigated not only from the limited perspective of the binding study.

Conclusions

Perhaps the limitations of binding studies have at this point been overestimated; if this impression has been created, it was done so in an attempt to offset the widespread abuse which this method has suffered. The information concerning the initial interaction of a molecule with its receptor and the subsequent relaying of the hormonal information to the cell are critical to the understanding of the mechanism of hormone action. This present study has provided a realistic view of the ACTH receptor on the

adipocyte. The data presented here has served to establish that this membrane entity has a high affinity for ACTH and that it binds this molecule rapidly and reversibly; that it is present in a reasonably finite number; that the binding and cell specific response are closely associated functions; that the loss of sensitivity incurred in the preparation of ghosts and membranes may not be due to loss of the receptor. This study has also fostered a respect for the difficulty in measuring the binding to a small site which has been placed in an artificial experimental environment, and consequently, the limitations of such an endeavour are well appreciated. In light of this, it would be unwise to propose models for the mechanism of hormone action which are based solely on information obtained from binding studies, and such proposals will await supporting evidence from additional experiments.

A great deal of work remains to be done before even this particular study is complete. Further characterization of the system should be carried out. The rate constants for the association and dissociation of ACTH with its receptor must be determined experimentally. In addition, several orders of magnitude of ACTH concentrations should be tested to find a minimal amount which will displace the bound hormone without increasing dramatically nonspecific adsorption. Also, the specificity of the binding must be rigorously tested by challenging the receptor with other

polypeptide hormones. Furthermore, the utility of this method should be tested in defining the differences between the rat and rabbit adipocyte response. In concluding, it must be stated that although this binding study has been an ongoing project during the past three years, the results presented at this time are nonetheless still preliminary.

CHAPTER 4

THE EFFECT OF GLUCOCORTICOIDS ON THE ADIPOCYTE RECEPTOR POPULATION AND ADENYLYL CYCLASE ACTIVITY

Introduction

As described in Chapter 1, the adipocyte offers a distinct advantage in the study of the mechanism of hormone action since it contains a well characterized membrane associated adenylyl cyclase whose activity can be monitored by measuring cAMP production; in addition, this cyclase appears to be shared by a number of hormones which are capable of activating it through distinctive receptor sites. The membrane-enriched adipocyte ghost preparation has consequently been employed by several investigators, and its use has provided important information about some of the earliest events involved in hormonal activation. One such study, which appeared in 1970, indicated that the ACTH sensitivity of adenylyl cyclase in rat adipose cell ghosts could be specifically regulated by administration of the synthetic steroid dexamethasone, whereas this effect was not apparent for either epinephrine or glucagon stimulated cyclase (Braun and Hechter, 1970). These results argued strongly for a steroid-induced synthesis of the ACTH receptor which, in light of ACTH's primary action on steroidogenisis in the adrenal gland, would be at least teleologically feasible. Since the ability to regulate and modulate the number of receptors would certainly be advantageous in studying the various interactions of ACTH with the adipocyte, Braun and Hechter's results had a special

significance for this dissertation study. The binding data of the previous chapter is a case in point; for example, an increase in the number of receptors would alleviate many of the problems involved in the detection of binding at lower concentrations, while the availability of a receptor-depleted preparation would provide the perfect control for nonreceptor binding, as discussed in Chapter 3. Furthermore, maximizing the number of ACTH receptors by Braun and Hechter's proposed steroid administration, would certainly facilitate any attempts to isolate this molecular entity.

Braun and Hechter's experimental protocol involved adrenalectomizing male Sprague-Dawley rats, allowing sufficient time for the clearance of residual steroids, and then effecting steroid replacement with dexamethasone (0.5mg/100g body weight) administered over a 48 hour period. By measuring the amount of cAMP produced by adipocyte ghosts, Braun and Hechter were able to demonstrate that the ghosts from adrenalectomized rats exhibited a reduced response to stimulation by ACTH, while the extent of stimulation effected by epinephrine or glucagon remained unchanged. They reported further that sensitivity to ACTH could be restored by dexamethasone administration, and additionally, that ghosts isolated from sham-operated rats who had received dexamethasone exhibited a super-normal response to ACTH stimulation. Finally, Braun and Hechter were able to

demonstrate that all the effects of dexamethasone on the enhancement of the ACTH response in either adrenalectomized or sham-operated animals could be blocked by actinomycin D or cycloheximide.

The Characterization of Adipocyte Ghost Adenylyl Cyclase Activity

A detailed description of the adenylyl cyclase structure and its relationship to the hormone receptor has already been presented in Chapter 1 and will not be repeated here; the reader is referred back to that discussion to clarify any points. However, before introducing the topic of steroid regulation of the ghost cyclase activity, it is appropriate to describe the characteristic cyclase behavior of the cell free ghost preparation which was used in this study.

The ghost preparations were obtained from isolated rat adipocytes according to the procedures of Rodbell (Birnbaumer et al., 1969) as described in detail in Chapter 3. Typically, stimulation of the cyclase activity was achieved with concentrations of ACTH from approximately 10^{-8} to 10^{-5}M, and also with epinephrene from 10^{-8} to 10^{-5}M; NaF from 1 to 10mM also stimulated the cyclase, presumably by directly activating the catalytic subunit. As anticipated, the kinetics of the ACTH-induced cyclase activation exhibited a rapid onset; cAMP production above basal could be measured as early as 3 minutes after the addition of hormone, reached a maximum at approximately 12 minutes, and remained stable for at least 15 minutes. This observation is in agreement with the work of Bar and Hechter (1969). It was also found that varying the amount of ATP present in the incubation medium had a significant effect on cAMP

production; a concentration of 3.2 was optimal, while a high concentration of 6.4mM was inhibitory (increased basal, decreased hormonal-induced activation), presumably because the ATP concentration exceeds the Mg^{++} present. Furthermore, the amount of hormonally-induced cAMP was enhanced approximately two-fold in the presence an ATP-regenerating system of phosphocreatine and phosphokinase, as was also observed by Birnbaumer et al. (1969) and Bar and Hechter (1969).

On further characterization of the adipocyte ghost cyclase, several investigators have also demonstrated a dependency on Ca^{++} for the ACTH-induced activity (Rodbell, 1969; Bar and Hechter, 1969; Schwyzer, 1975); the system to be used in this study was therefore tested in this regard as well. Upon the addition of 1mM Ethyleneglycol-bis-(β-amino-ethylether)N,N'-tetraacetic acid (EGTA) to the ghost preparation, it was observed that cyclase stimulation due to ACTH was greatly reduced, as indicated in Table 6; however, complete inhibition of cAMP (particularly at maximum ACTH levels) was never achieved, as has been claimed by other investigators. In this present study, nevertheless, a consistent increase of basal cAMP levels was always noted in the presence of the calcium chelator, a phenomenon which has been documented in the studies cited above. Our inability to inhibit entirely the ACTH-induced cyclase activity when EGTA is included in the incubation medium has not been resolved, although it is suspected that some calcium remained in the system, perhaps being present in our ATP preparation.

TABLE 6

The Effect of EGTA on Ghost Cyclase Activity

		nm cAMP/ mg protein/ 15 minutes	% Increase Over Basal
NO EGTA			
BASAL:		0.234	-
ACTH:	10^{-9}M	0.234	0
	10^{-8}M	0.252	8
	10^{-7}M	0.270	16
	10^{-6}M	0.432	85
	10^{-5}M	0.648	177
Epinephrine:	10^{-7}M	0.270	3
	10^{-6}M	0.315	35
	10^{-5}M	0.423	57
	10^{-4}M	0.558	106
1mM EGTA			
BASAL:		0.396	-
ACTH:	10^{-9}M	0.396	0
	10^{-8}M	0.396	0
	10^{-7}M	0.378	<0
	10^{-6}M	0.486	23
	10^{-5}M	0.846	114
Epinephrine:	10^{-7}M	0.396	0
	10^{-6}M	0.522	32
	10^{-5}M	0.630	59
	10^{-4}M	0.846	114

Alternatively, perhaps high concentrations of ACTH are able to overcome this inhibition and the ACTH preparation used in this present study might have been more potent than that used by other investigators; this is a somewhat reasonable hypothesis since the inhibition is least effective at our highest hormone concentration.

The question of guanyl nucleotide regulation of the adipocyte ghost cyclase was also examined, and it was determined that under normal assay conditions, that is 3.2mM ATP with regenerating system and 5mM $MgCl_2$, and 10mM theophylline, GTP ($2x10^{-5}$M) had no effect on either basal or hormonally-induced activation. Yamamura et al. have recently demonstrated a marked dependence of purified adipocyte membrane cyclase on GTP for activity (1977), as stimulation is significantly depressed in the absence of the nucleotide. It was observed that our ghost preparations were quite active despite the fact that no exogenous GTP was added. It is feasible, therefore, that the procedures involved in obtaining a purified membrane fraction have removed endogenous GTP stores, thereby necessitating their addition before full cyclase activity is observed. However, in view of the recent and exciting findings of the importance of this nucleotide in cyclase regulation, this aspect warrants a more detailed investigation.

The stability of the cyclase ghost preparations was also monitored and it was found that virtually no activity was lost on short-term storage (up to 2 days) at -20° C,

while storage for longer periods resulted in a gradual and parallel decrease in both basal and hormonally-induced cyclase activity; for example, after storage for 7 days both activities will show approximately a 50% decrease.

Having described the behavior and characterization of our ghost cyclase activity, we are now in position to return to the discussion of the effect of adrenalectomy and steroid replacement on hormonally-induced cAMP production.

Methodology

Materials:

For the determination of the ghost cyclase activity: cAMP, ATP, GTP were products of Schwarz/Mann; theophylline, creatine phosphokinase and creatine phosphate were obtained from Calbiochem. Tris(ultra pure grade) was obtained from Mann; all other salts were Analytical Reagent Grade. For the cAMP analysis: Norit A Decolorizing Charcoal was a product of Pfanstiehl Chemical Company and tritiated cAMP (specific activity 20-40ci/mmole) was purchased from New England Nuclear. Dexamethesone was obtained from Sigma, L-epinephrine from Calbiochem, highly purified ovine ACTH was prepared by the published procedure (Canova-Davis and Ramachandran, 1976). For the rabbit kinase isolation, New Zealand White rabbits were obtained from

Campbell (Los Gatos, CA) and DEAE-Cellulose was a product of Bio-Rad. For the DNA assay, indole was obtained from Fisher Chemicals and calf thymus DNA was a product of Sigma.

Methods:

Adenylyl Cyclase Assay in Ghosts: Adipocyte ghosts were prepared as described in Chapter 3. Adenylyl cyclase activity of the preparation was measured according to the method of Rodbell, (Birnbaumer et al., 1969), adapted as follows. The incubation medium consisted of 6.4mM ATP, 10mM $MgCl_2$, 20mM theophylline, 0.2% bovine serum albumin (w/v), 0.2mM$KHCO_3$ in 50mM Tris-HCl pH7.4 with an ATP regenerating system of 20mM phosphocreatine and 0.4mg/ml Creatine Kinase. A Stock Incubation Medium consisting of the $MgCl_2$ and theophylline in the Tris buffer was kept stored, and the ATP, albumin and ATP-regenerating system were added on the day of assay. Hormone (0.025ml) was added to the incubation medium (0.05ml) in a 12-75mm polystyrene tube, and the reaction was initiated by the addition of the ghosts (0.025ml) suspended in 1mM $KHCO_3$. The reaction was allowed to proceed at 37°C with gentle shaking for 15 minutes at which time it was terminated by the addition of 1ml ice-cold 0.1M Na acetate buffer pH 4.0 to each of the tubes, with subsequent placement in ice. Unincubated controls were generated by adding an aliquot of ghosts to the incubation mixture after the reaction was

stopped with the acetate buffer. Between 25-50μg of ghost protein (as determined by the Lowry procedure) were used per tube.

Cyclic AMP production was determined by a modidification of the Gilman protein binding assay (Gilman, 1970) as described by Johnson et al. (1972). Briefly, an aliquot of the incubation mixture (0.05 or 0.1ml) was incubated in 50mM Na acetate buffer pH 4.0 with rabbit muscle kinase/ inhibitor (0.025ml) and tritiated cAMP (approximately 25,000 cpm in 0.025ml) at 4°C for a minimum of 2 hours. Separation of bound from free tritiated cAMP was accomplished by charcoal adsorption of the free nucleotide as follows. All procedures were carried out at 4°C. An aliquot of Norit charcoal (0.5ml) suspended in 20mM Phosphate buffer pH 6.0 (4mgNorit/ml) with 0.5% BSA was added; each tube was vortexed, and after a minimum of 5 minutes and a maximum of 10 minutes, was centrifuged at 3000 rpm for 20 minutes. Aliquots (0.5ml) of the supernatant were removed and counted in 4.5.ml PCS to determine the kinase-bound tritiated cAMP. Nonradioactive cAMP was used as standard; an aliquot of the complete incubation mixture (the unincubated control can be used) was included in all standards to compensate for any medium-induced alterations in kinase binding.

Preparation of cAMP dependent Protein Kinase:

The cAMP dependent protein kinase was isolated and purified according to the method of Gilman (1970), adapted as follows. The leg and back muscles (approximately 800g)

from a New Zealand White male rabbit (4kg) were removed and placed in a chilled beaker until use. The muscle was then suspended in 4mM EDTA pH 7.0 (250ml/100g muscle) and homogenized at 4°C in a Waring blender. The resulting homogenate was centrifuged in a Sorval GSA rotor at 700rpm for 1 hour, and the supernate was decanted through glass wool to remove the lipid material. The pH of this supernate was adjusted to 6.1-6.2 and centrifuged again for 1 hour. The resulting supernate was brought to pH 5.5 and the precipitate which formed was removed by centrifugation. The pH of this supernate was adjusted to 6.8 with 1M potassium phosphate buffer pH 7.2, and the ammonium sulfate precipitate was obtained by the addition of 32.5g ammonium sulfate/100ml protein solution. The precipitate was collected by centrifugation and dissolved in 150ml of 5mM potassium phosphate buffer pH 7.0 with 2mM EDTA; the protein solution was dialysed extensively against the same buffer. Any precipitate which formed was removed by centrifugation.

Purification of the kinase was achieved by chromatography on a DEAE-cellulose column (35-4mm) equilibrated with 5mM potassium phosphate buffer pH 7.0; a stepwise gradient from 100mM to 300mM potassium phospahte buffer was employed. Both the optical density at 280nm of the fractions as well as the tritiated cAMP binding capacity were determined, and it was found that two major protein peaks exhibited binding activity. These two fractions were

pooled separately and dialysed at 4°C for 96 hours against 5mM potassium phosphate buffer pH 7.0 with 2mM EDTA. Characterization of the kinase indicated that the cAMP binding plateaued at a kinase concentration of 20% (v/v), and therefore, the kinase was used at a 1:5 dilution in the cAMP analysis. The purified kinase was stored in aliquots at -20°C and was allowed to thaw gradually in ice before use; thawed preparations were never re-used. Under these conditions, the binding activity was stable for approximately 2 years. Protein Kinase Inhibitor: The inhibitor was also prepared according to the Gilman procedure. Briefly, rabbit muscle was homogenized in 10mM Tris HCl pH 7.5 and boiled for 10 minutes. The protein was filtered through glass wool to remove the particulate material, and the inhibitor was precipitated with a 1:8 volume of 50% TCA. This precipitate was collected at 15,000xg, dissolved in water, and the pH was adjusted to 7.0 with 1N NaOH. This fraction was dialysed against water and any precipitate which formed was discarded. The preparation in a 1:5 dilution was found to be effective in increasing the binding of cAMP to the kinase. Therefore, in the analysis for cAMP the inhibitor, diluted 1:5 with water, was used to dilute the kinase. The inhibitor was stored at -20°C and found to be effective for at least 2 years, despite thawing and re-freezing.

DNA Analysis: Determination of DNA content of the

adipocytes was accomplished by the method of Ceriotti (1952) as follows. Cells were prepared for analysis by extraction of lipid in $CHCl_3$:CH_3OH (2:1). The indole reagent was prepared to contain 0.04% indole (w/v) in distilled water with 60mM $CuSO_4 \cdot 5H_2O$. This reagent (0.25ml) was added to each cell sample (0.5ml) and the mixture was acidified with 0.25ml concentrated HCl. The samples were vortexed, covered and boiled for 10 minutes. After cooling in ice, the samples were extracted by the addition of 1ml $CHCl_3$ with spinning to clarify the phases; the lower organic phase was removed and discarded. The extraction procedure was repeated 3 times, and the color which remained was read at 490nm. Calf thymus DNA in 0.1N NaoH was used as standard; the optical density of the standards was found to be linear from 1 to 10µg DNA/ml.

Dexamethasone Administration:

Male Sprague-Dawley rats (Simonsen) weighing 160-180gms were used in all experiments and when adrenalectomized, were maintained on drinking water supplemented with 0.9% saline. To insure that endogenous steroid stores had been cleared, adrenalectomized rats were maintained at least 5 days postoperatively before dexamethasone treatment was initiated. A total dosage of 0.5mg dexamethasone/100g body weight was injected intraperitoneally in 5 injections administered over a period of 48 hours. Because of the difficulties encountered in dissolving the dexamethasone in an aqueous solution, the following vehicle for injection

was adopted: 5mg DEX/ml dissolved in 95% EtOH; 1ml DEX/EtOH added to 4ml of a solution of 5% BSA in 0.9% saline to yield a final concentration of 1mg DEX/ml vehicle. The volume of each injection was maintained at less than 0.2ml. A control group of animals was given a series of injections of a placebo solution which consisted of 1ml 95% EtOH in 4ml 5% BSA/0.9% saline. In all experiments, animals were sacrificed 5 hours after the last injection.

Results

As already noted, our interest in Braun and Hechter's study originated in the possibility of modulating specifically the ACTH receptor population and thereby enhancing the insight into the nature of this receptor; consequently, an attempt was made to confirm the results of Braun and Hechter. Using the prescribed protocol of adrenalectomy and dexamethasone administration we were, however, unable to demonstrate a selective enhancement of the ACTH response in those preparations from the steroid treated rats; as illustrated in Fig. 18, the epinephrine-stimulated cyclase activity is increased by dexamethasone administration as well. It is interesting to note that the enhancement is more pronounced at the lower doses of hormone tested and appears to decrease at maximally-stimulating concentrations. In order to determine whether these results were in some way unique to the nature of the ghost preparation or to the high concentrations of hormone necessary for stimulation, the adrenalectomy and steroid replacement were repeated and the same experiment was conducted using whole adipose cells and measuring both cAMP production and lipolysis. These results are presented in Table 7; the extremely high basal levels for both cAMP and glycerol in those cells isolated from dexamethasone-treated rats complicate any comparison with the response obtained with the adipocytes from the steroid depleted animals. However, it is not difficult to see that the hormone-induced

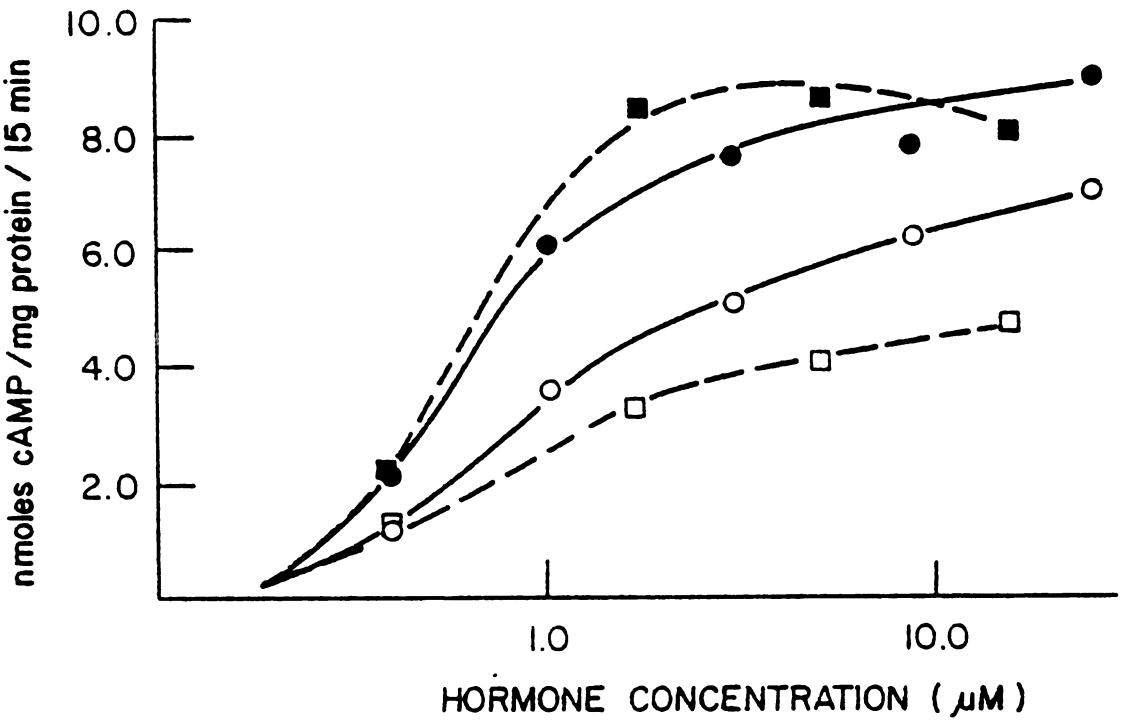


Fig. 18. Adenylyl cyclase activity of ghosts from adrenalectomized rats. (O-O), ACTH stimulation of placebo-treated group; (●-●), ACTH stimulation of dexamethasone-treated group; (□--□), epinephrine stimulation of placebo-treated group; (■--■), epinephrine stimulation of dexamethasone-treated group. Basal cAMP levels were 1.61 and 1.71nm cAMP/mg protein/15 min for the placebo and dexamethasone-treated groups respectively.

TABLE 7

The Effect of Dexamethasone Administration on Adipocytes from Adrenalectomized Rats

		cAMP Production[a]	% Increase Over BASAL	Lipolysis[b]	%
NO DEXAMETHASONE:					
BASAL:		0.15	-	1.81	-
ACTH:	10^{-10}M	0.18	20	1.84	2
	10^{-9}	0.33	115	1.84	2
	$3x10^{-9}$	0.65	367	1.84	2
	10^{-8}	3.75	2400	2.47	36
	10^{-7}	5.40	3500	8.26	356
Epinephrine:	10^{-9}M	0.20	33	1.61	<0
	10^{-8}	0.30	100	2.22	23
	10^{-7}	3.45	2200	4.63	156
DEXAMETHASONE TREATED:					
BASAL:		7.44	-	18.73	-
ACTH:	10^{-10}M	8.05	8	16.29	<0
	10^{-9}	15.10	103	18.86	1
	$3x10^{-9}$	23.20	212	20.13	8
	10^{-8}	39.39	429	26.61	42
	10^{-7}	51.64	594	26.26	40
Epinephrine:	10^{-9}	14.44	100	19.08	2
	10^{-8}	22.64	200	20.00	7
	10^{-7}	32.38	336	26.61	42

[a]Nanamoles of cAMP/g fat/15 min.; values are the average of duplicates.
[b]Micromoles of glycerol/g fat/2h.

cAMP production in the adrenalectomized group which had not been given steroid replacement is not depressed. In contrast, the lipolytic response of this group to ACTH is diminished dramatically, while for epinephrine there is some loss of sensitivity. These results are comparable to those of Exton et al. (1972). In the dexamathasone-treated adrenalectomized animals, both the ACTH and epinephrine stimulated rise in cAMP occurs, and considered as a percent increase over basal, this rise does not exceed that observed for the steroid deficient rats. In the dexamethasone-treated group, it is interesting to note that the cAMP production is dose-dependent although the lipolytic response shows no dose-dependence on hormonal concentration; from these results, it can be assumed the cyclic nucleotide is no longer the rate-limiting step in the lipolytic response when present at such high levles.

In light of the proposal that the guanyl nucleotides play an obligatory role in hormonally-induced cyclase activity, (Rodbell, 1975), adipocyte ghosts from the adrenalectomized rats were incubated in the presence of $5x10^{-5}$M GTP; however, no increase in ACTH-stimulated cyclase activity could be found over the controls which were not exposed to GTP.

Discussion

The apparent discrepancy between the data of Braun and Hechter and the results presented here has not been resolved.

Several explanations can be proposed - not for the purpose of laboring the point, but rather to draw attention to the complexity and difficulty of the problem. To begin with, an ethanol-BSA vehicle was devised in the present investigation for injection of the steroid to alleviate the poor solubility of the dexamethasone; Braun and Hechter make no mention of their vehicle. Therefore, the possibility of our treating the animals with different effective doses should be considered, particularly since the various effects of dexamethasone have been reported to be dose-dependent (Fain and Czech, 1975). Furthermore, the general health of the animals and variations in food intake can influence dramatically the metabolism of the adipocyte and its response to hormonal stimulation (Exton et al., 1972), presumably through the effect of circulating insulin levels. As far as experimental procedures are concerned, every attempt was made here to follow Braun and Hechter's protocol as closely as possible. However, the method employed in this study for analysing cyclase activity differed in the addition of theophylline, a phosphodiesterase inhibitor, to preserve the cAMP produced; by contrast, Braun and Hechter relied on exogenous cold cAMP to serve as a scavenger.

On the basis of the results presented here and the ACTH binding data to follow, it has been determined that adrenalectomy does not reduce the number of ACTH receptors on the adipocyte and that the lesion which prevents the

expression of the lipolytic response must occur after the initial hormone-receptor interaction. Additionally, steroid replacement with dexamethasone does not selectively induce ACTH receptors, but rather effects a more general and complex promotion of protein synthesis. Despite the fact that the initial interest in this steroid study was stimulated by the possibility of modulating the ACTH receptor, a closer analysis of the data which was collected in the course of the investigation would seem to be in order. Perhaps some light might be shed on the molecular basis for the so-called "permissive" effect which steroids are known to exert on a wide variety of tissues (Ingle, 1952).

The effect of the glucocorticoids on fat metabolism is a very extensive topic which is well beyond the scope of this discussion; however, the reader is referred to a general review in the Handbook of Physiology (Fain and Czech, 1975). Since it is well established that adrenalectomy results in a general depression of hormonally-induced lipolysis (Allen and Beck, 1972; Exton et al., 1973; Fain and Czech, 1975; Shafrir et al., 1960), perhaps the most appropriate approach for the present discussion would be to determine at which point(s) the lesion in the lipolytic response occurs. To begin with, the initial interaction of the hormone with the receptor does not appear to be influenced by adrenalectomy and/or steroid administration; this point will be discussed in greater detail below. The next major event in hormonal

activation, the generation of the second messenger cAMP, might very well be responsible for the decreased lipolytic response. However, the question of whether the level of steroids can regulate the amount of cAMP which is produced and whether this could account for the inhibition of lipolysis following adrenalectomy is an extremely complicated issue, and as such, it stands as an excellent testimony to our limited knowledge of the cyclase mechanism. Part of the difficulty stems from the necessity of dissociating and defining the various biochemical events which can influence cAMP levels. These events might be described in terms of a coupling mechanism through which the receptor activates the cyclase, and of equal importance, in terms of the degradation of cAMP to 5'-AMP by the phosphodiesterase enzymes whose activity will to a large extent determine the amount of cAMP that is measured and assumed to reflect the activity of the cyclase.

As far as the nebulous coupling or transducing step which precedes cyclase activation is concerned, this would certainly be a reasonable site for the adrenalectomy-induced lipolytic lesion to occur, particularly if the decreased cAMP production appears to be hormone specific, that is, if sensitivity is lost to one hormone more so than to another. However, the general lack of knowledge of the manner in which the interaction with the receptor is translated to the cyclase prohibits any real speculation about the coupling lesion; therefore, an empirical consideration

of published results must be relied on to perhaps shed some light on this aspect. To begin with, the work of Braun and Hechter obviously implies that steroids selectively regulate the ACTH sensitive cyclase in the adipocyte ghost; on the contrary, the data presented here indicate that both ACTH and epinephrine sensitive cyclases are affected. Fain and Czech have also used the adipocyte ghost preparation to investigate this problem and have reported an increase in both ACTH and epinephrine-induced cyclase activity after a 3.5 hour incubation of isolated adipocytes with dexamethasone (1975). However, this observed increase tends to dissappear when Fain and Czech's results are normalized in terms of a percent increase over basal level.

In reference to the coupling mechanism, it is worth noting that calcium ion appears to be necessary only for the ACTH-induced cyclase activity (Rodbell, 1969; Bar and Hechter, 1969) and that this requirement is expressed after ACTH-receptor interaction (Lefkowitz, 1970). Moreover, since all the lipolytic hormones share a common cyclase, these observations must indicate that calcium is needed for ACTH receptor coupling to the cyclase. Any lesion which appears to be specific for the ACTH sensitive cyclase, may therefore be explained at least in part by the possible steroid regulation of some calcium binding-protein.

In any discussion involving the regulation of hormonally-induced cyclase activity, the role of GTP should not be

overlooked. The possibility that the guanyl necleotide requirement for hormonal cyclase stimulation may be modulated by the level of steroids is an intriguing hypothesis. Although we have observed that GTP exerts no effect on the ACTH-induced cyclase in adipocyte ghosts from adrenalectomized rats, perhaps a more detailed study using various concentrations of guanine nucleotide, as well as the non-hydrolyzable GTP analogue $GPP(NH)_p$ is warranted before this idea is dismissed.

The effects of adrenalectomy on hormonally-induced cAMP production in whole adipocytes should also be considered, although this cannot be taken as a direct measurement of the cyclase activity. Our data would indicate that neither the ACTH nor the epinephrine stimulated cAMP levels are deficient after adrenalectomy; at all doses tested, a rise in cAMP production over basal is detectable. Dexamethasone treatment is observed to increase these levels, but to super-normal values which are well beyond the range within which cAMP would be rate-limiting for lipolysis. Allen and Beck (1972) have reported a decrease in both ACTH and epinephrine stimulated cAMP in whole adipose cells from adrenalectomized versus normal rats, but this supression tends to disappear (particularly for epinephrene induced production) when the assay is conducted in the presence of theophylline as in the experiments presented here.

From the results obtained with isolated whole adipocytes, it is becoming apparent that the rate of cAMP degradation is an important factor which must be considered

in determining the effects of adrenalectomy on the hormonal sensitivity of the adipocyte. In the interpretation of data which is expressed in terms of cAMP production, one must of course consider the role which the coupling mechanism has assumed in producing these levels; however, it is perhaps equally important to take into account the phosphodiesterase activity of the preparation. As indicated previously, all of our experiments which have involved cAMP measurements have been conducted in the presence of theophylline, and this inclusion might explain at least in part some of the discrepancy with Braun and Hechter. In any event the work presented here and that of other investigators would seem to imply that dexamethasone exerts an inhibitory effect on the phosphodiesterase activity. Indeed, it has been confirmed by Senft et al. (1968) that the phosphodiesterase activity of liver, kidney and adipose tissue was increased 5 days after adrenalectomy and that steroid treatment resulted in a lowering of activity in all the tissues. Manganiello and Vaughan (1972) have also observed a decrease in the phosphodiesterase activity of HTC hepatoma cells cultured with dexamethasone. It should be noted, however, that the postulation of a direct effect of steroids on phosphodiesterase activity would be imprudent at this time, particularly since Fain (1975) and Manganiello and Vaughn (1972) have been unable to demonstrate a dexamethasone-induced change in adipocyte phosphodiesterase in vitro. Enough evidence does exist

nonetheless, to draw attention to the activity of this enzyme when cAMP levels are measured in studies involving steroids, particularly at the pharmacological doses which we have employed.

Before completing the discussion on the effects which the phosphodiesterase activity might have on the adrenalectomy-induced lesion, a few words must be mentioned about the use of a subcellular fraction like the ghost in this context. As discussed in reference to the binding studies, fractionation of cells can result in the concentration of particular enzymes and in the generation of an artificial cellular environment. Recent reports of the existence of a cytoplasmic as well as a membrane-bound phosphodiesterase (Kono et al., 1977) complicate further the use of the membrane-enriched ghost preparation for these studies.

To summarize this portion of the discussion, suffice it to say that a great deal of confusion and controversy exists as to whether the depressed lipolytic response observed after adrenalectomy and restored with steroids can be attributed to the decreased levels of cAMP. Some evidence does indicate that the receptor coupling to the cyclase might be affected by glucocorticoids (Braun and Hechter, as well as this study), resulting in low hormonally-induced cAMP production. On the other hand, in isolated intact adipocytes depressed cAMP levels after adrenalectomy are not apparent, particularly when the assay is conducted in the presence of a phosphodiesterase

inhibitor; it is reasonable to speculate therefore that the results obtained with the ghost preparation might not be relevant to the problem.

Although there is no agreement concerning the effect of steroids on the events which occur between the hormone-cell interaction and the production of cAMP, there is little controversy over glucocorticoid regulation of the pre-lipolytic events occuring after the generation of the "second messenger". The current general consensus is that adrenalectomy imposes a loss of sensitivity on the target cell to the cAMP which is produced, so that in effect the "second message" is never received (Allen and Beck, 1972; Exton et al., 1972; Schaeffer et al., 1969). Schaeffer et al. have demonstrated that adrenalectomy results in a reduction of the amount of inactive glycogen liver phosphorylase and that the administration of hydrocortisone restores the level of enzyme as well as the hormonally-induced hyperglycemic effects. This same group has also observed a steroid-dependent conversion of inactive to active phosphorylase in muscle tissue (1969a). Furthermore, Miller (1961) has shown that adrenalectomy causes a decreased activation of phosphorylase b in the perfused heart. In agreement with these results, several investigators have postulated that adrenalectomy could result in a change in the adipocyte protein kinase phosphorylase and/or the triglyceride lipase which form the terminal portion of the lipolytic response. It is interesting to note that the

action of phosphorylase kinase from muscle has been shown to require calcium (Brostrom et al., 1971), and that tissue perfusion with calcium repairs the kinase lesion caused by adrenalectomy. Moreover, it should be pointed out that the phosphodiesterase has also been reported to be calcium-dependent (Kalkiuchi and Yamazaki, 1970; Teo and Wang, 1973). It is tempting to speculate that adrenalectomy causes a general intracellular calcium deficiency which can set in motion a sequence of events that may in part account for the far-reaching consequences of steroid insufficiency. In any event, it is evident that the depressed lipolytic response to many hormones can be attributed to a post cAMP deficiency, although the mechanism is not yet clearly defined.

Before ending the discussion on the adrenalectomized-induced lesions in the lipolytic response, a summary would seem to be in order. In retrospect, it appears that what was apparently a widespread controversy over the site of the lesion, reduces to a relative agreement if the results obtained on the adenylyl cyclase activity of the ghost preparation are discounted. Again, despite the utility of the ghost, the investigator must be wary of the artifacts which can be associated with this preparation. The dramatic loss of sensitivity to ACTH stimulation specifically in the rat adipocyte ghost has already been mentioned. It has been suggested that the effect of the glucocorticoids is not on the hormone receptors or

on the adenylyl cyclase itself, but rather on the stability of the membrane (Fain and Czech, 1975); therefore, the presence of certain steroids might reduce the loss of hormonally sensitive cyclase which occurs during fractionation. Along these lines, Jarett et al. (1971) have demonstrated that during the course of purifying adipocyte membranes, the ability of ACTH, epinephrine and glucagon to stimulate adenylyl cyclase can be lost in patterns distinctive from both one another and from the loss of total cyclase activity as well.

In the experiments presented here in which isolated whole cells have been employed, adrenalectomy does not have much of an effect on cAMP production, particularly when assayed in the presence of theophylline. In light of these observations and the number of reports in the literature implicating dexamethasone as a phosphodiesterase inhibitor, it is likely that the changes in cAMP levels observed after steroid manipulation are secondary to changes in phosphodiesterase activity. Furthermore, the lesion in the lipolytic response may not be involved in the production of cAMP, but may reside in a general affect on the later steps which result in activation of the lipase. At this point, the occurrence of multiple lesions cannot be eliminated, particularly since ACTH-stimulated lipolysis appears to be suppressed more than the epinephrine-induced response. The reported changes in calcium metabolism following adrenalectomy and the return to normalcy after

steroid replacement (Miller, 1961), as well as the evidence for a dexamethasone-induced calcium transport across intestinal membrane (Harrison and Harrison, 1960), make it tempting to speculate that the ACTH-induced adipocyte responses, which are known to be calcium dependent, are affected at multiple sites after adrenalectomy.

The Effect of Adrenalectomy and Dexamethasone Administration on ACTH Binding to Isolated Adipocytes:

After this all too lengthy discussion of the effects of adrenalectomy and glucocorticoids on the response of the adipocyte, it is probably difficult to recall the initial purpose of this study - namely, to effect a steroid-induced selective modulation of the ACTH receptor. In light of our failure to demonstrate a selective effect of adrenalectomy or steroid replacement even in the ghost preparation, and the confirmation from subsequent work as described above, we questioned any specific alteration in the ACTH receptor population. However, it is evident now that monitoring the cyclase activity through measurement of cAMP production is too complicated a process, and need not reflect the state of the receptor population. As a more direct indication of receptor number, the binding of tritiated ACTH to adipocytes isolated from adrenalectomized and adrenalectomized/dexamethasone-treated rats has been carefully measured and compared to the binding of normal cells. Within the limitations and

sensitivity of the binding measurements (as discussed in Chapter 3), the results illustrated in Fig. 19 indicate that there are no differences in the binding to the various preparations. The magnitude of the binding at each concentration of tritiated ACTH tested is essentially the same in all three cell populations, when these are normalized to DNA content. A comparison cannot be made on the basis of the dry weight of the cells, since this parameter will reflect primarily the fat content. A large discrepancy between the weight/DNA content of the normal versus the adrenalectomized populations was found - that is, cells from the adrenalectomized animals were about $^{1}/3$ the weight of those from normal animals. However, the weight/DNA content was found to be comparable for the cells isolated from the adrenalectomized and adrenalectomized/dexamethasone-treated groups.

A few words concerning the shape of the binding curves are appropriate at this time. In his excellent chapters on the theory and mathematics of hormone receptor interactions, Rodbard (1973) indicates that a change in the number of receptors on a target tissue will be reflected in a shift in the sensitivity of the dose response. For example, if the binding or biological response is expressed as a function of hormone concentration, an increase in the number of receptors will effect a shift to the left of the curve to express this increased sensitivity. This is clearly not the case with the binding data presented here; these results are therefore,

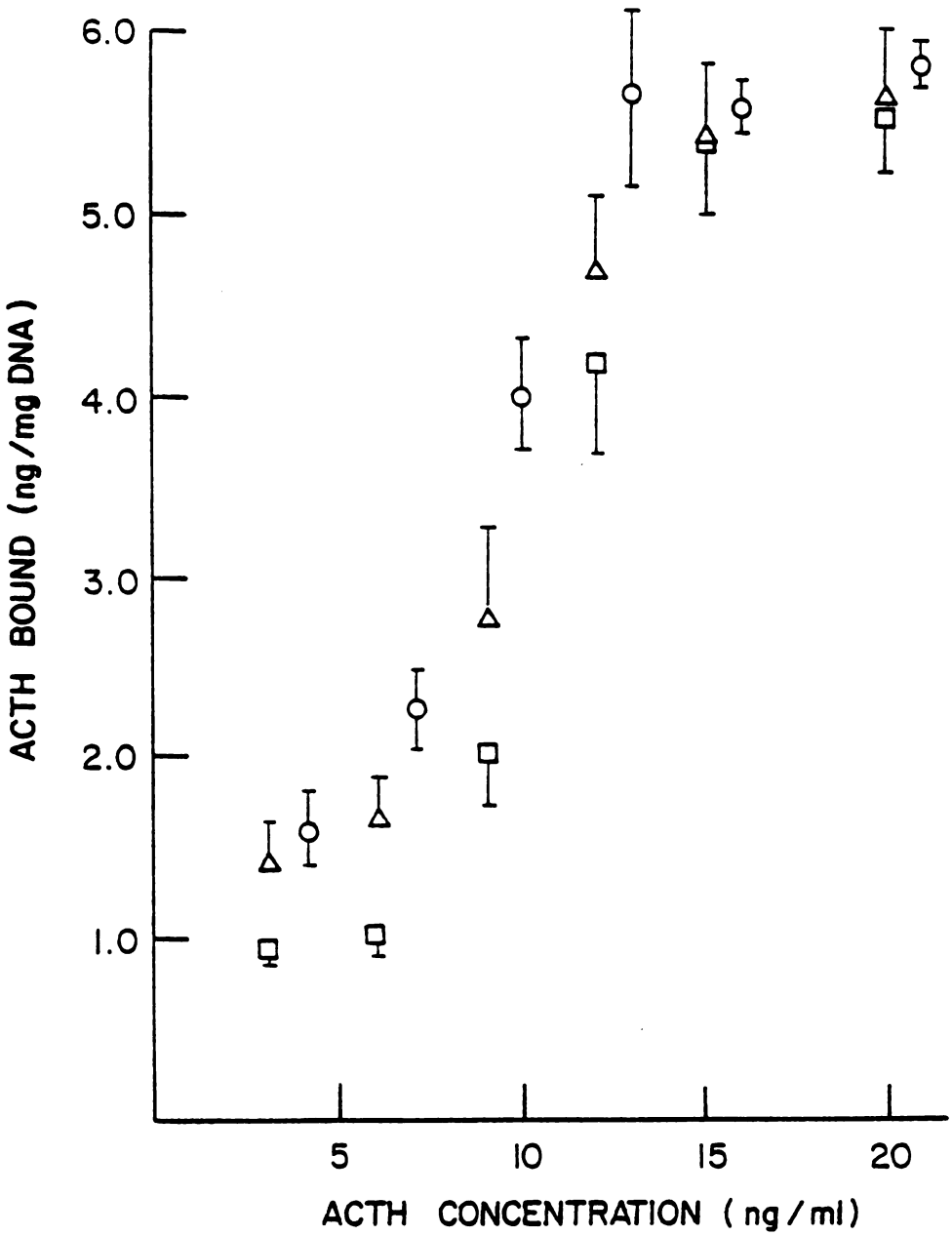


Fig. 19. Binding of tritiated ACTH (90ci/mmole) to isolated adipocytes from normal (O), adrenalectomized (Δ) and adrenalectomized/dexamethasone-treated (□) rats. Binding was measured as described in Chapter 3.

confirmed in a qualitative sense, which is perhaps more credible than the quantitated binding. It is also interesting to recall that the results with cyclase stimulation in the ghosts (Fig. 18) and those of Braun and Hechter as well, do not show a shift in the dose response curve of the adrenalectomized versus the adrenalectomized/dexamethasone-treated populations for either ACTH or epinephrine stimulation. Although the ghosts from the two populations may differ in the magnitude of the cyclase response, the concentration of hormone required to effect half-maximal stimulation remains essentially the same, thereby implying by Rodbard's standards that the number of receptors has not been altered.

It can be concluded from the results presented here that within the limits of the binding analysis, compared to the normal state, we can observe no alteration of the ACTH receptor population on the adipocyte following either adrenalectomy or adrenalectomy/steroid replacement. The demonstration that adrenalectomy fails to affect the binding of a hormone to its target cell, while the response is apparently altered, is not unprecendented; Rajerison et al. (1974) have shown this to be the case for the interaction of vasopressin with the rat kidney cyclase system.

From this limited data the possibility that the binding has been altered in a more subtle way- for example, in the rates of association or dissociation, or perhaps in the function of cooperativity among binding sites cannot be eliminated. However, the constancy of the ACTH receptor

population concurs with the results of the cAMP production in whole adipocytes as presented previously. The binding to the ghost population was not attempted in view of the high concentrations of tritiated ACTH which would have been required and the concomitant difficulties, as discussed in Chapter 3. However, the failure to observe a selective enhancement of the ACTH-induced cyclase activity after dexamethasone treatment even in the ghost preparation (Fig18) more or less discredits the theory that steroids are capable of effecting specifically an ACTH receptor modulation.

Hormone Regulation of Receptor Populations

Before concluding this chapter, some mention must be made concerning the recent trend of investigating the possibility that a hormone regulates its own receptor population. The literature is replete with reports of circulating levels of hormones which can either increase (Posner et al., 1974 for prolactin) or decrease (Gavin et al., 1974 for insulin; Lesniak et al., 1973 for growth hormone) their receptor number. Indirectly, the binding results presented here give some insight into whether ACTH exerts this type of influence on the adipocyte. As a consequence of adrenalectomy, the negative feedback effect of the adrenal steroids has been removed, and this state would result in very high levels of circulating ACTH. However, this condition was determined not to effect a change in the ACTH receptors

on the adipocyte. On the other hand, the administration of dexamethasone, which will inhibit ACTH secretion, again appears to effect no alteration in the number of adipocyte receptors as determined by our binding measurements. The possibility cannot be eliminated, however, that the receptor population has been altered to an extent which is not detectable by the level of sensitivity of our analysis. Nevertheless, perhaps this approach to regulating and modulating ACTH receptors on the adipocyte would prove to be more straightforward and challenging than would the manipulation of steroid levels which, as has been demonstrated, will introduce a series of complicated metabolic changes.

CHAPTER 5

THE CHARACTERIZATION OF THE ADIPOCYTE MEMBRANE BY ONE- AND TWO-DIMENSIONAL POLYACRYLAMIDE SDS ELECTROPHORESIS

Introduction

The difficulties of exploring the mechanism of hormone action on a molecular level cannot be underestimated; indeed, Chapters 3 and 4 have undoubtedly documented this claim already. The problems involved with binding studies certainly limit the information which can be obtained about the initial hormone-cell interaction, and the pitfalls of monitoring receptor activity by measuring a far-removed consequential event have already been overemphasized in the preceding chapter. These circumstances necessitated the introduction of a more direct chemical means for investigating cellular events and the molecular components involved. For these purposes, we adapted a highly sensitive sodium dodecyl sulfate polyacrylamide gel electrophoresis system (SDS PAGE) which could be implemented to investigate the protein components of the adipocyte membrane; the information obtained in this manner could then be used, for example, to complement and hopefully clarify the binding results and the steroid regulation study which have been described previously.

The use of PAGE to investigate detergent-solubilized cell components is certainly not unprecedented. In prokaryotic systems, for example, this procedure has been employed extensively by Studier (1972) to study phage protein

biosynthesis and also by Ames (1974) to identify the histidine transport system in typhimurium. In mammalian systems, the components of the rat adipocyte have been characterized by SDS PAGE by Czech and Lynn (1973) and Kawai and Spiro (1977), as has the turkey erythrocyte by Caldwell (1976). The technique has also been applied to measure protein synthesis by monitoring the uptake of radioactive amino acids in specific proteins (Gudas, 1976), hormonally-induced phosphorylation of cell components (Benjamin and Singer, 1975), and the induction of specific enzymes (Ford and Engel, 1974). In view of these many successful applications, this facet of the study was undertaken with the confidence that the information obtained would be an asset to the overall research project. In order to increase the sensitivity of the system and to improve the resolution, the study was also extended to the use of a two-dimensional isoelectric focusing (IEF)/SDS PAGE system modeled after the Ames technique (1976), a procedure similar to that described by O'Farrell (1975).

The Theoretical Basis of SDS Polyacrylamide Electrophoresis and Isoelectric Focusing

Before approaching the experimental aspects of this study, a brief discussion of the principles behind the SDS PAGE and IEF will be presented. Although a detailed treatment is beyond the scope of this discussion, excellent reviews on the theoretical implications of PAGE can be found

in the original work of Ornstein (1964) and Davis (1964) and more recently by Chrambach and Rodbard (1971), and for IEF, Finlayson and Chrambach (1971) should be consulted.

In general, the separation of proteins in an SDS PAGE system depends on the ability of a protein to bind the anionic detergent SDS in a fixed amount which is proportional to the protein's length or Stoke's radius. The detergent's obliteration of the protein's intrinsic charge implies that the mobility of the protein in an electric field will be a simple function of its molecular weight; that is, the separation will be accomplished on the basis of protein size only. Several exceptions to this idealized situation do exist, and should be at least mentioned, particularly when using this technique to characterize membrane components. To begin with, although it has been determined that most proteins bind 1.4g SDS/g protein when saturated (Pitt-Rivers and Impiombato, 1968), there are many exceptions; most notable among these are the glycoproteins which have been reported to bind up to 10g SDS/g (Grefarth and Reynolds, 1974). Additionally, the intrinsic charge of the protein may affect binding, as polyglutamic acid has been found not to form a complex with SDS (Fasman et al., 1964). Moreover, proteins allied to membranes, that is, lipophilic proteins, also exhibit anamolous SDS binding characteristics (Simons and Kaarianien, 1970).

To return to other repercussions of the assumption that SDS PAGE separation is accomplished exclusively on

the basis of protein size, one must consider whether all SDS-protein complexes are of constant diameter (that is, varying only in length), a condition which would eliminate the necessity of considering the effect of frictional drag on mobility. Experimental evidence indicates to the contrary that small molecular weight polypeptides (less than 15,000 daltons) do not form complexes of typical constant "protein" diameter, and this phenomenon may explain why they do not display mobilities which are proportional to their molecular weight (Williams and Gratzer, 1971).

The question of whether the bound SDS completely obliterates the intrinsic protein charge is also an important issue, and at least in the presence of very basic proteins (for example, histones), this does not appear to be the case (Panyim and Chalkley, 1970). As might be anticipated, charge obliteration has also not been demonstrated for small peptides which, because of their relatively short chain length, are unable to bind as much SDS as a larger protein (Swank and Munkres, 1971).

Another point concerning the binding of SDS to proteins which should be considered is that in certain instances SDS may not disrupt all non-covalent protein interactions. Aggregations which are known to be resistant to SDS dissociation have been reported, as has been a dismutase enzyme which still retains its activity in the presence of 10M urea and 4% SDS (Abernethy et al., 1974). These

observations have a particular significance when the SDS PAGE technique is applied to membrane proteins, which are thought to form numerous and complex interactions.

The above points are not intended to discourage the application of SDS PAGE, but rather to caution against the rampant use of this technique for absolute molecular weight determinations. Its application to the characterization of membrane and cell components unquestionably provides a useful tool for promoting the understanding of these proteins, provided the limitations of the method are appreciated; a detailed theoretical analysis of many of these problems has been published (Maddy, 1976).

Having accepted the fact that not all proteins form SDS complexes which conform to theoretical size and charge characteristics, and are of constant diameter, we are prepared to discuss how the "ideal" complexes would be separated on polyacryamide gels. To begin with, acrylamide gels are considered to have an average pore size which is inversely related to the concentration of acrylamide and crosslinking. When macromolecules such as protein-SDS complexes are forced through gels by an electric field, the relative mobility of the complex in the gel is retarded as a function of its size. In theory, therefore, it is possible to effect the separation of various protein-SDS complexes by judicious selection of a gel with the appropriate pore size.

For reasons to be described in the Methodology, in our SDS PAGE we elected to utilize a discontinuous stacking system which would insure a sharper band resolution. A detailed theoretical discussion of this system can be found in the Ornstein review (1964); however, for the sake of clarity, a brief description will be given here. As its name implies, this system promotes the concentration or "stacking" of proteins in a small gel area, and achieves this effect through both physical and chemical means. To accomplish the physical aspect of stacking, the proteins in the sample are introduced into a large pore area which allows them to migrate without being retarded; however, at the interface of this stacking gel and the smaller pore separating gel, the proteins are arrested abruptly and essentially "stack up" in very narrow bands before entering the area of separation. The chemical stacking is achieved by a discontinuous buffer system which consists of a "leading" ion, or one that will migrate faster than any protein and be insensitive to pH changes, as well as a "trailing" ion, or one whose pK dictates that its mobility will be less than that of the slowest protein. It is fairly easy to appreciate the feasibility of setting the pH of the buffer system in such a way as to "sandwich" the protein in between the leading and trailing ions, thereby effecting the chemical stacking. In the system adopted for this particular study, chloride was selected as the leading ion and glycine (pK 9.6) to trail. The

pH of the stacking gel was set at 6.8, so that under these conditions glycine is not very negatively charged and will not migrate very quickly toward the anode. However, the pH of the separating gel is set at 8.8, which provides an ionic environment in which most of the glycine molecules will be negatively charged. Under these conditions the mobility of glycine will increase toward the anode, thereby sharpening the boundary between the glycine and the chloride, and effecting a stacking of the protein in the process. It should also be emphasized that the concentration of protein which is achieved with this technique not only allows for an improved resolution, but renders the separation independent of sample volume as well.

As mentioned previously, this investigation was expanded to a two-dimensional separation which involved isoelectric focusing in the first dimension, followed by a conventional SDS PAGE for the second. Since IEF (or pH gradient electrophoresis) resolves molecular species differing in net charge only, the initial fractionation with IEF afforded the advantage of separation on the basis of both size and charge, therefore overcoming a limitation of SDS PAGE. Excellent papers on this method have been published (Finlayson and Chrambach, 1971; O'Farrell, 1975); consequently, no attempt will be made to discuss IEF in detail here. Suffice it to say that the technique again involves the use of polyacrylamide gel as a solid support, but the separatory capabilities

rely primarily on the inclusion of ampholytes of a selected pH range within the gel. When subjected to an electric field, proteins will then migrate through the gel to their isoelectric point. Because of the difficulty encountered in the solubility of the membrane preparation used in this study an efficient focusing of these proteins could not be accomplished. To circumvent this problem the two-dimensional method described by Ames (1976), which involves solubilizing the protein with SDS before focusing was adopted. Although this would appear to be impossible since SDS should obliterate intrinsic protein charges, the separation of SDS-solubilized proteins does occur when they are subjected to IEF. Ames attributes this somewhat surprising event to incomplete charge obliteration under the detergent conditions used, or alternatively, to the dissociation of the detergent from the protein; this latter phenomenon has been confirmed by other investigators as well (Weber and Kuter, 1971; O'Farrell, 1975). Although the isoelectric separation of SDS-solubilized proteins cannot at the present be explained definitively, the inclusion of the anionic detergent does permit an otherwise impossible fractionation of the adipocyte membrane preparation.

Methodology

Factors Involved in Insuring Maximum Protein Resolution

In the early phases of this work some difficulty was encountered in obtaining homogeneous gels, in controlling

the gelation time, and in effecting well defined protein bands. It was found in time ,however, that each of these problems could be rectified at least partially by using only electrophoresis purity reagents. The initial attempts at forming gels involved the use of inferior grade acrylamide and bis-acrylamide from Eastman which were recrystallized according to the method of Leonig (1967). It was observed that the recrystallized reagents polymerized almost instantaneously when mixed with the ammonium persulfate solution, and it was assumed that some inhibitor was eliminated during the purification. A reduction in the amount of ammonium persulfate delayed the gelation time to approximately 10 to 15 minutes, but a further reduction of the initiator failed to result in any polymerization. The use of electrophoresis purity reagents from Bio-Rad (Richmond, CA) allowed polymerization to take place within a reasonable amount of time, and this could be easily manipulated by altering the concentration of ammonium persulfate added. We preferred a polymerization time of approximately 45 to 60 minutes for the separating gel, as this allowed adequate time to overlay the gel carefully, to disperse any trapped air bubbles, or to handle any unforeseen emergencies; this gelation time could be insured by the addition of 0.025% ammonium persulfate (w/v) in a 7 - 10% gel with 0.017% TEMED (w/v). In addition, the Bio-Rad electrophoresis purity reagents consistantly provided homogeneous and reproducible gels.

Another difficulty encountered early on was the failure to obtain well defined sharp bands. This problem was alleviated by using the highest purity SDS available. It is easy to understand how the quality of the SDS would affect resolution if one recalls that protein separation on the basis of molecular weight is achieved by the protein's ability to bind an amount of SDS which is proportional to its size. It stands to reason, therefore, that if the SDS contains carbon chains with 11 carbons or 13 carbons, or at the extremes even 8 or 15, the SDS-protein complex could appear to be a diffused area, rather than a sharp, clearly resolved band. This becomes particularly critical when attempting to separate a large number of proteins of similar molecular weight, as is the case when characterizing membrane components.

The special case of resolving a heterogeneous mixture of several proteins warrants additional comment in respect to whether discontinuous stacking gels need be employed. Although some investigators have observed little improvement in resolution when using disc gels (Hjerten et al., 1965), they were dealing primarily with the resolution of a single species. In our experience, the type of sorting and resolution which the membrane mixture requires can only be achieved by a discontinuous stacking; this simple gel modification (and of course the use of highly purified SDS) insures the sharpest of bands, and thereby improves

the possibility that several proteins of similar molecular weight will not overlap.

The resolution of a protein mixture on any type of gel or under any circumstances will ultimately depend on the integrity of the sample. Incompletely solubilized proteins give rise to "streaking" which can be perhaps attributed to denatured material that does not enter the gel, thus causing irregular background staining and obliteration of the final pattern.

Materials:

Electrophoresis: For the buffer systems, Tris (2-amino-2(hydroxymethyl)-1,3-propanediol) was obtained from Eastman, glycine (amino acetic acid) from Swarz/Mann, and glycerol from Merck. For the acrylamide gels, Acrylamide, Bis-acrylamide (N,N-methylene-bis-acrylamide), ammonium persulfate and TEMED (N,N,N',N'-tetramethylethylene-diamine) were all Electrophoresis Purity Reagents from Bio-Rad. Acrylamide and Bis from Eastman when repurified were found not to provide acceptable gels, as described in the text. Sodium dodecyl sulfate (SDS, sodium lauryl sulfate) which gave optimum results was obtained from British Drug House (Specially Pure) or Pierce (Sequanal Grade); SDS from Sigma (inferior grade) did not give acceptable resolution. For staining gels, Coomassie Brilliant Blue R-250 was a product from Bio-Rad; for the Schiff reagent, periodic acid was

purchased from G. Frederick Smith Chemical Company and basic fuchsin from Matheson Coleman and Bell.

The gel apparatus was designed after the Hoeffer Slab Apparatus. Glass plates were either purchased from Hoeffer (San Francisco, CA) or made to order to U.C. Glass Company (Berkeley, CA). Sample combs, spacers and clamps for the apparatus were obtained from Hoeffer. Lubriseal from Arthur H. Thomas was used for fastening together the glass plates; all plates were treated with Photoflow solution from Eastman Kodak before use.

Isoelectric focusing: For the gels, Acrylamide, Bis, ammonium persulfate and TEMED were as was used for the SDS PAGE. Urea was purchased from Mallincrodt (Analytical Grade) and Nonidet P-40 (NP-40) was a product of Particle Data Research (Elmhurst, IL). Ampholines (40%) were obtained from LKB and were stored at 0°C. For imbedding the focusing gel in the second dimension electrophoresis gel, Agarose was purchased from Bio-Rad. For the auto-radiographic detection of proteins, ^{35}S-methionine (specific activity 500 ci/mmole) was obtained from New England Nuclear.

Methods:

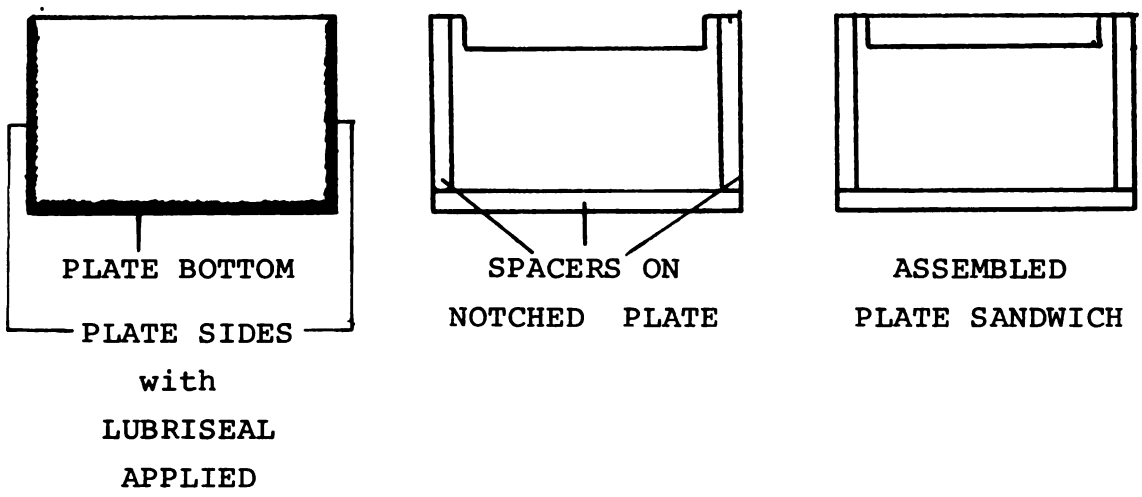
Assembly of the Slab Electrophoresis Apparatus:

The slab apparatus was designed according to the model proposed by Studier (1973), and consists essentially of two

glass plates which will "sandwich" the gel and which are clamped onto an acrylic box support. The plates are separated by polyvinylchloride spacers, and these spacers can vary in width to achieve the desired gel thickness. One plate is notched and is directed inward against the support, the top of which also serves as the upper buffer chamber. The gel is poured between the plates and sample wells are then formed by a teflon comb which is inserted at the top of the plates. After polymerization of the gel, the acrylic support to which is clamped the gel sandwich is submerged in a tank which serves as the lower buffer chamber. The components of the apparatus are illustrated in Fig. 20.

In the electrophoresis system described here, polished glass plates, 7"x5½", and 1/8" thick were used; the dimensions of the notched plate were also 7"x5½", with a 5½" long and 6/8" deep notch. In order to conserve the amount of reagents used and also to achieve maximum sensitivity in protein detection, we used a thin gel formed with 0.8mm thick spacers.

The apparatus was assembled as follows: (See Fig. 20) Both plates were greased along their perimeter with a narrow band of Lubriseal, care being taken not to apply more than the width of the spacer. The bottom spacer was positioned on the notched plate by moving it back and forth horizontally several times to insure a tight seal. The side spacers were then placed on the notched plate in a similar manner; an extra dab of Lubriseal was applied in



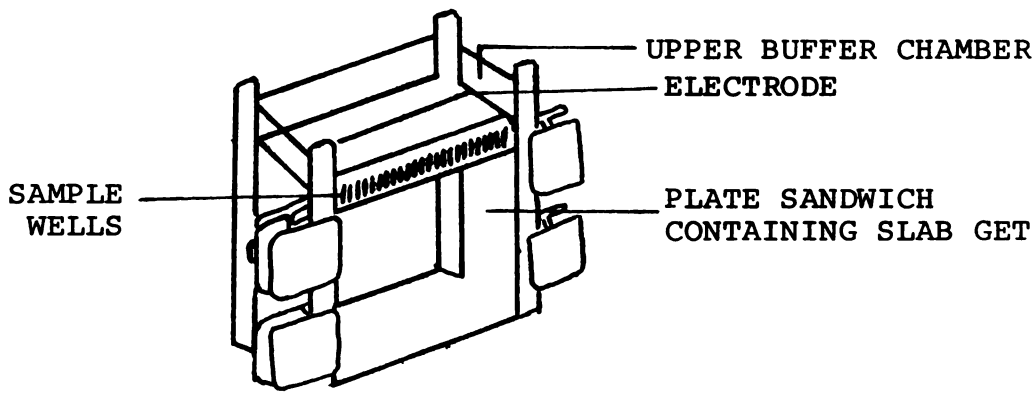


Fig. 20. Slab gel electrophoresis apparatus.

the corners where the side spacers meet the bottom one, as this area is particularly prone to forming leaks. The greased unnotched plate was lifted carefully and its greased side placed on top of the notched plate so that the spacers would be sandwiched in between. Pressure was applied all along the edged until a continuous seal could be seen between the plates and the spacers. The plate sandwich was then placed on the support and clamped down in six positions until the comb fit snugly when test-inserted. The plate was marked just below the bottom of the comb and the comb was removed.

Preparation and Pouring of Gel Solutions:
Caution: The monomeric form of acrylamide is a potent neurotoxin; unpolymerized solutions should therefore be handled with great care. If skin contact should occur, the area should be washed off immediately with running water followed by soap and water.

The gel system is adapted from the original Laemmli procedure (1970) and has been described in detail by Ames (1974).

Separating Gel Buffer: 0.563 M Tris-HCl, pH 8.8 with 0.1% SDS.

Stacking Gel Buffer: 0.139 M Tris-HCl, pH 6.8 with 0.1% SDS.

Gel Rinse Solution: 2 volumes of Separating Buffer and 1 volume of 0.1% SDS.

Acrylamide Stock Solution: 30% acrylamide, 0.8% bis, 0.1% SDS (w/v in deionized water).

Electrode Buffer: 3.03g Tris, 14.41g glycine, 1.0g SDS/l, pH 8.3.

All solutions were stored at 0°C and found to be stable over several months of use; the acrylamide stock was stored in a dark glass bottle.

The separating gel was mixed according to the following schedule:

% Gel Desired	30% Acryl. Stock	.1% SDS	Separating Gel Buffer
10	10 ml	0	20 ml
9	9 ml	1 ml	20 ml
8	8 ml	2 ml	20 ml
etc.			

The above reagents were combined with approximately 8µl TEMED and 0.16ml ammonium persulfate solution (5% w/v in water, prepared fresh), and the reagents were swirled gently to mix. The solutions were not deaerated and air bubbles were never found to be a problem in the gels. The stacking gel was prepared in a similar manner as follows: for a 3% gel, 1ml of Stock Acrylamide was added to 9ml of Stacking Buffer, followed by 5µl TEMED and 0.2ml ammonium persulfate solution (5% w/v).

Immediately after combining the reagents, the separating gel solution was pipetted between the plates to the previously marked height, and any bubbles which were introduced during the pipetting were carefully tapped out. As soon as possible, the gel rinse solution (0.5ml) was then

gently overlayed along the top of the gel and the comb was inserted at this point (insertion at this time was found to give a smoother interface between the separating and stacking gels; insertion just before pouring the stacking gel sometimes distorted the polymerized separating gel, possibly due to a "bowing" of the glass plates). Gelation of the separating gel could be detected as a difference in the refractive index between the polymerized and unpolymerized solutions; using the formula described above, approximately 45 to 60 minutes were required. When gelation was confirmed, the unpolymerized solution was removed by tipping the apparatus and allowing drainage into the sink. This should be done as soon after gelation as possible since unpolymerized solution will eventually dissolve into the gel; for best results, it should be removed within 30 minutes after gelation. The top of the gel was then washed twice with the gel rinse solution and drained completely. If not inserted previously, the comb was positioned at this time, the stacking gel reagents were combined as described above, and were then pipetted to the top of the apparatus, care being taken not to introduce air bubbles in the sample wells; polymerization should take place within 15 minutes.

After the stacking gel was polymerized, the spacer was removed from the bottom of the gel and the excess Lubriseal wiped off the plates with a Kleenex. The lower buffer tank was then filled with the electrode buffer, and the slab apparatus was placed into the tank; one end was positioned

first and the other was then gently lowered to force out any air trapped between the plates at the bottom of the gel. (If air bubbles are trapped, they must be removed to insure that an even current is delivered across the entire gel; this may be accomplished with a pasteur pipette whose tip has been curved to form a U-shape.) At this time, the electrode buffer was added to the upper buffer chamber and the comb was removed carefully; the presence of the buffer when removing the comb serves to flush out any unpolymerized gel from the channels and also to support the walls of the channels. The gel could be stored at this point (either at 4°C or room temperature) for use on the following day.

Preparation and Application of Sample Solutions to the Gel:

Sample Solubilizing Solution Stock: 0.0625 M Tris-HCl, pH 6.8 with 2% SDS and 10% Glycerol(v/v), On the day of use, β-mercaptoethanol was added to the stock solution to give a final concentration of 5% (v/v).

Proteins were diluted with the desired volume of solubilizing solution, mixed and boiled for 3 minutes; solutions should be clear, and must be diluted further if turbid. Samples could be frozen for future use, and were then heated to clarify before being used.

Samples should be added only after the apparatus has been set up completely and positioned for electrophoresis. A Hamilton syringe with a piece of polyethylene tubing attached to the needle simplified sample addition, and the

tubing eliminates having to draw the protein into the syringe barrel. Alternatively, disposable micropipettes were also found to deliver samples easily and accurately. Although we did not routinely add tracking dye to samples (as this tends to mask solubility problems), it is useful to pipette some Bromphenol blue dissolved in solubilizing solution into an unused channel.

To avoid any protein diffusion, electrophoresis was begun as soon after sample addition as possible. For SDS electrophoresis, the electrode in the upper buffer chamber is always designated as the cathode or negative electrode. The electrophoresis was usually run at constant current, approximately 10 to 15 mamp/slab (this was doubled if two slabs were run simultaneously). A low current was found to be optimal for the efficient separation of complex mixtures of heterogeneous proteins, but this advantage must be balanced with the time element; no run was allowed to proceed for more than 5 hours, as convection and diffusion could then become a factor. In reference to the problem of heating, it should be stated that the apparatus described here contains no cooling element. Therefore, for those runs in which heating could be detrimental, the electrophoresis was performed in the cold room at 0°C. Alternatively, addition of a large volume of lower buffer to the tank dissipated heat and improved resolution, particularly of the low molecular weight proteins.

The electrophoresis was generally terminated when the tracking dye reached the bottom of the gel. To remove the slab, the plates were pried apart to expose the gel; it was found that the optimal way to handle the slab and avoid breakage, was to grasp the gel along the bottom of its long side (gloves should be worn) and gently lift it from the plate.

Staining of the Gel:

Coomassie Blue Stock Solution: 0.25% Coomassie Blue (w/v in water).

Staining Solution 1: 25ml isopropanol, 10ml glacial acetic acid, 55ml water, 10ml Coomassie Stock.

Staining Solution 2: 10ml isopropanol, 10ml glacial acetic acid, 79ml water, 1ml Coomassie Stock.

Staining Solution 3: 10ml glacial acetic acid, 90ml water, 0.1ml Coomassie Stock.

Destaining Solution: 10% acetic acid.

Protein staining was best accomplished in a Coomassie Blue/isopropanol/acetic acid solution; although most procedures employ a methanol mixture, it was found that the Coomassie is more soluble in isopropanol (Fairbanks et al., 1971). Slabs usually stained rapidly (1 to 3 hours) in the concentrated Coomassie solution; however, it was observed that the time required for staining was dependent on the type of SDS used, and this has subsequently been confirmed by other investigators (Matheka et al., 1977). We generally stained

the slabs for 1 to 3 hours in Solution 1 (in the absence of SDS, gels stain almost instantaneously), and then for 1 hour in both Solutions 2 and 3. The rationale for transferring the slab to increasingly lower concentrations of Coomassie is that the staining of the proteins by the dye is irreversible, while that of the gel is reversible; therefore, by gradually decreasing the concentration of Coomassie the protein should continue to stain while the gel itself begins to destain, thus establishing a very efficient system. Gel destaining was completed in 10% acetic acid; this could generally be accomplished within 5 hours if the acetic acid was changed often and the slab was agitated. It should be noted that the gel could be left in any of the solutions as long as overnight if necessary.

The procedure for staining glycoproteins for their carbohydrate content was adapted from the method of Zacharius et al. (1969), executed stepwise as follows:

1. Immerse slab gel in 12.5% trichloroacetic acid (500ml) for 30 minutes. Rinse in distilled water; replace in fresh 12.5% trichloroacetic acid for an additional 30 minutes.
2. Rinse lightly with distilled water.
3. Immerse in 1% periodic acid (w/v in 3% acetic acid) for 60 minutes.
4. Wash thoroughly for 10 minutes in distilled water with stirring. Repeat 6 times.
5. Immerse in Fuchsin Stain in the dark for 60 minutes.

6. Wash with freshly prepared 0.5% metabisulfite (w/v) for 10 minutes. Repeat 3 times.
7. Wash in distilled water with frequent changes and stirring until destained.

Gels may be stored in 3 to 7.5% acetic acid.

Fuchsin Stain: Potassium metabisulfite (16g) was dissolved in 2 liters of water to which was added 21ml HCl (conc). Finely powdered basic Fuchsin (8g) was added and the solution was mixed gently for 2 hours at room temperature (or until the dye is in solution). After standing for an additional 2 hours, the solution was treated with a small amount of decolorizing charcoal and filtered within 15 minutes; the reagent was stored at 0°C.

Two-dimensional Electrophoresis:

1. Isoelectric Focusing:

To permit protein detection by autoradiography, ^{35}S-methionine was incorporated into the samples in the following manner. Cells were isolated from 160g male Sprague-Dawley rats as described previously and were suspended in KRB/4%BSA with 0.01% LTI (10ml/8 animals). Cells were preincubated at 37°C under an atmosphere of 95% O_2/5% CO_2 with gentle shaking for 30 minutes, at which time 0.5mci ^{35}S-methionine was added. The incubation was continued for 3.5 hours; at the conclusion of the incubation, the cells were washed thoroughly two times with fresh KRB/BSA buffer, and ghosts were prepared as described previously.

The samples for isoelectric focusing were solubilized according to the procedure of Ames and Nikaido (1976), briefly as follows. For 1.5mg protein: The protein was placed in a microcentrifuge tube, and was diluted with 10µl 0.5 M Tris-HCl (pH 6.8), 20µl SDS (10%), 5µl 0.01 $MgCl_2$, 10µl β-mercaptoethanol, and water to 100µl. The tube was capped and boiled for 3 minutes, followed by centrifugation at 40,000xg for 60 minutes in a Spinco centrifuge. The supernate was removed and diluted with 2 volumes of a dilution buffer containing 9.5M urea, 2% ampholines (gradient pH 4-6:5-8:7-9 in a 1:1:1 ratio), 5% β-mercaptoethanol and 8% NP-40 (w/v).

Isoelectric focusing was performed according to the O'Farrell protocol (1975). Gels were made in glass tubing (130mm x 2.5mm inside diameter) which were stoppered at one end with parafilm. The gel mixture (10ml) was prepared as follows: urea (5.5g) was added to a 125ml side arm flask, followed by 1ml water and 2ml NP-40 (10% w/v in water). The flask was swirled and heated to facilitate dissolving the urea. Acrylamide stock (1.33ml of 28.38% acrylamide and 1.62% bis) was then added, followed by 0.4ml ampholines (pH range 4-6;5-8;7-9 in a 1:1:1 ratio). When the urea was completely dissolved, ammonium persulfate (10µl of a 10% solution) was added and the solution was degassed for 1 minute. The glass tubes were then filled to within 5mm from the top and overlayered with 8M urea. After setting for 1 to 2 hours, gelation was confirmed and the

parafilm was removed from the tubes and replaced with dialysis membrane (to prevent the gels from sliding out in the presence of the detergent); the tubes were then fitted into a standard electrophoresis chamber. The lower reservoir was filled with 0.01M phosphoric acid. The gel overlay was removed and samples were pipetted onto the top of the gel; sample overlay (10µl of 9M urea/1% ampholines) was added, followed by extensively degassed 0.02M sodium hydroxide, and the upper chamber was then filled with the sodium hydroxide as well. The electrophoresis was conducted with water cooling at 300 volts for 14 hours, followed by 500 volts for 2 hours.

Gels were easily removed from the tubes by attaching a syringe and gently extruding the gel with pressure. Gels were equilibrated in 10ml of the SDS PAGE solubilizing solution for 1 to 2 hours with shaking, and could be stored frozen until use. The pH gradient of the focusing was determined by slicing a blank focused gel into 1cm pieces, diluting the 1ml deionized water, and allowing the slices to incubate at room temperature for 18 hours; the pH was then read on a Metrohm pH meter with a glass electrode. A typical gradient obtained with the ampholine system pH 4-6:5-8:7-9 in a 1:1:1 ratio was as follows:

Base: 8.80, 8.45, 7.84, 7.48, 7.26, 7.00,
6.70, 6.36, 6.08, 5.72, 5.35, 5.20: Acid

2. SDS Polyacrylamide Electrophoresis:

The second dimension of our system was conducted as previously described for SDS PAGE with minor modifications. For the two-dimensional system, the separating gel was poured to 25mm below the plate notch, was overlayered with 1ml of water, and was allowed to polymerize for 1 hour. The overlay was replaced with separating gel buffer diluted 1:4 and allowed to stand overnight. The overlay was removed the following day, and the gel was rinsed with stacking buffer. The stacking gel solution was poured to right below the notch and was overlayered with stacking buffer.

The focusing gel was imbedded in the polyacrylamide slab apparatus (unmodified) as follows. Agarose (0.5% w/v) was dissolved in stacking buffer and a small amount was pipetted onto the top of the stacking gel. The focusing tube gel was gently placed on the Agarose solution (one end first to allow extrusion of trapped air), and once centered properly, was covered with more Agarose. It was helpful to shine a heating lamp on the apparatus while the focusing gel was being imbedded, as this prevented the Agarose from gelling during these manipulations.

The electrophoresis was then run as previously described. A small amount of Bromphenol Blue could be added to the upper buffer to make the solvent front more visible.

Drying of Slab Gels for Autoradiography:

Slabs which were to be subjected to autoradiography were dried in the following manner. The focusing gel and

Agarose imbedding were removed and the slab was sandwiched in between two large sheets of dialysis membrane (Bio-Rad) and placed on a clean glass plate. All wrinkles and air bubbles were smoothed out, and spacers were buttressed against each of the four sides of the gel, care being taken to extrude as much water as possible. Once positioned properly, the spacers were clamped down; the gel was then placed under a hood and allowed to dry for at least 18 hours. This procedure rarely resulted in cracking of the slab, provided that no "nicks" were initially present along the sides of the gel.

Results and Discussion

The gel represented in Fig. 21 illustrates the typical protein pattern of the SDS-solubilized rat adipocyte ghost and membrane; this pattern was found to be highly reproducible from one preparation to another. The ghost is seen to be comprised of at least 30 proteins which range in molecular weight from 200,000 to approximately 17,000 daltons. It should be noted that the major difference in the ghost versus the membrane pattern is in the loss of the low molecular weight components, which are apparently purified out on the sucrose gradient; additionally, an intensification of the 70,000 and 85,000 molecular weight (MW) is apparent in the membrane preparation. As indicated in Fig. 21, these two bands stained positively for glycoproteins in both ghost and membrane, but again were found to be more intense in the latter.

The results presented here are in general agreement with those obtained by Czech and Lynn (1973). However, the SDS PAGE system described here appeared to yield superior resolution and it is difficult to compare the more complex pattern presented here with that of Czech and Lynn which indicated 11 protein bands ranging from 168,000 to 22,000 MW. It does appear, nevertheless, that the carbohydrate-staining bands of the present study do correspond to Czech's, although we have failed to observe the additional 62,000 MW Schiff-positive glycoprotein. The results of the study with the adipocyte ghost and membrane also compare favorably to that of Kawai and Spiro (1977) who used a highly efficient discontinuous sucrose gradient (as compared to our linear gradient) to obtain several membrane fractions from mechanically dispersed adipocytes. In the lighter membrane fractions, Kawai and Spiro observed 5 major bands (85,000 to 35,000 MW) which are analogous to some of ours; moreover, their heavier membrane preparations reveal a complex pattern which is similar to what we have observed. Kawai and Spiro also demonstrate the presence of 2 glycoprotein bands at 74,000 and 88,000 MW, and this appears to be in almost precise agreement with our results.

A comparison of the protein pattern of the ghosts obtained from normal versus adrenalectomized rats was executed in an attempt to identify a protein entity which could be responsible for the adipocyte lesion observed after steroid withdrawal. As indicated in Fig. 22, no

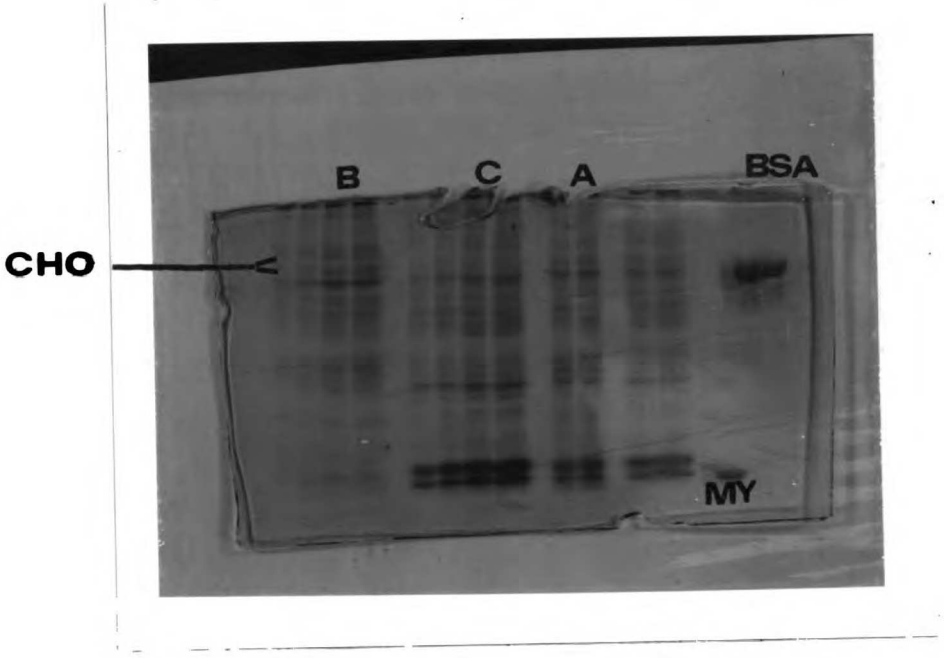


Fig. 21. SDS PAGE pattern of the adipocyte ghost (A), purified membrane (B), and the mitochondrial fraction (C). The carbohydrate-positive bands are indicated (CHO). The electrophoresis was performed as described in the text. Approximately 25µg/20λ of sample was applied to the gel. Bovine serum albumin (BSA, 5µg) and myoglobin (MYO, 5µg) were included as 68,000 MW and 17,000 MW standards respectively.

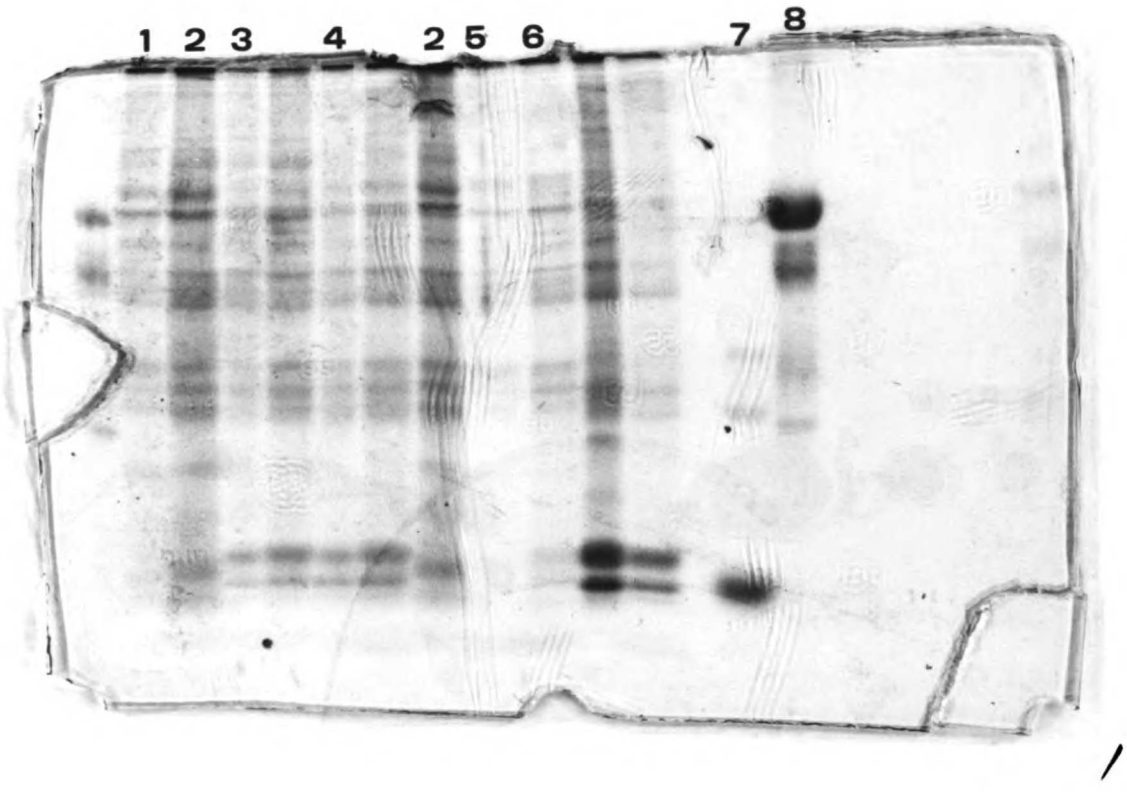


Fig. 22. SDS PAGE pattern comparing adipocyte fractions from the rat and the rabbit, as well as the normal rat versus the adrenalectomized rat. Key: 1 = rat membrane; 2 = adrenalectomized rat membrane; 3 = rat ghost; 4 = adrenalectomized rat ghost; 5 = rabbit membrane; 6 = rabbit ghost; 7 = myoglobin; 8 = bovine serum albumin. Approximately 25µg of adipocyte fraction was applied.

major differences between the two patterns could be detected at least within the sensitivity of our system. A comparison of the pattern of the rat versus the rabbit adipocyte was also attempted, and despite the known differences in their response to ACTH stimulation (Ramachandran and Lee, 1976), no apparent changes in membrane protein composition could be found.

Although the SDS PAGE system provided a rapid, consistent and sensitive characterization of the adipocyte membrane proteins, it soon became obvious that it could not offer the degree of resolution which we required. For this reason, the SDS fractionation was coupled to an isoelectric focusing technique to provide a two-dimensional system which possessed an even greater degree of sensitivity. In order to promote the level of detection to the improved level of sensitivity, the adipocytes were preincubated with ^{35}S-methionine so that autoradiography could be used to identify the protein components. Fig. 23 demonstrates a two-dimensional pattern.

The two-dimensional system was found to be more difficult to implement than the one-dimensional SDS PAGE. The necessity of including SDS to effect complete solubilization of the membrane preparation limited the pH range within which the proteins could focus. As illustrated in Fig. 23, most of the proteins are concentrated in the basic region, with none found in the acidic extrementy; Ames (1976) and O'Farrell (1975) report the same observation when focusing

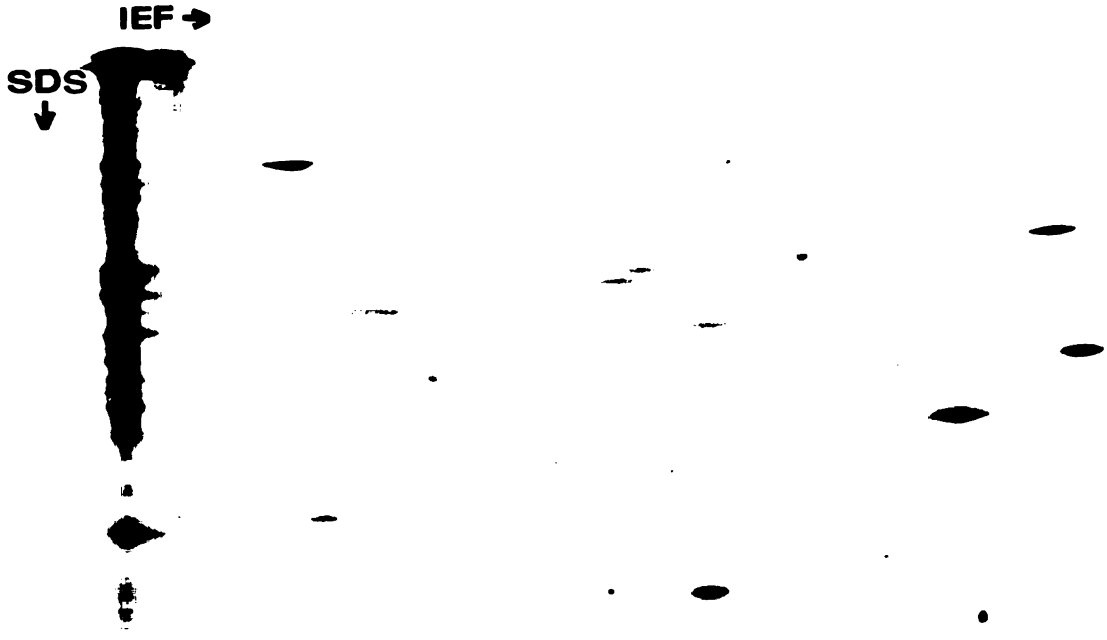


Fig. 23. Two-dimensional electrophoresis of the rat adipocyte ghost. The pH gradient is as described in the text, with the basic end at the left side of the pattern and the acidic at the right. Between 200,000 and 300,000 cpm were applied to the focusing gel, and the autoradiograph was exposed for 27 days.

in the presence of SDS. This phenomenon has been attributed to the migration of the detergent to the low pH region of the gradient, rendering it even more acidic and incapable of focusing proteins. A combination of ampholines which gave the broadest distribution of proteins was eventually found; however, this gradient occurred over an extremely narrow pH range (pH 8-5), and such a situation is not ideal for obtaining sharply focused, well defined spots. Some difficulty was also encountered with local heat convection which, when not controlled, resulted in poor resolution of the lower molecular weight components. In addition, the same autoradiographic efficiency as reported by O'Farrell (1975) was not achieved in this study. Although the incorporation of ^{35}S-methionine was efficient enough to allow the application of approximately 400,000 cpm, it was found necessary to expose our gels for long periods of time (3-4 weeks) to effect detection; such long exposures resulted in the generation of artifacts and diffused spots. This depressed efficiency was attributed either to a loss of counts during the focusing or equilibration, or possibly to quenching caused by the dialysis membrane which was used to coat the dried gel. Some of the additional problems which can limit the resolving power of this delicate two-dimensional technique are discussed in the excellent study of O'Farrell (1975).

The protein components of the adipocyte ghosts prepared from normal and adrenalectomized rats were subjected to the

two-dimensional separation, and the autoradiographs are presented in Fig. 24. Although the resolution in this experiment was not the best which could be achieved, there are again no apparent differences, with the exception that the adrenalectomized sample in general contained more counts. It must be noted, however, that the pattern represents not the total ghost protein content, but rather the newly made proteins which were synthesized during the 4-hour incubation with the labeled methionine. In order to check what proportion of the total proteins this represented, another sample was subjected to SDS PAGE, and was then both stained and radiographed. On the basis of this experiment, it was observed that approximately 80% of the total proteins had been synthesized to some degree in those four hours; however, it was difficult to determine the relative amounts synthesized for the various proteins. This observation emphasizes the difficulty of analyzing the autoradiograph, particularly if any changes which occur are not so dramatic, so that they are seen not as the presence or absence of spots, but rather as alterations in their relative intensities. Even if the radiographs are scanned with a densitometer to determine relative intensities, this procedure assumes equivalent specific activities for each protein, a somewhat erroneous assumption particularly in mammalian cells in which the protein turnover is less dramatic than in bacterial systems.

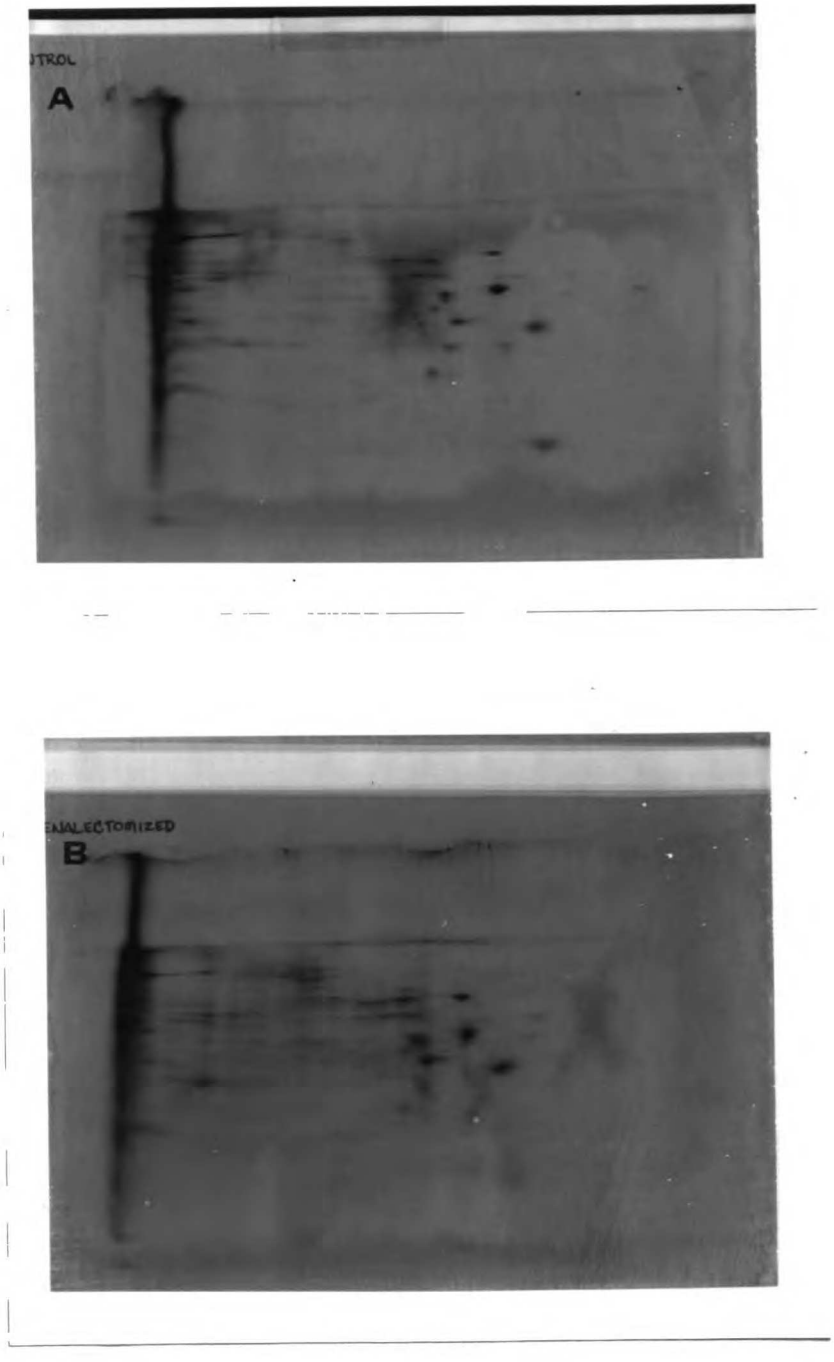


Fig. 24. Comparison of the two-dimensional electrophoresis patterns of the normal rat adipocyte (A) versus the adrenalectomized rat (B). The pH gradient is as described in the text. Approximately 400,000 cpm of each sample was applied to the focusing gel, and the autoradiograph was exposed for 21 days.

In view of these problems it is perhaps premature to conclude that the two-dimensional system revealed no changes in the protein patterns of the adipocytes prepared from normal and adrenalectomized rats. A more detailed analysis of the radiographs should at least be attempted, perhaps by the microdensitometer technique suggested by O'Farrell (1975). In addition, the lesion caused by the adrenalectomy might be of a more subtle nature; for example, the rate of synthesis of some critical protein(s) could be altered. This possibility might be investigated by exposing the cells to shorter pulses of radioactive amino acid. It must also be considered that the adrenalectomy-induced lesion (or even the differences between the rat and rabbit adipocyte) does not lie in an alteration in the protein components of the ghost of membrane; the results of Chapter 4 would indicate that the cyclase activity of the ghost preparation is misleading, and that the lesion could be associated with components located in the cytoplasm. Furthermore, both the species specificity of the adipocyte and the differences in response seen after adrenalectomy may be ascribed to a non-protein cellular inclusion, and could for example involve a factor such as the guanine nucleotides.

In summary, although the electrophoretic techniques described here have not yet been able to identify differences in protein membrane components which could account

for changes in hormonally-induced adipocyte response, these methods are still useful in the characterization of protein content and in the monitoring of membrane purification.

CHAPTER 6

CONCLUSIONS

The interaction of adrenocorticotropin with its putative receptors on the isolated adipocyte has been investigated from several experimental vantage points. Structure-function studies with analogues of ACTH which were synthesized to contain diiodinated tyrosine selectively at position 2 or 23 (or both) indicated that the integrity of tyrosine 2 is essential for the expression of ACTH's full biological activity; this was demonstrated by the fact that diiodinated tyrosine in this position yields a molecule with less than 5% of the potency of the native hormone. On the other hand, diiodination of the tyrosine at position 23 effected little or no loss of activity, and additionally, when combined with diiodination at position 2 caused no greater decrease in potency than the halogenation of the tyrosine 2 alone. The immunological reactivity of the iodinated ACTH analogues was also tested with a highly specific radioimmunoassay in which tritiated ACTH possessing complete biological activity was used as the radioligand. These experiments indicated that the ACTH analogue containing diiodinated tyrosine at position 2 could compete as effectively as native ACTH in displacing the radioligand from its antibody, while diiodination at tyrosine 23 caused a highly significant decrease in immunological reactivity of the analogue.

The results of these structure-function studies indicated clearly that a radioiodinated analogue of ACTH would not be an acceptable probe in studying the molecular basis of hormone-receptor interactions; as described in detail in Chapter 2, some compromise in either biological potency or immunological reactivity would always accompany the use of the iodinated analogue in such investigations. Consequently, the preparation of a tritiated ACTH molecule with high specific activity and complete biological and chemical integrity was attempted. Utilizing the method of reductive dehalogenation of iodinated ACTH analogues accomplished with pure tritium gas in the presence of a catalyst, tritated ACTH of specific activities of 45ci/mmole (from the 3,5-I_2Tyr^{23}ACTH) 90ci/mmole (from the 3,5-I_2Tyr^{2,23}ACTH) were prepared. The tritiated hormones were found to behave identically to native ACTH in all chemical characterizations, and to possess indistinguishable biological potencies relative to the unmodified hormone as well. The achievement of maximal theoretical specific activities was found to be highly dependent on conducting the catalytic dehalogenation in an aprotic solvent in which no protons would be available for exchange with the iodine atoms on the tyrosine ring; this latter reaction was observed to occur quite readily in the presence of a catalyst and could consequently compete with the reduction by tritium gas for the removal of the tyrosyl iodine atoms, thereby diluting out the specific activity. To insure maximum

specific activities for the tritiated ACTH the following conditions were adopted: the solvent system consisted of 0.1N acetic acid: hexamethylphosphoramide: dimethylformamide (1:10:90 v/v); the catalyst was palladium oxide on calcium carbonate.

The tritiated ACTH of 90ci/mmole has been employed to investigate the binding of the hormone with its putative rat adipocyte receptor. As described in Chapter 3, the binding reaction to isolated cells was observed to be rapid and reversible, and to occur over a concentration range which parallels quite closely that which elicits a functional lipolytic response ($K_D = 2 \times 10^{-9} M$). These high affinity receptors were also found to be finite in number, with approximately 8500 sites calculated to be present per cell. The problem of non-functional binding and/or adsorption which was encountered in these measurements cannot be overemphasized; ACTH was found to bind with high affinity to a number of artificial materials with a magnitude that could often mask the receptor binding. However, careful selection of experimental conditions and the use of low physiological hormone concentrations has made possible the measurement of the ACTH binding which appears to be responsible for triggering the functional lipolytic response.

The binding of ACTH to adipocyte membranes and membrane-enriched fractions has also been investigated. These results indicated that the loss of sensitivity to hormonal stimulation which occurs during the preparation of these fractions

(as determined by adenylyl cyclase stimulation) does not appear to be attributable to deficiencies in binding. The same low hormone cencentrations could be bound to the membrane fractions as were bound to the highly ACTH-sensitive isolated adipocytes. Since this membrane binding could not be correlated directly to a function (as could the adipocyte binding be related to lipolysis) further investigations must be conducted to determine whether this binding interaction is truly involved with the functional receptor or whether it merely represents a high affinity nonspecific binding.

In order to facilitate the measurement of the interaction of ACTH with its receptor and ultimately to isolate this molecular entity, an attempt was made to regulate and modulate selectively the ACTH receptor population of the adipocyte ghost by the administration of dexamethasone to adrenalectomized rats, as had been reported in the literature (Braun and Hechter, 1970). The extremely complicated consequences of steroid administration largely obscured the attempt to identify the loss or restoration of receptor populations by monitoring cAMP production. Nonetheless, it was observed that in the rat adipocyte ghost, adrenalectomy resulted in a loss of both ACTH and epinephrine-induced cAMP production, and that this deficiency was somewhat restored after dexamethasone administration; however, no selective loss/induction of the ACTH-induced cyclase stimulation could be confirmed. In order to determine whether

these results were unique to the ghost preparation, both hormonally-induced cAMP production and lipolysis were also measured in isolated adipocytes from adrenalectomized rats. In the whole cells, adrenalectomy caused no apparent loss of response to either ACTH or epinephrine stimulation; furthermore, dexamethasone administration, although it caused both a super-normal production of cAMP and an extremely elevated lipolytic response, did not increase these responses to levels higher than those seen in the steroid-deficient rats when the magnitude of the stimulation was expressed relative to the basal state. The binding of tritiated ACTH to isolated adipocytes from normal, adrenalectomized and adrenalectomized/dexamethasone treated rats was also measured and confirmed that no detectable changes occur in the receptor populations of adipocytes from these three metabolic states. The apparent decrease in hormonally-induced cAMP production in adipocyte ghosts from adrenalectomized animals has been attributed to steroid regulated alterations in phosphodiesterase activity, calcium transport, or even the structural integrity of the subcellular fraction; more thorough investigations would be necessary to determine the precise site of the lesion.

The final aspect of this study has involved the characterization of the protein components of the rat adipocyte membrane by one- and two-dimensional SDS polyacrylamide gel electrophoresis. The high resolution of this method has

allowed the identification of over 30 membrane proteins ranging in molecular weight from 17,000-200,000; two carbohydrate containing moieties (with apparent MW 70,000 and 85,000) have also been identified. The one-dimensional electrophoresis system has also been utilized to compare adipocyte membranes from the rat versus the rabbit species, but as yet has indicated no apparent differences in the protein components which could account for the species specific hormonal response which has been reported (Ramachandran and Lee, 1976). In addition, the adipocyte membrane from the normal versus the adrenalectomized rat has been analyzed on the one- and two-dimensional systems; again, however, no gross differences in the protein components of the membranes have as yet been found. The SDS polyacrylamide gel electrophoresis system has nonetheless provided an important tool for characterizing and monitoring adipocyte membrane purifications and modifications.

It is hoped that this study has provided some insights into the investigation of the mechanism of hormone action, particularly at the level of the membrane receptor. The importance of selecting a radioactive probe of chemical and biological integrity has been emphasized, and a workable general method of the preparation of such a tritiated molecele has been presented. A realistic investigation of the binding of a radiolabeled hormone to its functional receptor

has also been conducted and has indicated the difficulties and pitfalls which are inherent to the procedure. Additionally, the modulation and regulation of a specific receptor has been attempted; this aspect of the study has served to underscore the complexity of receptor regulation, particularly when events which are removed from the initial hormone-receptor interaction are monitored and assumed to reflect directly the state of the receptor population.

The elucidation of the mechanism of hormone action presents a constant challenge to endocrinologists and biochemists. The availability of a well characterized tritiated ligand of high specific activity will make possible more precise measurements of hormone-receptor interactions. The knowledge obtained from such studies may then be utilized to design an intelligent approach to the isolation and characterization of the receptor moiety.

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