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# BIOCHEMICAL CHARACTERIZATION OF ONE LOCUS OF CORTICOTROPIN ACTION:

# THE ADRENOCORTICAL CYTOSKELETON

by

ROGER ANDREW CHEITLIN

**DISSERTATION**

**Submitted in partial satisfaction of the requirements for the degree of**

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**in**

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## ABSTRACT

In addition to the rapid stimulation of adrenal steroidogenesis, the pituitary hormone corticotropin (ACTH) has other effects on the adrenocortical cell which require hours to be manifest. These long-term, or "trophic", actions include changes in adrenocortical cell shape and morphology. This dissertation further characterizes an earlier observation that ACTH induces changes in adrenocortical cell shape. It was found that chronic administration to isolated rat adrenocortical cells in culture causes cell rounding and retraction when observed at the light microscopic level. This retraction was hormone-specific, qualitatively dose-dependent, and completely reversible upon hormone removal. When cytoskeletal microfilaments were visualized by indirect immunofluorescence using an antibody raised against rabbit skeletal muscle actin, it was apparent that in addition to its effect on gross cell shape, ACTH also induced a loss of actin stress cables coincident with cell rounding. Using densitometric scanning analysis of SDS-PAGE gels of adrenocortical cell lysates from ACTH-treated and untreated cells, a specific loss of actin was observed upon hormone administration. This loss was confirmed using an enzymatic assay based on the binding of actin to the pancreatic enzyme deoxyribonuclease I (DNase I). Finally, a modification of an existing radioimmunoassay, also based on the actin:DNase I interaction, was employed to confirmed both gel and enzymatic analyses. These approaches all confirmed that ACTH induces a specific loss of 35-50% of the total adrenocortical actin without significant changes in total cellular protein or DNA. As with changes in cell shape, this effect is hormone-specific, dose-dependent, and follows a time course which parallels that of hormone-induced cell retraction. Pusle-labelling experiments established that the loss of actin in response to ACTH was due to an increase in the rate of actin degradation. Lysosomotropic agents such as chloroquine and ammonium chloride could completely block ACTH-induced loss of actin, offering evidence for lysosomal involvement in hormone-induced actin loss. These results strongly suggest that cytoskeletal content and organization are under hormonal control, and in particular, implicate microfilaments in the hormonal regulation of adrenocortical cell shape and cytoskeleton.

"A WORK OF ART IS GOOD IF IT HAS SPRUNG FROM NECESSITY.
IN THIS NATURE OF ITS ORIGIN LIES THE JUDGEMENT OF IT:
THERE IS NO OTHER...
FOR THE CREATOR MUST BE A WORLD FOR HIMSELF AND FIND
EVERYTHING IN HIMSELF AND IN NATURE...."

RAINER MARIA RILKE, 1903

THIS DISSERTATION IS DEDICATED

TO

JENNIFER NICOLE CHERRY

THE NEWEST MEMBER OF THE FAMILY

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## Preface to Chapter 1

The ultimate purpose of this dissertation is, in my opinion, to demonstrate an acquired ability to apply a scientific approach to fundamental questions in cell biology. To this end, I have undertaken the study of one chronic effect of the pituitary hormone, corticotropin (ACTH), on the adrenocortical cell in culture. In particular, I have addressed the molecular mechanism of action of ACTH on adrenocortical cell shape. I believe that an important part of my graduate education has been the acquisition and appreciation of the literature relevant to my field of study. As a begining to this dissertation, I present here a review of the literature in the field of the cytoskeleton. In the following pages, I hope to demonstrate a familiarity with the work collected thus far in this field. As the study of the adrenocortical cytoskeleton has been a new direction for my laboratory, I leave this chapter as a legacy to the Hormone Research Laboratory of Dr. C.H. Li.

# CHAPTER 1. REVIEW OF THE CYTOSKELETON

## Introduction

The past several decades have witnessed the birth and childhood of a new field in cell biology. Early explorations into cellular biochemistry portrayed the archetypal cell as an inert, watery bag of soluble enzymes. This view has persisted through the the last century of scientific investigation. As recently as the middle of the twentieth century, the cell cytoplasm was regarded as rather like "mother's work basket-a jumble of beads and buttons of all shapes and sizes, with pins and threads for good measure, all jostling about and held together by 'colloidal forces'" (1). Research in the past few decades has clearly shown this view to be fundamently incorrect. In fact, the cell consists of an extensive matrix of proteinaceous structures, an architectural superstructure which collectively has become known as the "cytoskeleton." A detailed biochemical characterization of the cytoskeleton is only now under way. With every new scientific publication, evidence mounts for an intimate role of cytoskeletal components in the underlying molecular mechanisms of diverse cellular activities. In this chapter, I will introduce the generally-accepted concepts concerning the cytoskeleton, with particular focus upon one component, the actin microfilament, which will be the focus of the research presented later in this dissertation.

Since the introduction of the earliest microscopes, biologists have been fascinated with the motile nature of isolated cells. They have meticulously and accurately catalogued a diversity of cellular motions, from cellular locomotion across artificial surfaces to circadian rhythms of beating cilia, from the frenetic swimming of sperm to the plodding ingestion of food particles. With the advent of the electron microscope, anatomists have probed deeper into the cell cytoplasm to discover the intricate structures of the organelles within. Early on it was recognized that cells of diverse origins contain rodlike structures of varying physical dimensions which were often associated with areas of the cell and subcellular organelles involved in motile behavior. These structures could be seen in the best light microscopes when viewed under phase contrast. In conjunction with improved techniques for fixing and sectioning cells, these elongated structures developed considerable personality when viewed under the electron microscope. Elongated tubes, 100-140nm in diameter, were found in peculiar 9 + 2 arrays lining the lumen of sectioned cilia, and as parallel bundles of tubes in the mitotic spindle of dividing cells. Long tubes of similar dimension were found running parallel and immediately beneath the plasma membrane and closely approaching intracellular organelles in many cells. Other tubular structures of distinctly smaller diameter (40-70nm) were described, often in areas in the cell devoid of the larger tubes. These smaller tubes were commonly found as bundles coursing through the cytoplasm the entire length of the cell, or as cytoplasmic anastomoses of

filaments very close to the plasma membrane.

In concert with microscopic explorations of the anatomy of the cell, biochemical analyses of subcellular activities revealed that the cytoplasm hosted a multitude of organic and inorganic molecules involved in a fantastic array of chemical reactions which were precisely arranged both in time and space, and exquisitely sensitive to changes in the internal milleu. The cytoskeleton became viewed as an organelle unto itself whose functions involved the spacial separation of these biochemical processes, a sort of physical framework upon which subcellular events were organized.

The observation that tubular structures were located in close proximity to subcellular organelles lead to the hypothesis that these elements were involved in, and ultimately responsible for, movements associated with organelles. In the 1940s intensive work was initiated to understand the underlying molecular mechanism of muscle contraction. It was already recognized that the skeletal muscle myofibril consisted of a complex, highly organized pattern of overlapping filaments of the same dimensions as those described in nonmuscle cells. It was discovered that the organization of these filaments changed dramatically upon muscle contraction. This observation added strength to the notion that such mechanical structures somehow dictated the movements involved in muscle contraction. A dynamic concept of the cytoskeleton began to replace the static nature which the name originally implied.

Early attempts to correlate structure with function in cases

of nonmuscle movement relied heavily on anatomical evidence gleaned from the electron microscope. The original nomenclature of cytoskeletal structures was based entirely on attempts to classify different sizes of cytoplasmic filaments according to their anatomical location. The implicit expectation, of course, was that by grouping cytoskeletal structures by the subcellular organelles in which they were found and according to the types of movement these structures displayed, cause-and-effect relationships between cytoskeletal structures and the organelles with which they were associated would become apparent. With the advent of more sophisticated techniques with which to analyze both the structure and composition of cytoskeletal systems, in particular immunological approaches, it has become apparent that this original classification was quite perceptive, and has stood the test of time surprisingly well.

## Structure and Function of Skeletal Muscle

Though it may seem strange to begin a discussion of the cytoskeleton of nonmuscle cells with an analysis of skeletal muscle, I feel this is important for so much of our present day understanding of the cytoskeleton is based on early work on muscle contraction. Vertebrate striated skeletal muscle consists of multinucleated cylindrical myofibres, which in turn consist of a system of elongated myofibrils (2). Contained within each myofibril is a series of repeating units known as sarcomeres, the functional units of muscle contraction. The basis of the myofibril banding seen in muscles sections under the electron

microscope lies in the highly ordered arrangement of myofilaments of two kinds - thick filaments (10-12nm in diameter) and thin (5-8nm) filaments - which intercalate with one another in such a way that in resting muscle thick filaments overlap adjacent thin filaments in a precise arrangement. Proteinaceous crossbridges between thick and thin filaments can be seen in the electron microscope which are in fact extensions or "heads" of thick filaments. Each sarcomere is separated from its next neighbors by a line of electron dense material known as the Z line.

Upon contraction, the organization of the sarcomere undergoes pronounced changes. Thin filaments apposing one another from opposite Z lines of each sarcomere move toward one another longitudinally past the thick filaments, shortening the entire length of the sarcomere without changing the length of individual thick or thin filaments. Muscle relaxation involves a reverse sequence of events to bring the sarcomere filaments back to their original respective positions, and the sarcomere itself back to its original dimensions. Work by Huxley and coworkers (3) clearly showed that the force generated during muscle contraction was proportional to the extent to which thick and thin filaments overlapped. Furthermore, the thick filament crossbridges change angle relative to the thin filaments upon movement of filaments past one another. The hypothesis which emerged from these studies, the now famous "sliding filament" theory of muscle contraction (3), was that force generation is the result of the sliding of thick filaments past thin filaments powered by rotational "strokes" of thick filament heads over adjacent thin

filaments.

Biochemical analysis of thick and thin myofilaments has clearly defined the molecular basis of myofilament organization and function. Thick myofilaments are composed exclusively of a single protein, myosin, which comprises some 50% of the total structural protein in muscle (4). Myosin is a large, asymmetrical protein of 470,000 daltons, composed of six polypeptide chains, two of 200,000 daltons and four about 20,000 daltons each. When extracted from muscle in a native conformation, myosin exists as a rod-shaped molecule in which the heavy subunit chains comprise the tail of the molecule and the light chains constitute the "head" of the thick filament. It is the myosin "head" of light chains which interacts with neighboring thin filaments (4). The light myosin chains contain intrinsic ATPase activity (3); it is ATP hydrolysis at this site which is thought to supply the power for head rotation (5). Under physiological conditions, purified muscle myosin molecules undergo spontaneous, reversible ionic aggregation to form polymers of myosin subunits which retain the anatomical characteristics of thick filaments extracted intact from muscle.

The thin muscle filament contains no myosin; instead it is composed of several other proteins - actin, tropomyosin and troponin - one of which, actin, forms the the structural backbone of the filament. Skeletal muscle actin is a single chain, globular protein of 375 amino acid residues (molecular weight $\tilde{=}$ 45,000) whose complete amino acid sequence has recently been elucidated (6). One unique compositional feature of this protein

is the presence of the rare amino acid N-methylhistidine in position 73, a feature which has been useful in the identification of actin in nonmuscle cells. Muscle actin has been crystalized in the presence of the actin-binding protein deoxyribonuclease I (7). Molecular models derived from electron density maps display an elongated molecule with dimensions 67 x 40 x 37Å, consisting of two domains, each domain containing a central B-pleated sheet surrounded by regions rich in alpha-helices (7).

As with myosin, monomer actin (termed "globular" or "G-actin") can polymerize in vitro in the absence of other proteins (6). Polymerization involves ionic association of actin monomers into individual filaments, followed by spontaneously twisting of two filaments together to form a stable, right-handed double helix (filamentous or F-actin). In vitro actin polymerization is sensitive to ionic conditions; favorable polymerization demands concentrations of greater than 50mM KCl as well as the presence of ATP and $CaCl_2$ (7). Each actin molecule contains a binding site for one ATP located within the cleft between the actin domains and a separate site for a divalent cation (7). Actin polymerization is accompanied by hydrolysis of one ATP to ADP for each monomer added (8); however, ATP hydrolysis may not be used to drive polymerization but instead to prevent actin denaturation upon assembly into repeating subunits (9).

Tropomyosin and troponin specifically associate with actin filaments and assume regulatory roles in the association of thick and thin myofilaments. Tropomyosin is a rod-shaped protein of

70,000 daltons, composed of two identical subunits which separately polymerize into filaments which then intertwine to form a coiled coil (4). The tropomyosin coil lies in the groove between the actin filaments along the entire length of the F-actin helix. Troponin, on the other hand, does not form filaments; instead troponin molecules attach to single tropomyosin molecules at evenly spaced intervals along the actin filament (10). This 76,000 dalton protein is composed of three distinct subunits of varying sizes: tropomyosin-binding subunit (TnT), ATPase-inhibiting subunit (TnI) and the calcium-binding subunit troponin C (TnC). Each subunit plays a distinct role in the regulation of tropomyosin-actin interactions. Troponin C, a low molecular weight protein with physicochemical properties strikingly similar to other calcium-binding proteins (11), binds troponin I and undergoes dramatic conformational changes upon binding calcium. Troponin I strongly inhibits actomyosin ATPase activity while at the same time increasing the affinity of tropomyosin for actin. Troponin T binds tightly to tropomyosin and functions to bind troponin to tropomyosin.

From a vast number of in vitro studies on the interactions of troponin with tropomyosin and actin filaments, a well developed model for how troponin regulates the tropomyosin-actin associations has emerged (12). In this schema, binding of calcium to troponin initiates a reduction in the affinity of TnI for tropomyosin. This in turn inititates a slight physical shift of the tropomyosin molecule in the actin filament groove, enough to expose sites on the actin molecule which then interact with the

light myosin chains comprising the thick filament heads. Thus, troponin, in conjunction with tropomyosin, is responsible for regulating thick and thin myofilament associations via myosin crossbridges.

This model implies that ultimate control of muscle contractility is due to the calcium which bathes the myofilaments. Indeed, membrane-bound channels which surround sarcomeric structures, together called "sarcotubular system" (12), contain large amounts of calcium which is released upon appropriate external stimuli. Electrical stimulation of the muscle fiber promotes depolymerization of membranes associated with the sarcotubular system, release of stored calcium, and subsequent activation of the cascade of molecular events which results in filament movement. Muscle relaxation is dependent on recall of this released calcium by activation of sarcotubular membrane-bound $Ca^{++}$-stimulated $Ca^{++}$-ATPases (13).

I have described the molecular mechanisms by which skeletal muscle contracts in order to demonstrate how the complex cytoskeletal elements within the sarcomere interact to produce muscle contraction. In this case, the organization of the cytoskeleton defines the nature of the subcellular movements observed. In my subsequent discussion of nonmuscle cell cytoskeletal architecture, the concept of a structure-function relationship between cytoskeletal structures and cellular movement will repeat itself in endless thematic variations. Though in each system described the individual proteins involved may change and the organization of cytoskeletal components will differ, the idea of

the cytoskeleton as concertmaster of movement will remain a central theme in the interpretation of cytoskeletal function.

## Classification of Cytoskeletal Structures

The elongated tubes seen in the light and electron microscopes are ubiquitous in nonmuscle cells. These filaments have been classified into two major groups, the 25nm diameter tubes or microtubules and the smaller 7nm diameter microfilaments. Microtubules and microfilaments each consist of a polymer of one major protein in equilibrium with a pool of monomer subunits. Microtubules and microfilaments are dynamic structures whose length and spacial assembly are precisely regulated by associated proteins and other environmental factors. In addition to microtubules and microfilaments, a third class of cytoskeletal structures, intermediate filaments, have been catalogued in many eukaryotic cells. Together these filaments form the structural framework of many subcellular organelles. As I will detail in the following sections, these cytoskeletal elements are in fact the mechanical modus operandi for many of the characteristic movements associated with cells in vivo and in vitro.

## Microtubules

The 25nm diameter tubes found in the cytoplasm of all eukaryotic cells appear in electron microscope as hollow cylinders of varying lengths with electron dense walls approximately 5nm thick. These structures, first named micro-

tubules in 1963 (114), are found ubiquitously in eukaryotic cells. Microtubules constitute the underlying cytoskeletal framework of cilia and flagella in motile cells (15); they comprise the architecture of the mitotic spindle upon which chromosomes attach and separate during metaphase (16); they comprise the proteinaceous superstructure of specific cytoplasmic organelles such as centrioles and basal bodies (17). In cultured cells, microtubules are found as long, seemingly rigid tubes which swirl in dense networks around the nucleus (18), and as meshworks of filaments adjacent to and parallel with the plasma membrane (19).

Biochemical analyses of intact microtubules isolated from cells, in concert with immunological analyses of microtubule constituents, have revealed that microtubules from diverse sources are polymers of one protein, tubulin. Tubulin isolated under nondenaturing conditions from cytoplasmic microtubules or from isolated cilia consists of a heterodimer of similar but nonidentical subunits with individual molecular weights of 55,000 which show differences both in amino acid composition and in net charge (20). Extensive amino acid sequence homology exists between the tubulin subunits supporting the notion that they diverged from a common ancestral protein (21). Tubulin heterodimers of alpha- and beta-tubulin spontaneously associate into microtubules under proper conditions of salt, magnesium and calcium ions. The physicochemical properties of tubulin polymerization are strikingly similar to those of muscle actin polymerization. Like actin filament formation, tubulin polymer-

ization requires a critical concentration of tubulin monomers (22). Polymerization involves ionic association of tubulin heterodimers and is accompanied by hydrolysis of one bound nucleotide for every heterodimer added, though in the case of tubulin the nucleotide is GTP rather than ATP (23). Polymerization in vitro reflects in vivo tubulin assembly in that it is reversible upon treatment with agents such as colchicine or low temperatures which are known to depolymerize microtubules in living cells (23). For example, treatment of mitotic cells with the vinca alkaloids vinblastine or vincristine to metaphase cells initiates reversible disintegration of the mitotic spindle (24). These same agents promote microtubule diassembly in vitro (24). Furthermore, both microtubule states of assembly in vitro and in vivo are sensitive to calcium (25), and chemical modifications including phosphorylation and sulfhydryl oxidation which alter microtubule integrity in vivo also affect microtubule polymerization in vitro (26). Such protein modifications have in fact been proposed as control mechanisms for microtubule assembly (26).

Studies of the reformation of microtubular arrays in vivo following removal of microtubule-disrupting agents from mitotic cells suggest that microtubule growth and elongation is a polarized process which first involves nucleation of new microtubules from discrete regions of the cell (27). In interphase cells, microtubules are found in dense swirls around the nucleus and radiate centripedally toward the plasma membrane from one or a small number of starlike sites, or asters, near the nucleus

(28). In dividing cells, microtubules radiate from electron dense material which surrounds each centriole (29) and inset into the chromosomal kinetochores of the mitotic spindle (29). Recent evidence has shown that isolated kinetochores and pericentriolar material extracted from isolated centrioles can in fact act as nucleating sites for microtubule assembly in vitro (30). These observations have given rise to the hypothesis that microtubule growth in vivo is initiated from such sites, known as microtubule organizing centers or MTOCs (28). If MTOCs act as nucleating sites for microtubule polymerization, then they may be important in directing the three-dimensional organization of microtubules within the cell. Whether cells such as plant cells which do not have centrioles in their mitotic spindle contain analogous MTOCs to direct microtubule growth and orientation is not yet known.

Microtubules purified from brain cell extracts polymerize in vitro much more efficiently than highly pure preparations of tubulin, due to the presence of contaminating proteins. These proteins, known collectively as microtubule associated proteins, or MAPs (31,32,33), specifically associate with tubulin polymers. MAP proteins have been classified into two major groups: high molecular weight proteins of 200,000-300,000 daltons (HMW-MAPs) and the tau proteins of 60,000-70,000 daltons. Both classes of proteins promote net polymerization of purified tubulin in vitro and bind to microtubules extracted from cells and to newly formed microtubules (34). The precise function of these proteins in vivo, however, has not yet been determined. However, both immuno-

fluorescence localization data and and in vitro properties of MAP proteins strongly suggest that at least some of these proteins are involved in physically cross-linking microtubules to other cell components (see below).

At present there is disagreement as to how ubiquitous microtubule-associated proteins are in nonneuronal cells. Antibodies raised to specific MAP proteins or to mixtures of MAP proteins have either identified (35,36) or failed to identify (37,38) crossreactive proteins in other cell types examined. On the other hand, microtubules isolated intact from nonneuronal cells do contain proteins which copurify with tubulin and which promote tubulin polymerization in vitro (37), suggesting that other cell types do contain proteins which can serve MAP-like functions in vivo.

The highly ordered arrangement of microtubules in cilia and flagella of motile cells clearly illustrates how the structure of microtubules and their associated proteins relates to the characteristic movememts of these organelles. Cilia and flagella are motile appendages found on a wide variety of plant and animal cells. Well known examples include the flagella of spermatozoa (39), cilia on intestinal epithelial cells (40), and the cilia on the surface of unicellular green algae (41). A variation on the structural theme of the intestinal cilium are the axostyles of certain parasitic protozoa (10) which power these organisms through their environment. Cilia are responsible for movement of the liquid environment past the cell; flagella are responsible for movements of organisms through their fluid environments.

Though both types of movement vary considerably in their cyclic rhythms, both structures contain a similar cytoskeletal framework which is responsible for these movements.

The functional unit of the cilium and flagellum is the axoneme. In transverse sections viewed under the electron microscope, the axoneme consists of a cylinder of nine equally spaced outer doublet microtubules surrounding two single central microtubules, the classical 9 + 2 pattern (10,44). There are variations on this theme found in nature (9 + 9 + 2 and 9 + 0 configurations) but these are exceptions to the rule of 9 + 2. The axoneme is anchored to the cell by attachment to a basal body which lies beneath the cell surface and is itself composed of nine microtubutle triplets, groups of three microtubules arranged in a cartwheel-like array around the circumference of the basal body. The outer doublet microtubules of the axoneme are continuous with the inner two microtubules of each triplet while the central pair microtubules arises from an amorphous region at the distal end of the basal body. A conspicuous system of striated fibres extends from the basal body into the cell.

The outer doublets of the axoneme each consist of a complete microtubule of 13 closely packed polymers of tubulin (A tubule) with an incomplete microtubule of 10 polymers (B tubule) attached longitudinally to it. The A tubule of one doublet is attached to the B tubule of its next neighbor doublet by "arms" equally spaced along the A tubule. These arms are conspicuously hooked when viewed in transverse section. Radial spokes extend from the inner surface of the A tubule and connect via spoke heads to

projections off the surface of the inner microtubule pair. The outer doublets are also connected circumferentially by links which join A tubules with adjacent A or B tubules. All components mentioned have characteristic periodicities relative to one another.

It is quite straightforward to biochemically purify constituent proteins of the axoneme by various protocols which selectively extract specific axoneme components. These protocols make use of the differential sensitivities of A and B tubules to ionic conditions, e.g., the presence or absence of calcium, to nonionic detergents and to temperatures. Tubulin polymers isolated from A and B tubules are each composed of polymers, or protofilaments, of alpha and beta-tubulin heterodimers (45). Like tubulin extracted from cytoplasmic microtubules, this tubulin will reform complete A and B tubules in vitro; however, doublet formation seems to require some sort of template or nucleating center such as the basal body (46).

In addition to the tubulins, the axoneme contains a unique protein, dynein, which constitutes the crossbridge arms of the axoneme. Dynein is actually four separate associated proteins denoted A,B,C and D (47). Though the location of component A in the outer doublet arm is known, the precise locations of the other polypeptides in the axoneme are not. Component D contains intrinsic ATPase activity (48), shown experimentally by selective extraction of dynein subunits from intact, demembranated cilia (47).

The one hundred odd other axoneme proteins identified by

SDS-PAGE are minor components relative to the tubulins and dynein. Though most have not yet been characterized in terms of chemistry, location or function, one protein, nexin, has been extensively analyzed. Nexin constitutes approximately 2% of the total axonemal protein and forms the links between outer microtubule doublets seen in electron micrographs of transverse sections of axonemes. Nexin functions to hold the A tubules together and can keep A tubules in their original relative positions even after all other associated proteins have been removed (10,44).

This very complex organization of microtubules and associated proteins makes functional sense only when the movements of cilia or flagella are analyzed in terms of microtubule distribution, in a similar way as was done for the muscle sarcomere. The experimental approach has been to freeze these organelles in motion at various times to compare the position of cytoskeletal components relative to one another as a function of time. Through much work primarily on the cilia and flagella of insect and sea urchin spermatozoa, it is now well established that the rhthymic bending of these microtubules depends entirely on the sliding of specific doublets relative to one another. Movement of doublets in one region of the microtubule causes contraction of that aspect of the microtubular wall and consequent bending of the whole cilium or flagellum in the direction of that contraction (51). Those radial spokes in contact with the sliding microtubules change angles with respect to stationary spokes in a manner analogous to the movement of the myosin head crossbridges

over the actin filament in skeletal muscle during contraction. Radial spokes attach firmly to the central microtubules in the leading edge of the bend but detach at the trailing edge of the end. This precise cycle of attachment and detachment controls the extent of microtubule sliding and hence the degree of bending. Selective extraction of spoke arms from intact, demembranated cilia has revealed that hydrolysis of nucleotide triphosphates by dynein ATPase activity supplies the energy required for "stroke" of the radial spoke arm (52).

Experimental proof that microtubule sliding is in fact the structural mechanism underlying ciliary motion has come from studies on isolated sperm in vitro. Cilia and flagella detached from sperm cells can be induced to resume normal motility ("reactivated") when supplied with exogenous ATP (53). Detergent-treated sperm in which the plasma membrane has been completely removed can also be reactivated by addition of ATP to the medium in which they are bathed (54). In both cases, the degree of dynein ATPase enzymatic activity is tightly coupled to ciliary and flagellar movement. Moreover, conditions which produce optimal movement are identical to those which favor activation of isolated dynein ATPase (54). Extraction of crossbridge arms with high salt from intact, demembranated sperm has shown a direct correlation between the frequency of flagellar beats and the number of interdoublet connections (39). If isolated axonemes are gently trypsinized, they retain their morphological integrity; however, upon addition of ATP, these axonemes immediately disintegrate into individual doublets and axonemes extend up to

eight times in length (55). Finally, conditions which favor axonemal disintegration directly match those which reactivate intact, untrypsinized axonemes. These observations strongly support a model of ciliary or flagellar movement based on sliding of microtubules in which ATP hydrolysis powers the movement of microtubule crossbridges.

## Microfilaments

The 4-7nm diameter tubular structures seen in the electron microscope in many nonmuscle cell types bears striking resemblance to the thin actin myofilaments found in the skeletal muscle sarcomere. In all cells examined, over a broad spectrum of organisms, these filaments, or "microfilaments", have been observed crisscrossing deep in the cytoplasm as stress cables (56), concentrated in dense meshworks underneath the plasma membrane (19,57), and as tight bundles of filaments as concentric rings of fibers in the epithelial belt desmosomes (58) and in the contractile ring underlying the cleavage furrow in dividing cells (59,405). Microfilaments have been found in organized filament arrays in microvilli of the epithelial brush border (10,57), and in very specialized organelles such as stereocilia of hair cells (120) as well as in microspikes and filopodia of cultured cells (56). In all these cases, both biochemical anlaysis of microfilament constituents and anatomical location of these filaments argue for a role of these microfilaments in cellular motion, an hypothesis for which I will present evidence in the following sections.

Biochemical analyses of isolated microfilaments have identified the major protein constituent of these filaments as a protein with striking similarities to muscle actin. Nonmuscle actin is another ubiquitous protein, and a major cellular protein which often comprises up to 15% of the total cellular protein (62,63). Nonmuscle actin shares many physicochemical properties with skeletal muscle actin. Nonmuscle actin is a globular protein of 42,000-45,000 daltons with a very high degree of alpha-helical content (64) which, like skeletal muscle actin, can spontaneously form F-actin polymers both in vivo and in vitro. In vitro actin polymerization requires specific conditions of salt (0.1mM KCl) and magnesium ions (1-2mM) which match those conditions found within cells. Globular actin (G-actin) binds ATP (65), and actin polymerization is accompanied by hydrolysis of this nucleotide in a manner identical to muscle actin. In comparative biochemical studies of nonmuscle actins, the critical concentrations for polymerization were found to be very similar to muscle actin (66). Furthermore, nonmuscle actins, like their muscle counterparts, are sensitive to chaotropic agents such as 0.6M KCl which promote depolymerization (69). All nonmuscle actins both bind muscle myosin heads and activate skeletal muscle myosin $^{2+}$ATPases (387). However, the kinetics of ATPase activation differ between muscle and nonmuscle actins as well as among the nonmuscle actins themselves.

From animal cells to plant cells, from protozoa to fungi, the chemical similarities between muscle and nonmuscle actins are also striking. Though to date nonmuscle actins have not been as

thoroughly chemically analyzed as skeletal muscle actin, comparisons of partial amino acid sequences of nonmuscle and muscle actins have revealed a high degree of homology between these proteins (67). As a group, the cytoplasmic actins are more similar in sequence to one another than to muscle actin (68,70). Sequence heterologies are found throughout the molecule, but concentrated in the $NH_2$-terminal region. The minor sequence and compositional differences in the first few $NH_2$-terminal residues account for the distinctive isoelectric points of the different isoactin classes (68). All purified skeletal muscle actins analyzed thus far focus as a single isoionic species, termed alpha-actin, in isoelectric focusing gels. Actins from brain, Dictyostelium discoideum, Physarum and a host of nonmuscle cell types focus at two other more alkaline pH bands, termed beta- and gamma-actin, as well as at the alpha-actin position (66,68). At least one report presents evidence for an even more basic isoionic actin species (69). The ratio of alpha-, beta-, and gamma-actins in nonmuscle cells is quite variable, and as yet no evolutionary patterns have been noticed. Differences in net charge among nonmuscle actins have been succesfully used in attempts to purify actins, and has become a useful means of identifying purified proteins as actin.

Nonmuscle actins can also be distinguished immunologically from one another and from muscle actins (70). Though generation of anti-actin antibodies is quite difficult, most likely due to high evolutionary conservation of the molecule, several laboratories have reported success in raising low-titre anti-actin

antibodies (71). In immunoassays using radioactive actin (70) or enzyme-antibody conjugates (393,396), it has been found that muscle actin contains antigenic determinants which are not present in nonmuscle actins, and vice versa. Some muscle and nonmuscle actin determinants are dependent on tertiary structure; others are not (71). Such immunological differences have made it difficult to utilize radioimmunoassays to positively identify and to quantitate nonmuscle actins.

The sequence position of amino acid differences between nonmuscle and muscle actins predicts that different actins are coded for by separate genes. Hybrid screening of DNA libraries from bovine, human and fly sources have confirmed the existence of families of actin genes (394,395). Studies using Drosophila melanogaster DNA libraries (394) have located multiple actin genes of different chromosomes and have clearly shown that these genes are responsible for the synthesis of different cytoplasmic actins. There is additional evidence that different genes code for different isoionic species within the same cell (395).

Upon realizing that nonmuscle cell filaments contained actins which were quite similar to muscle actin, many investigators began to search for other muscle cell cytoskeletal components in nonmuscle cells. Although myosin is found widely distributed among a variety of nonmuscle cell types, it is present in much lower amounts than in muscle, and often at one one-hundreth the actin concentration (10). In some nonmuscle cells, myosin is not detectable at all (398). As with nonmuscle actins, cytoplasmic myosins resemble their muscle counterpart in

molecular weight (roughly 470,000 daltons), subunit composition and in physical dimensions (44). They bind reversibly to actin filaments (405), and have intrinsic $Mg^{2+}$ATPase activity which is actin-activatable and, at least in some systems, is calcium-sensitive (74). Like smooth muscle myosin, and in contrast to skeletal muscle myosin, cytoplasmic nonmuscle myosins are auto-phosphorylated by a Ca++-sensitive myosin light chain kinase (403). In vitro phosphorylation of isolated myosin leads to bipolar aggregation of myosin molecules into clusters of 10 to 20 molecules held together at their tail regions. This is in striking contrast to the extensive head-to-tail assembly of muscle myosin subunits in the thick sarcomeric filaments. Nonmuscle myosin can polymerize into filaments in vitro under physiological conditions; however, the resulting filaments are much smaller and more labile than the thick myofilaments found in muscle.

In contrast to the state of myosin in the muscle sarcomere, nonmuscle cell myosin is never found as long polymers as in skeletal muscle. Instead, cytoplasmic myosin is found decorating actin stress cables (100), within actin filament networks beneath the plasma membrane (63), in the contractile rings of dividing cells (56), and possibly in regions of the membrane in close contact with the substratum (63). In all these cases, myosin is closely associated with actin microfilaments and the general distribution of myosin matches that of the filaments. In other regions of the cell, such as in the advancing lamellipodia and membrane ruffles of locomoting cells (237), myosin is notably

absent. The distribution of nonmuscle myosin and its physicochemical similarities to muscle myosin have been used to support the hypothesis that organelles which contain both actin filaments and myosin move by a contractile mechanism of sliding filaments similar to that in muscle (see part V, section F).

Biochemical evidence for other cytoskeletal muscle or muscle-like proteins in nonmuscle cells is fragmentary. There is immunofluorescence data to suggest that a tropomyosin-like protein may decorate actin stress cables in some cells (75). Tropomyosin has been convincingly demonstrated in blood platelets and brain tissue (75). Tropomyosin may also reside in adhesion plaques of cultured cells (75,76). On the other hand, there is no evidence that nonmuscle cells contain muscle troponin. However, the abundance of a structurally and functionally related calcium-binding protein, calmodulin (400,402), may negate any requirement for troponin in nonmuscle cells.

Many other actin-associated proteins have been isolated and characterized from nonmuscle cell extracts. Pioneering work in this field began in the 1960s with the experiments of Kane et al. (63) who found that when homogenates of sea urchin eggs were prepared in the cold in the presence of ATP and warmed to 37°C, a semi-solid consisting of actin filaments quickly formed. With time, this gel spontaneously contracted to form a solid pellet. Isolation of the factor responsible for this gelation revealed a 58,000 dalton protein, named "fascin", which later was shown to cross-link purified actin filaments in vitro (77). An antigenically related protein has been found associated with actin

filament bundles of the filopodia of sea urchin coelomocytes (78), and in the actin filaments of the acrosomal process of Limulus sperm (79). A number of other proteins, similar to fascin in their ability to initiate actin filament aggregation in vitro, have been described (80). These gelation factors, or "gelactins", vary widely in size (23,000 up to 38,000 daltons) and have now been isolated from many cell types (80).

In addition to fascin and the gelactins, a host of other actin-associated proteins have been identified which crosslink can actin filaments. These proteins have been classified based on their ability to from cross-linked microfilament matrices, and whether this ability is sensitive to calcium (80). Crosslinking factors initiate the formation of two types of actin filament associations: formation of isotropic, random three-dimensional networks of filaments crosslinked by high molecular weight factors, and of long, closely packaged, highly cross-linked bundles of filaments (81). In the case of the sea urchin egg extracts mentioned above, fascin links filaments into needle-like bundles; another protein is responsible for cross-linking them together into a network (77). Crosslinking proteins all bind to F-actin in specific stoichiometries, usually at a molar ratio less than 1 mole of factor to 10 moles of actin. Calcium-insensitive actin filament binding proteins include actin-binding protein from macrophages and leukocytes (82), and filamin, a 500,000 dalton, rod-shaped dimer found in smooth muscle (83). Calcium-sensitive crosslinking proteins include actinogelin from various ascites tumor cells (84), and villin, a

95,000 dalton protein in intestinal epitheilial cells (85). Recently, a calcium-sensitive, calmodulin-binding protein called "caldesmon" was isolated from chicken gizzard muscle, and was shown to interact with purified F-actin filaments in the absence of calmodulin (86). Included in the catagory of calcium-sensitive actin-binding proteins are those which inhibit gelation either by severing actin filaments or by inhibiting filament crosslinking: these are Acanthamoeba capping protein (87), Physarum fragmin (88) and macrophage gelsolin (89). Severin, an actin-binding protein isolated from Dictyostelium discoideum (90), binds intact actin filaments in the presence of calcium and fragments them into small pieces. Deoxyribonuclease I is a low molecular weight protein which has been shown to bind to G-actin in such a way that it cannot add to growing actin filaments (105). Profilin, a 16,000 dalton protein isolated from platelets and spleen, also forms a stable complex with G-actin (91), and has been shown to restrict actin nucleation and growth from cross-linked sperm actin bundles in vitro (91,92). The function of these actin-binding proteins as possible microfilament-regulatory proteins in vivo has been proposed (92).

In addition to actin-binding proteins involved in gelation, other actin-associated proteins have been identified in a wide variety of cell types. Alpha-actinin is a protein found in motile and nonmotile cells which can bind to and cross-link actin filaments (93). By immunofluorescent staining of cultured cells, alpha-actinin has been localized in regions of active movement in association with actin filaments: in the leading edge of

locomoting cells (94), adjacent to the plasma membrane immediately under caps of surface receptors in lymphocytes (95) and at sites of close cell contact with the substratum (69). Along with myosin and tropomyosin, this protein decorates cytoplasmic stress cables with a characteristic periodicity (69,96). Other microfilament-associated proteins such as vinculin (97) and fibronectin (356) may also interact directly with actin filaments. In the special case of the human erythrocyte, both spectrin and band 4.1 have been shown to link to actin filaments in erythrocyte ghost cytoskeletons (316), and to bind to and cross-link F-actin in vitro (98,99)(see also Part V, section C).

Actin Polymerization and Treadmilling

An attempt to define models for cell movement which recognize involvement of actin microfilaments is requisite upon an understanding of how actin monomers associate to create actin filaments. It is generally believed that actin polymerization involves at least two, probably three, distinct events, each of which is completely reversible (81). The first step is a slow nucleation step in which several actin monomers aggregate to form a nucleus out of which actin filaments will grow. The second step involves elongation of nascent filament chains by addition of actin monomers preferentially to one end of the filament. A third step would be association of short filaments end-on-end to form longer filaments. In contrast to microtubules, virtually nothing is known about microfilament nucleation sites in vivo and no structures analogous to microtubule-organizing centers have been

described, with perhaps the exception of the actomere in the acrosomal process of Thyone sperm (100). There is electron microscopic evidence that actin filaments may elongate from as yet undefined sites located in or on the plasma membrane (101). Using heavy meromyosin and myosin subfragment S-1 binding to actin filaments to determine filament polarity, it has been conclusively shown that filament growth in vitro proceedes unidirectionally out from nucleation centers by preferential addition of actin monomers to one end, the so-called "barbed" end of the filament (102). At some time during elongation, the ATP molecule bound to each actin molecule is dephosphorylated; however, it is not known how tightly monomer addition and nucleotide hydrolysis events are coupled. In fact, in vitro studies of the kinetics of actin polymerization suggest that dephosphorylation lags behind polymerization (103) and therefore cannot be responsible for monomer addition. Cooke and others (9,42) have suggested that adenosine diphosphate bound to actin in a microfilament may serve to stabilize the molecule against denaturation.

While elongation occurs at both ends of an actin filament, growth is biased, with monomers adding to one end at a faster rate than to the other (104,105). Thus, at each end of the filament there must be a unique equilibrium between actin molecules in the filament and those free in solution. For filament growth to occur, net addition of actin monomers at the barbed end must exceed loss from the other end. Elongation then must be a consequence of unequal flux of monomers at the ends.

This concept led Wegner in 1976 (106) to coin the term "treadmilling" to describe the polarity of actin polymerization which is a consequence of unequal actin monomer addition to the two ends of the actin filament.

In vitro studies of filament growth have demonstrated that actin monomers associate and dissociate from each end of the growing filament (81). At each end of the filament, actin molecules bound to the filament are in equilibrium with actin molecules free in solution. Moreover, each filament end has a characteristic critical monomer concentration, defined as the concentration of unbound actin molecules at which the rate of actin addition to the filament balances the rate of dissociation (81). Treadmilling theory predicts that filament growth will occur only when the critical concentrations at the two ends of a filament are different. Theoretically, if the critical concentrations are the same, then the net subunit flux at each end will be zero, and no net addition or subtraction will result, regardless of the concentration of unbound actin. If, on the other hand, the critical concentrations of the filament ends are different, then net addition or loss of actin subunits from the filament will depend on the concentration of free actin. This has the important consequence that at concentrations of unbound actin intermediate to the critical concentrations of the filament ends, the filament will exist in a steady state in which molecules come off predominantly (but not exclusively) at one end and are put on predominantly at the other, i.e., treadmilling will be achieved. In this state, even though the net length of the polymer does not

change, individual molecules will be translocated from one end of the polymer to the other in a continuous process. Pulse-chase studies using radioactive G-actin (42) and fluorescence energy transfer studies in conjunction with $^{35}S$-methionine-labelled actin incorporation into unlabeled filaments (81) have confirmed this prediction by demonstrating actin movement of labelled actin through the filament with time.

Plots of microfilament growth rate as a function of monomer concentration, using isolated intestinal epithelial microvilli as nucleation centers, show that in physiological buffers containing 20mM KCL and less than 1mM $MgCl_2$, the critical concentrations at both ends of the actin filament are the same, and the filament does not change length (63). However, when the concentration of KCl is raised to 75mM in the presence of 5mM $MgCl_2$ and ATP, conditions which favor actin polymerization in vitro (107), the critical concentrations for actin addition shift to different values such that now the concentration of unbound actin monomers lies between the critical concentrations of the two ends, and net filament growth occurs. In this state, actin molecules will add to the filament end with the lower critical concentration (the "barbed" end) faster than at the other end (the "pointed" end). As the rate of addition of actin molecules is higher at the "barbed" end than the rate of loss from the "pointed" end, net subunit addition is achieved, and the filament grows in length coincident with the initiation of treadmilling. Treadmilling, then, owes its existence to the fact that the critical concentrations for actin addition at the two ends of the

actin filament are different. Any change in the ionic environment of an actin filament, therefore, can have a great influence on filament growth by virtue of its effect on the state of monomer flux.

Not only can treadmilling theory clarify the sensitivity of actin polymerization to alterations in the ionic environment of the actin filament, it also offers a simple explanation for the mechanism of action of agents which promote microfilament depolymerization. Drugs like cytochalasin B and some of the endogenous capping proteins are known to bind to the "barbed" end of growing filaments and to inhibit further actin subunit addition (314) and to promote microfilament depolymerization in living cells (314). Treadmilling theory predicts that inhibition of actin addition at this end would lead to net subunit loss with time as a result of continued dissociation of subunits from both ends of the filament. In other words, by blocking subunit treadmilling, these agents would initiate microfilament dissolution.

If actin treadmilling functions in the polymerization of microfilaments in vivo, how then is filament length controlled? The situation in the cell is somewhat different than in the test tube where microfilaments and microtubules are free-floating structures. There is abundant evidence that in vivo, microfilaments are anchored to the plasma membrane and to membrane-bound organelles at one, and possibly both, end of the filament (19,57). This attached end is presumably unavailable for monomer exchange. In this case, actin polymerization would depend entirely on the critical concentration at the free end, and on

the concentration of unbound actin molecules. In this case, according to treadmilling theory, filament growth should proceed until the concentration of free monomer drops to the critical concentration of the unattached end. However, as there is good evidence that as much as fifty percent of the total actin in cells exists as G-actin (108), simple calculations further predict that at the concentrations of monomer actin found in the cell, actin filaments should grow to enormous lengths, sizes which vastly exceed those found in nature (44,63). The fact that this situation is not found in vivo is evidence that some other factor(s) is involved in the regulation of actin filament assembly in the cell. Moreover, in vitro experiments in which microfilament growth rates were determined using filament formation from isolated Limulus actin bundles (109) have clearly demonstrated that treadmilling alone cannot account for the rapid changes in filament organization observed in living cells, again arguing for involvement of additional factors in the control of actin assembly.

The most obvious candidates for a role in regulating actin polymerization are the host of actin-binding proteins mentioned above which can bind to G- or F-actin in vitro. Actin-associated proteins such as Acanthamoeba capping protein (110), beta-actinin (111), and profilin (112) are known to bind to monomer actin or actin filaments and to initiate filament depolymerization in vitro. If these proteins bind to the ends of actin filaments in a manner similar to cytochalasin B, then they would promote filament depolymerization by inhibiting subunit addition to

filaments undergoing treadmilling. Thus, by timely synthesis and/or release, such proteins might be able to control filament integrity through this inhibitory effect on treadmilling. Several observations support this hypothesis. First, by virtue of the fact that capping proteins operate at the ends of the actin filament, this model predicts that only minute amounts of such regulatory factors would be necessary to control filament length Indeed, naturally occurring capping proteins are found in trace amounts relative to actin in cells (399). Second, studies have shown that purified capping proteins can prevent actin polymerization in vitro (400). There is some biochemical evidence that actin-associated proteins regulate actin polymerization in vivo in the case of the sperm acrosomal reaction (73). Finally, known capping proteins have been localized by immunofluorescence to actin microfilaments in cells (43), demonstrating a close physical association of these proteins with filaments in vivo.

It has been suggested that intracellular pH, which is known to have a profound inhibitory effect on actin polymerization in vitro (100), might be another factor involved in the regulation of microfilament assembly. The observation that changes in pH can alter the binding of actin-associated proteins to microfilaments in vitro (100) further suggests that pH regulation of filament integrity might be mediated indirectly through its effects on the interaction of regulatory proteins with actin filaments.

Though the treadmilling model is here described in terms of actin filaments, it can be applied with equal ease to microtubule polymerization. In fact, much of the original in vitro work to

determine association/dissociation rates was done on tubulin polymerization using purified tubulin and isolated MTOCs (109). Pulse-chase experiments with podophyllotoxin, an agent which blocks microtubule assembly at steady state (109), have been used to demonstrate in vitro treadmilling of microtubules (115). Microtubule assembly in vitro is greatly influenced by several of the high molecular weight MAPs mentioned earlier. These proteins enhance microtubule nucleation and increase the rate and extent of polymerization by stabilizing microtubules (116). Under depolymerizing conditions they promote the formation of microtubule rings (117). MAP proteins are also the major substrates for cAMP-stimulated phosphorylation both in vivo (118) and in vitro (117). As phosphorylation of MAPs inhibits both the rate and extent of microtubule assembly in vitro (117), phosphorylation of these proteins might be another important regulatory locus in microtubule assembly. In addition, nascent chains of nonmuscle actin are also phosphorylated in vivo (119), suggesting the possibility that microfilament formation might also be controlled by a similar mechanism.

## Higher Orders of Microfilament Organization

Investigation of the state of actin microfilaments in vivo has revealed several distinct forms of three-dimensional filament organization: closely packed filament bundles, long helices of filaments known as "stress cables" or "stress fibers", and loose, random networks of filaments. These higher orders of filament organization can be typical of specific cell types; more

commonly, though, two or three classes of filements are found in different regions of the same cell. In all cases, filaments are cross-linked by actin-associated proteins. The precise molecular mechanism(s) of filament cross-linking has not yet been determined; however, with improved techniques of biochemical analysis for the isolation of actin-binding proteins, and for in vitro reconstitution of purified filament components, several systems have been worked out in detail. In the succeeding sections, I will briefly describe and comment on these systems.

The packing of actin filaments into tight bundles has been thoroughly investigated by Tilney and others (120) using isolated microfilament bundles from the acrosomal process of the horseshoe crab (Limulus) sperm as a model system. These bundles are found in vivo as parallel arrays of filaments known as paracrystals (120). By negative staining and thin-sectioning of these bundles, cross-bridges between closely apposed filaments can be visualized (120). Crossbridges are composed of actin-associated proteins, including myosin (120) and fascin (121), and can be seen in register throughout the filaments, giving bundles of these filaments a crystalline appearance in one axis when stained for immunofluorescence with specific antibodies (120). Similar bundles have been identified in epithelial microvilli of the intestinal epithelial brush border (57), and in the contractile ring of mitotic cells (122). So-called "needles" of actin filaments with the same geometry have been produced with purified actin filaments in vitro (123). In all these cases, without exception, filaments are packed together with identical polarity

as revealed by HMM or S-1 decoration of actin subunits (120). The helical nature of the actin filament dictates where the cross-bridges can form. Since different actin symmetries are found in nature, it follows that crossbridges between actin subunits must be at slightly different positions along the length of the filament in bundles of different symmetries (120). This prediction has been borne out in imaging studies of actin needles formed in vitro (124).

There are, however, clear examples in nature in which actin filaments are not packed in a crystalline array. The actin filament bundle in the stereocilia from lizard hair cells is one such example (120). Here filaments are aligned parallel and very close to one another longitudinally but filament cross-bridges are distributed in a seemingly random fashion throughout the bundles. How filaments can assume the same three-dimensional packing arrangement yet display such different cross-linking modes is not known, but the answer most certainly resides in the chemical nature of the cross-links involved.

The obvious function of cross-bridges is to cross-link actin filaments. The not so obvious consequence of ordered cross-bridging is, on thermodynamic grounds, the great stability of such arrays of filaments. When actin filaments are mixed in solution under proper ionic conditions, filaments rapidly arrange into hexagonal matrices which retain a liquidlike state. With time, however, these bindles rearrange into rigid needles which show an increased viscosity coincident with a greater number of cross-bridges (120). Such needles are extremely stable. These

observations have led to the suggestion that assembly of bundles of varying degrees of rigidity involves establishing varying numbers and arrangements of cross-bridges (120). Rapid formation of bundles of varying rigidity would be very useful in different cellular settings where different subcellular movements would demand rapid formation of microfilaments of different strengths. Analysis of specific in vivo situations in which subcellular movements involve rapid reorganizations of microfilaments into bundles of varying rigidity, such as during formation of the acrosomal process (120) and during changes in bundle rigidity in limp steriocilia upon sound stimulation (120), supports this view.

The microvillus offers an excellent example of tight microfilament packing. The luminal surface of intestinal epithelial cells, the so-called "brush border", is covered with densely packed projections called microvilli (10,57). Electron micrographs of microvilli reveal that they are membrane-bound projections of the plasma membrane 1-2um long and 0.1um in diameter (10). Through the core of each microvillus is a bundle of 20-30 microfilaments. These actin-containing filaments insert into a matrix of electron-dense material adjacent to the plasma membrane at the tip of the microvillus, and extend down to the base of the microvillus into a second set of loosely arranged microfilaments running perpendicular to the microvillus in a region called the terminal web (57). In the terminal web, microvillar filaments splay out and contact microfilaments which reside there. Microvillar core microfilaments are closely packed

together in parallel register and are held together by cross-bridges which connect adjacent filaments to one another and to the plasma membrane (57).

In addition to actin present in microvillar microfilaments, other proteins have been identified and localized using specific antibodies as immunofluorescent probes. The microvillus itself contains myosin, nonmuscle tropomyosin, and the calcium-dependent regulatory protein, calmodulin (125), as well as a host of unidentified proteins (125,126,386). Brush border microvilli can be isolated by mechanical shearing of epithelial linings and differential centrifugation (127). Treating isolated microvilli with Triton X-100 leaves the internal cytoskeletal core of the microvillus intact and free of plasma membrane and terminal web components (127). It has been reported that such preparations of microvillar cytoskeletons do not contain myosin or tropomyosin (128), suggesting that these proteins are specifically localized at the base of the microvillus. Electron microscopic analysis of the terminal web region in concert with immunofluorescence staining for myosin has revealed that the terminal web microfilaments does in fact contain myosin (129).

Isolated microvillar cytoskeletons contain several other proteins with varying molecular weights: 110K, 95K and 68K daltons by SDS-PAGE (126). The 95K protein lies in the proteinaceous cross-bridges between actin filaments seen when isolated microvillus cores are negatively stained for electron microscopy (130) and when microvillar cytoskeletons are stained with specific antibodies to this protein for immunofluorescence

microscopy (131). Early immunological evidence suggested that the 95K protein might be alpha-actinin, a protein often localized at focal points of cell attachment to the substratum (95). Several lines of evidence have since shown this to be incorrect. While alpha-actinin resides exclusively in the terminal web region, the 95K protein was found to decorate microfilaments at the cross-bridges between core filaments (125). Furthermore, although this protein shares some immunological reactivity with alpha-actinin (as well as with the 110K protein), peptide mapping has shown that the 95K protein is distinct from muscle alpha-actinin (132). The 95K protein has since been isolated and purified and has been given the name villin (133). Villin has been shown to produce gels from solutions of F-actin, strongly suggesting a role in cross-linking microfilaments (133).

Biochemical studies on isolated microvilli have suggested a function for the 110K and 68K proteins. When demembranated microvillar cores are treated with ATP and magnesium, microfilaments lose their cross-bridge projections and SDS-PAGE analysis shows concomitant loss of the 110K protein (130). Treatment of the cores with the nonionic deoxycholate, on the other hand, causes microfilaments to lose their tightly bundled appearance without loss of cross-links. Deoxycholate specifically released the 95K and 68K proteins. These results suggested that the 110K protein constituted the microfilament-membrane bridges, and that the 95K and 68K proteins comprised the links between the microfilaments. The 68K protein has recently been purified (134). Immunofluorescence studies have shown that this protein, now called

fimbrin, is found in many cells localized within regions of great motile activity such as membrane ruffles and filopodia (134).

## Stress Cables

In addition to the tightly packed bundles of actin filaments in organelles such as microvilli and the acrosomal process, microfilament also exist in large bundles of bundled microfilaments, so-called "stress cables" or "stress fibers" (19,56,57,96), which run throughout the cytoplasm of cultured cells. Interference reflection, Nomarski, polarizing and electron microscopy (135) have revealed that these microfilament cables correspond to the stress fibers observed years earlier under phase microscopy. Stress cables are sometimes seen swirling around the nucleus, as is typical of microtubules, but are more characteristically found close to the ventral surface of the cell immediately underneath and parallel to the plasma membrane adjacent to the substratum (19). Electron micrographs often reveal splitting of stress cables near the membrane, one branch continuing parallel and close to the membrane, the other coursing deeper into the cytoplasm. These filaments are quite long and often run the entire longitudinal length of asymmetrical cells (57). Stress cables sometimes appear to be in continuity with the plasma membrane at focal contact regions which can be visulaized in sectins parallel to the substratum (136), or by interference reflection microscopy (137), or by electron microscopy of replicas of the cell underside (138). In other cases, stress cables seem to interact with the membrane indirectly through

actin filaments networks (139). Stresscables often concentrate and focus at the adhesion plaques (140,141). In some cells, stress cables are seen in conjunction with microtubules; in others, these cables lie in areas devoid of microtubules (19).

Ultrastructural studies have shown that parallel arrays of actin filaments are aligned along the entire length of each stress fiber with adjacent microfilaments often opposite in polarity to one another (142). Immunohistochemical studies have confirmed the presence of actin (135), myosin (143), tropomyosin (144) and filamin (145) in stress cables. Indirect immunofluorescence using specific antibodies to purified cytoskeletal proteins has shown that in addition to actin (56,96), stress cables contain myosin and tropomyosin (146), alpha-actinin (142) and tropomyosin (142). These proteins co-localize along the length of the stress fiber in a periodicity which characteristically appears banded when visualized by indirect immunofluorescence. Microinjection of florescently labelled proteins into living cells results in uniform distribution of fluorescence along stress fibers when actin is injected (142), but in a banded distribution when either alpha-actinin (142) or tropomyosin (142) is injected. Electron microscopy frequently reveals that stress cables contain patches of osmiophilic dense material, analogous to the intracellular dense bodies of smooth muscle, spaced along the fibers (19). Alpha-actinin and tropomyosin have been localized by immunofluorescence to these densities (142,148). Myosin-like filaments have also been detected in register with these densities along the cables (57).

There is a great deal of evidence that actin-containing stress cables are intimately involved in a host of cellular events. The location of stress fiber ends in adhesion plaques has led to the hypothesis that these structures are vital to cell attachment (19), cell locomotion (57) and maintainence of an asymmetrical cell shape (19). The polarity of microfilaments within stress cables as well as the presence of other muscle cytoskeletal proteins, i.e., myosin and tropomyosin, along the length of these cables, strongly suggests that stress cables might be cytoskeletal structures which produce movement by some sort of actomyosin-like contractile mechanism (19). Strong evidence for such a model comes from in vitro experiments in which stress cables isolated by laser dissection were shown to contract in response to ATP (149). Evidence for this hypothesis will be presented in later section.

Microfilament Networks

Three-dimensional networks of actin filaments and actin-associated proteins constitute a third spacial organization of microfilaments. Random arrays of actin-containing filaments are found as dome-like meshworks near the dorsal surfaces of cultured cells (57) as well as within actively motile regions of the cell such as the lamellipodia of locomoting cells (44,57,150) and in the growing tips (the "growth cone") of developing nerve axons (44). Networks of microfilaments often concentrate in regions devoid of microtubules, such as under the plasma membrane beneath caps of surface receptors (400,402). Actin networks frequently

lie in continuity with the plasma membrane in specific motile structures such as ruffled membranes (19,151), membrane microspikes (19,152), and extending phagocytotic (19,153) and locomotor (19) pseudopods.

In addition to actin contained within filaments, regions containing these networks also contain other cytoskeletal proteins including myosin (154), alpha-actinin (144), fimbrim (57) and filamin (57,155). Membrane ruffles contain actin, alpha-actinin, fimbrin, and filamin, but myosin and tropomyosin are absent (57). Macrophage actin-binding protein is also concentrated in the plasma membrane regions of great motility (156).

Mixtures of purified actin filaments with certain actin-binding proteins spontaneously form cross-linked networks of filaments. In vitro gelation of actin with alpha-actinin (157), macrophage actin-binding protein (158), gelation factors (159) or filamin (160) has been demonstrated. Individually, these proteins do not gel. ATP appears to be required for actin polymerization but not for gelation as nonydrolyzable analogues of ATP can substitute for ATP only in the latter step (81). Networks created in vitro resemble meshworks in vivo in their sensitivity to external factors. Gels of actin and any of these actin-binding proteins are liquified by incubation at $0^{o}C$, a property which mimics the response of the intact cell to cold in terms of the consistency of its cytoplasm (19). Concentrations of cytochalasin B which disrupt cortical networks and inhibit movements in living cells also inhibit or reverse gelation of actin and actin-binding

protein in vitro (161). Furthermore, although gels of purified actin and actin-binding proteins do not contract, addition of cell myosin to such gels causes contraction (158). Myosin added to cell extracts also induces gelation and contraction (162). Contracted actin-based gels contain myosin (19), implying that the myosin participated in the contraction. These results parallel observations of microfilament networks in situ during cellular movements which suggest that these networks also contract (19). Finally, self-association of pure actin filaments is sensitive to many of the same conditions -pH as well as KCl and $CaCl_2$ concentrations- that affect gelation of cytoplasmic extracts (81). These observations give credence to the belief that in vitro gelation reflects in vivo network formation.

In vitro gelation of purified actin in the presence of gelation factors produces a three-dimensional filament network which is randomly arranged, as viewed by polarized-light (81) or electron microscopy (163). Mixtures of purified muscle actin and macrophage actin-binding protein, when examined in the transmission electron microscope after fixation, critical point drying and rotary shadowing, display a uniform mass of short, straight filaments organized in a random, branching network which show numerous side-to-side contacts between actin filaments (163). The viscosity (a physicochemical property of such matrices which is a reflection of the extent of filament self-association) of such gels is quite low and can easily be disrupted by mechanical stirring (164). The chemical nature of the cross-bridges between actin filaments seen in such gels has not yet been defined but

certainly involves the cross-linking proteins as different cross-linkers yield slightly different spacial arrangements of cross-linked actin filaments (81).

Cytoplasmic extracts represent a more complex type of gelation. Here, in addition to actin filaments, a number of endogenous actin-associated proteins are present which physically cross-link actin filaments upon gelation. Work on extracts from a number of different cell types has established that actin-filament gels can be constructed from the cytoplasms of most cells (165). Conditions for cytoplasmic gelation in vitro match those necessary for gelation of purified actin filaments (166) except for the notable calcium-sensitivity of many cell extracts not seen with purified actin filaments (81). Unique gelation proteins have been isolated from different cell sources (167). Many of these proteins, including actinogelin (168), filamin (169), actin-binding protein (170) , fascin (171) and host of Acanthamoeba gelation factors (172), have been purified and can initiate gelation of actin filaments in vitro. Although these proteins are quite different chemically, they all produce cross-links among actin filaments which yield gels of similar properties. All these proteins raise the low shear viscosity, i.e., increase filament crosslinking, in a concentration-dependent fashion (81). Curves relating network formation to cross-linker concentration typically show an abrupt transition from a viscous liquid to a gel, at a cross-linker concentration called the "critical gelling concentration" at which all the filaments are cross-linked to at least one other filament to form

a continuous network (81).

The molecular basis for cross-linking is only now being investigated. It has been difficult to firmly establish that a putative gelation cross-linker is soley responsible for cytoplasmic gelation for a number of endogenous, basic macromolecules such as ribonuclease, aldolase, histone and lysozyme (173), all of which are unlikely to have access to actin in the cell, can promote cytoplasmic gelation by cross-linking actin filaments at very low concentrations (i.e., with high specific activities). In addition to specific gelation proteins, a number of nonspecific molecules such as polylysine (81) can also produce filament cross-links (81). Finally, there is some evidence that purified actin filaments can cross-link in the total absence of any gelation factors, presumably by self-association of actin filaments (164). Actin-filament self-association is influenced by a variety of factors such as $CaCl_2$, $MgCl_2$ and KCl concentrations, and acid pH (173). Experimental evidence to demonstrate self-association of purified actin filaments has come from work using cytochalasin B and capping protein (174). These agents are known to bind to the "barbed" ends of actin filaments and to inhibit actin polymerization (175). Moreover, these agents inhibit subunit addition to growing actin filaments at the same concentrations that inhibit filament cross-linking (81). This suggests that a common binding site at the barbed end of the filament is responsible for the effects of these proteins on both polymerization and network formation. These agents act at very low mole ratios relative to actin molecules (81), suggesting that

inhibition of gelation must focus on the end(s) of the filaments. These observations led Pollard et al. (175) to hypothesize that actin-filament self-association was due to binding of the "barbed" ends of filaments to the sides of other actin filaments. In this model, cytochalasin B and capping proteins inhibited gelation by preventing the formation of these T-junctions. That observation that cytochalasin B dismantles structural networks of microfilaments in living cells (19) and inhibits subcellular movements associated with these networks (19) has been used as supporting evidence for the proposal that actin self-assembly might be important in cytoplasmic gelation in vivo (19).

The regulation of actin filament gelation is an active area of current investigation. In principle, formation of actin-filament gels could be controlled directly at the level of the cross-linking proteins, or at the level of the actin filaments themselves by modulating the concentration, length, and degree of self-association of the filaments. Experimental evidence exists for control at the level of the actin filament. For example, the cross-linking protein gelsolin can inhibit gelation by shortening actin filaments, presumably by a direct action on the filaments (167,176). Macrophage capping protein inhibits gelation of actin by a variety of cross-linking proteins by raising the concentration of any gelation factor required to promote gelation (177). The effect of capping protein must be due to its effect directly on actin, and not by competition with gelation proteins for specific actin-binding sites, for the nature of the gelation factor is irrelevant (81).

Transformation of cytoplasmic extracts from a viscous liquid to a rubbery gel are quite sensitive to calcium concentrations. For example, micromolar $Ca^{2+}$ inhibits gelation of cytoplasmic extracts from Acanthamoeba (166) and Dictyostelium (178). On the other hand, calcium concentrations greater than $10^{-7}$M initiate gel-to-sol transitions of other cytoplasmic extracts (80). In contrast to cytoplasmic extracts, purified actin filaments in the presence of specific gelation factors such as filamin are not sensitive to calcium and will promote gelation in the absence of calcium (80). These results imply that some other endogenous cytoplasmic factor(s) must confer calcium sensitivity to cytoplasmic extracts. As cytoplasmic extracts contain a host of actin-associated proteins which either cross-link actin filaments or prevent such associations (80,81,378), and many of these protein either bind calcium and/or show a marked calcium-sensitivity in their ability to initiate gelation in crude extracts (81) or of purified actin filaments (167), the exciting possibility exists that that calcium may be a key regulator of cytoplasmic gelation in vivo (81).

Many of the actin-associated proteins involved in gelation of cytoplasmic extracts have been purified, characterized and classified in terms of their calcium-sensitivity (80). Calcium-insensitive cross-linking proteins include macrophage actin-binding protein (82), filamin (83), and several of the gelation factors from Acanthamoeba (179). These proteins link actin filaments into isotropic gels in the absence of calcium; calcium added to cytoplasmic extracts has no effect on the rate or extent

of gelation (80). The bulk of purified gelation factors, however, show at least some calcium sensitivity. Actinogelin (84) and Acanthamoeba GP-85 (180) both initiate formation of weak gels of purified actin filaments only in the presence of calcium. Actin-associated solation factors which prevent gelation of cytoplasmic extracts, or of purified actin filaments in the presence of cross-linking proteins, are also calcium-sensitive (80). Fragmin is one such protein which inhibits gelation by fragmenting actin polymers into short oligomers in a calcium-dependent fashion (88). Macrophage gelsolin is a calcium-binding protein which accounts for most of the calcium sensitivity of gelation in macrophage extracts (89,176). This protein binds to and shortens F-actin (176), and can inhibit gelation of purified actin filaments by macrophage actin-binding protein (89). In the presence of calcium, Dictyostelium severin binds to and fragments F-actin into small pieces (90). All of these proteins influence microfilament organization in a calcium-dependent manner.

Villin illustrates an interesting example of an actin-associated protein with dual functions displaying different calcium sensitivities. At calcium concentrations less than $10^{-6}$M and in the absence of calcium, villin can cross-link actin filaments into tight bundles (80,404). However, at calcium concentrations above $10^{-6}$M, villin converts from an F-actin-bundling factor to an F-actin-severing protein and can fragment F-actin into shorter polymers of defined length as well as restrict growth of new actin filaments to this length (80). In this case, calcium regulates filament association by controlling

the expression of villin functions.

Calcium regulation of microfilament integrity may take place at loci other than cross-linking sites. One candidate for calcium control is the polymerization event itself (176). Several of the calcium-sensitive, actin-associated proteins already mentioned bind directly to actin filaments and inhibit polymerization. Both villin (85) and fragmin (88) bind to F-actin and inhibit actin polymerization. Recent studies using isolated acrosomal bundles of Limulus sperm as nucleating sites for skeletal muscle actin filaments (109) have clearly shown that loss of actin monomers from the nonpreferred end of the actin filament increases dramatically in the presence of millimolar amounts of calcium. In this cases, filament assembly is regulated by an effect of calcium on subunit off-rate.

Conditions for gelation of cytosplasmic extracts suggests another locus for control of network formation. Gelation requires MgATP at concentrations in excess of that necessary for actin polymerization (81). As pointed out earlier, gelation is an energy-requiring event which depends on ATP hydrolysis. Moreover, myosin-associated ATPase activity has been localized to actin microfilament networks in situ (63); this activity is known to be F-actin-activatable (63). These results suggest the presence of some ATP-requiring enzyme in the gelation process. This may be a kinase as many of the cytoskeletal proteins found in gels of cytoplasmic extracts are phosphorylated during the gelation reaction (119). Furthermore, many of these same proteins - tubulin, filamin, desmin and intermediate filament protein -

have been shown to be phosphorylated in vivo (119). Analogs or inducers of cyclic AMP have been shown to mediate phosphorylations of cytoskeletal proteins, including actin, in cells which respond to these agents by changes in microfilament organization and gross cell shape (119). Taken together, these observations support the hypothesis that control of cytoplasmic gelation may be mediated by phosphorylation of cytoskeletal proteins (80). The fact that many endogenous kinases are calcium-dependent, calmodulin-binding proteins (81) further supports the hypothesis that calcium may regulate network formation via phosphorylation events.

Microfilaments organized as networks are often found in distinct regions of the cell. For example, filament networks are most prominent in the advancing edges and membrane ruffles of motile cells, and in regions near the plasma membrane on the side of the cell facing away from the substratum (113). Microfilament bundles, on the other hand, are more often found on the ventral surface of the cultured cells and within specific motile organelles such as microvilli. Though cables and networks are most often found in different regions of the cell, this does not imply that these structures have strictly separate functions with no intercommunication. There is anatomical and biochemical evidence to suggest that cables and networks do interact and may in fact be interconvertable. Sea urchin coelomocyte pseudopods are flat structures filled with a network of thin actin filaments (181). Upon certain stimuli, these organelles reorganize into pointed stuctures known as filopodia. Metamorphosis of pseudo-

podia into filopodia occurs concomitant with reformation of actin filaments into tightly packed bundles (182). Disorganized arrays of microfilaments are converted into parallel arrays during formation of the ring of filaments underlying the plasma membrane upon capping of surface receptors (183), with different phases of contraction in Physarum (19), upon retraction of pseudopodia (19), and with construction of the contractile ring during cytokinesis (10). Merger of microfilament cables with networks of microfilament bundles is often observed in electron micrographs (19). Loose bundles of actin filaments exist in the endoplasm of Nitella internodal cells undergoing cytoplasmic streaming (10). When these filaments are prepared for electron microscopy by the freeze-fracture, deep-etch technique, reticular "webs" of thinner filaments (ca 4 nm) are seen which directly interact tail-to-tail with F-actin filaments. Biochemical evidence for cable-network interconversion has come from in vitro gelation studies which demonstrate that under suitable conditions, networks of actin filaments in the presence of filamin will spontaneously form loose microfilament bundles (184).

## The Microvillus

At this point, I would like to return for a moment to a discussion of the microvillus to illustrate how the three-dimensional arrangement of cytoskeletal filaments within the microvillus defines and regulates the movement of this organelle. We have seen that the microvillus contains a complex array of parallel microfilaments which attach to the plasma membrane at

the tip of the microvillus and which connect with a network of microfilaments in the terminal web region. The terminal web also contains intermediate filaments that appear to be connected to spot desmosomes on the membrane (185). Terminal web microfilaments connect bundles of adjacent microvilli and insert into the cell membrane at intermediate junctions (zonula adherens) that completely encircle the cell (185). Ciliary microfilaments are crosslinked to one another and to the plasma membrane at regular intervals by lateral cross-bridges (129). Associated with microfilaments in the terminal web, but lacking from the interna of the cilium, are myosin and possibly tropomyosin.

The organization of microvillar microfilaments bears a striking resemblance to the assembly of thin filaments in the skeletal muscle sarcomere. Both microvillar and sarcomeric microfilaments are organized in parallel arrays: the microvillar microfilaments in tight bundles orginating from the tip of each microvillus, and the actin filaments in the sarcomere as parallel arrays of filaments extending in both directions from the Z line. Electron microscopic analysis of HMM-decorated microvillar microfilaments shows that microvillar filaments are arranged with the same polarity, such that they all point away from attachment sites at the tip of the microvillus toward the terminal web region (57). In the sarcomere, actin filament are similarly polarized with the their "barbed" ends pointing away from the Z line. Both the microvillus and the muscle sarcomere contain alpha-actinin in association with microfilaments: at the tip of

the microvillus and in the terminal web in the case of the microvillus, and in the Z line in the case of muscle. Finally, the microvillus contains proteins which share chemical and structural properties to muscle myosin and tropomyosin. In the microvillus, myosin lies in the terminal web region and in the sarcomere, and in the sarcomere it is the major constituent of the thick myofilaments.

Microvilli can be isolated from brush borders by mechanical shearing and differential centrifugation which exhibit many of the structural and functional characteristics of microvilli in vivo. Demembranated microvilli so prepared contract rapidly upon exposure to ATP and calcium or magnesium (186), the core of each microvillus plunging toward and spaying into the terminal web region without any change in microfilament length (10). Isolated intact brush borders treated with ATP and calcium also contract, the membranes in the region of the zonula adherens pinching together, causing the brush borders to round up with the microvilli on the outside (186).

Based on these observations, Mooseker and Tilney (129) and Rodewald et al. (186) have proposed models for microvillar movement which rely on a sliding filament mechanism of contraction reminiscent of that in muscle. In these models, microfilaments of adjacent microvilli are connected by bipolar mysoin filaments in the terminal web. Contraction is a consequence of core microfilaments sliding past terminal web filaments powered by the "strokes" of the myosin heads in a manner identical to that which takes place between thin and thick

myofilaments in the sarcomere during contraction. Presumably, the tensile strength necessary for contraction would result from pulling of terminal web filaments against the immobile cytoskeletal components of the zonula adherens. Observation of the position and polarity of these microfilaments in situ in fact predicts that they would direct the movement of microfilaments toward the terminal web (57). In these models, the cyclic nature of microvillar beating would be a consequence of a rythmic repetition of microvillar contractions and relaxations. These models do not address questions regarding the control of microvillar contraction and relaxation. However, the observations that isolated brush borders contract in the presence of calcium and ATP (125), and that tropomyosin and calmodulin are found in the terminal web (125), suggest that calcium (via myosin phosphorylation?) might provide one means of regulation of contraction. These models nonetheless provide a working hypothesis for the underlying molecular mechanism of microvillar movement.

## The Acrosome Reaction

The acrosome reaction of Thyone sperm supplies another clear example of the way in which the organization of microfilaments defines the movements of a specific organelle. Many invertebrate sperm rapidly form a long, thin process called the acrosomal process to penetrate the protective layers of the egg during fertilization (10,57). In Thyone sperm, the acrosomal process forms from a region of the sperm, known as the periacrosomal cap,

located just anterior to the nucleus. Microscopic analysis of periacrosomal cap material has revealed an amorphous substance resembling a pellet of G-actin (10). Biochemical analysis has confirmed that this material is unpolymerized actin at millimolar conentrations in association with several minor proteins which are thought to keep actin in monomer form (113). No myosin or tubulin is present in the periacrosomal region. Formation of the acrosomal process (sperm activation) is the result of an explosive, directed polymerization of this actin (187) followed by discharge of actin microfilaments outward from the apical region of the sperm head. Staining of acrosomal microfilaments with myosin fragment S-1 reveals that these microfilaments, like those found in microvilli, are unidirectionally polarized (188). Growth of microfilaments appears to be toward the plasma membrane with actin monomers adding preferentially to the ends of the filaments at the apical membrane (57). This is in contrast to the case of microvillar microfilaments which grow away from the membrane and toward the cell center. Electron microscopic examination of the periacrosomal cup has shown that actin polymerization originates from a dense body in the center of the periacrosomal cup, consisting of 20-25 filaments embedded in a dense matrix (114). It has been suggested that this structure, called the actomere, acts as a nucleating body for actin polymerization in much the same way as MTOCs do for tubulin polymerization (114).

Study of conditions that initiate the acrosomal reaction and control actin polymerization, using isolated Thyone sperm and the

calcium ionophore A23187, suggest that calcium is necessary for formation of the acrosomal process and that changes in intracellular calcium levels might be the trigger for periacrosomal actin polymerization in vivo (189). In fact, these observations correlate well with an earlier finding that acrosomal actin will dissociate from its associated proteins when sperm are incubated in medium at pH 8.0 (57). If these accessory proteins do inhibit spontaneous filament growth by preventing bound G-actin from polymerizing, then perhaps changes in intracellular pH might regulate actin polymerization by monitoring the association of these proteins with actin.

The horse shoe crab Limulus also generates an acrosomal process similar to that in Thyone sperm but by a very different mechanism. Here, acrosomal microfilaments are already assembled prior to discharge, and stored as a tight, twisted coil around the base of the nucleus (10,79). Upon activation of isolated, intact Limulus sperm with calcium, this coil of microfilaments unravels from the anterior of the sperm and rotates so that the acrosome literally corkscrews its way forward. In the final acrosomal process, microfilaments are parallel to each other and packed in a hexagonal array (79), with cross-bridges between adjacent filaments in perfect register when viewed in the electron microscope (79). As there is no overall change in microfilament length before and after discharge, this dramatic movement must be brought about by alteration in the packing of the microfilaments (10). As with Thyone, acrosomal actin from Limulus is associated with other proteins, in particular a 55,000

dalton protein in 1:1 mole ratio with actin, and a 95,000 dalton protein whose function may be to cross-link adjacent filaments in the bundle (190).

Regardless of the differences in the mechanisms by which Thyone and Limulus sperm create acrosomal processes, experimental evidence from studies of both these sperm overwhelmingly supports the proposal that changes in the organization of actin microfilaments are responsible for formation and movement of this organelle. In the case of Thyone, actin polymerization and bundling of microfilaments produces the acrosomal process; in the case of Limulus, changes in the packing and position of preformed microfilaments create this structure. As with actin filaments in the microvillus and microfilaments in the cilium, the underlying cytoskeletal structure of the sperm acrosomal process defines the movements characteristic of the structure, i.e., structure cannot be dissociated from function, and vice versa. In essence, cytoskeletal structure predicts movement. The other motile events discussed in subsequent sections of this review - cell attachment, cell locomotion, and the changes in cell shape which accompany them - will all serve to re-emphasize this important point.

## Intermediate Filaments

In addition to microtubules and microfilaments and their associated proteins, a third distinct cytoskeletal system has emerged and gained much prominence in recent years. As their characteristic diameter of 10nm is between that of the smaller actin filaments (6nm), and the larger microtubules (25nm) and muscle myosin filaments (15nm), these filaments have become known as the "intermediate filaments." Intermediate filaments are extremely heterogeneous in chemistry, structure and location. Recently, they have been classified into five major groups on the basis of biochemical composition, immunological characteristics and tissue origin: (1) keratin (tono) filaments, found in cells of epithelial origin (subunit MWs, 40-65K); (2) desmin (or skeletin) filaments found predominantly in smooth, skeletal and cardiac muscle cells; (3) vimentin filaments (or decamin) filaments found in cells of mesenchymal origin (subunit MW, 57K); (4) neurofilaments, found in neurones (subunit MWs, 68K, 150K and 200K); (5) glial filaments, found in glial cells (subunit MW, 51K)(44,57,191,192,390). This classification is, however, not rigid; intermediate filaments of more than one class are sometimes found together in cells (57,389). Comparison of amino acid sequences of representative proteins from different intermediate filament groups reveals that they differ substantially in sequence, although showing some sequence homology within certain regions of these molecules (193). The high degree of sequence heterology among intermediate filament proteins stands in striking contrast to the relatively minimal

evolutionary divergence among actins or tubulins.

Little is known about the chemistry of intermediate filaments; however, some generalizations can be made. Intermediate filaments are polymers of chemically identical monomers much like microfilaments and microtubules. However, in contrast to both of the latter filaments, intermediate filaments are very insoluble under physiological conditions (194), do not bind myosin S-1 or HMM (194), and are of very high tensile strength (191). When viewed in the electron microscope, intermediate filament proteins appear as irregular threadlike molecules, in contrast to globular actin and tubulin (191). Thus they are distinct from microfilaments and microtubules. Immunological evidence supports this conclusion (195). There is evidence that intermediate filaments are composed of trimers of individual polypeptide chains wound about each other such that each domain of the holofilament exhibits a highly alpha-helical nature (44). Though in most cases, intermediate filaments are stationed as closely packed bundles in the cell, in at least two cases - in cultured fibroblasts and in white blood cells - these filaments are instead present as random networks (196).

Recent studies have attempted to determine functional roles for intermediate filaments. As with microfilaments and microtubules, filament structure and spatial organization have been analyzed in search of possible function. Intermediate filaments are predominantly found in those parts of the cell that are subject to mechanical stress, often in close association with specific organelles (44). For example, in epithelial cell sheets,

keratin filaments insert into strong intercellular junctions known as spot desmosomes which serve to hold adjacent cells together (19,191). Here, keratin filaments may serve to strengthen the physical bonds between cells in these regions. Another type of keratin filament actually passes through the desmosome (44); these filaments may be the "glue" which tightly seals these cells together into a continuous monolayer. Intermediate filaments are main protein constituents of cells which cover external surfaces in higher animals, e.g., in nail, hair and skin epidermal cells, which die and leave tough, durable cytoskeletons as protective coverings. Thus, in these cells, intermediate filaments serve to protect the organism from potentially destructive environmental factors (44). In skeletal muscle, a role for intermediate filaments in bringing Z lines into register and in linking Z disks to the plasma membrane has been suggested (191,196). Such observations have led to the general conclusion that this class of filaments provides strong structural support for cells in vivo (44,191). Intermediate filament proteins have also been found cultured cells such as HeLa cells (44) and chick embryo fibroblasts (44), cells which have no significant structural role in the tissues from whence they derive. There is evidence that in these cells, intermediate filaments may serve structural roles involved in cell spreading and shape formation, and possibly also in cell locomotion (191).

Even less is known about the regulation of intermediate filament assembly. As the proteins of these filaments are insoluble in physiological solutions, it has been difficult to

study intermediate filament assembly in vitro. However, all intermediate filament proteins examined so far seem to be phosphorylated in vivo (57,191,390). As phosphorylation is known to affect microfilament assembly in vivo (117) and microtubule polymerization in vitro (197), it may be that this chemical modification functions to control intermediate filament assembly (or disassembly). There is no evidence that intermediate filament proteins exist in pools of unpolymerized subunits, as is the case with actin and tubulin; therefore, regulation of assembly by control at the level of polymerization is not obvious. Discovery of class-specific intermediate filament proteinases (197) has led to the hypothesis that intermediate filament integrity may be regulated primarily through filament degradation.

## Other Cytoskeletal Structures: The Nuclear Matrix and Microtrabecule

With the advent of new techniques to isolate and prepare cytoskeletons for microscopic and biochemical evaluation, two other cytoskeletal matrices have been recognized. The early work of Mayer and Gulick (198) in the 1940s demonstrated a class of nuclear proteins which resisted extraction with buffers of high ionic strength. Further electron microscopic analysis revealed that these proteins constituted an extensive fibrogranular network which has become known as the "nuclear protein matrix" or "nuclear matrix" (199,200). With improved techniques for membrane solublization using nonionic detergents, studies have shown that in addition to proteins, this matrix consists of an intact

nuclear envelope (or lamina) and protein and lipid components of the resident nucleolus (200). The proteinaceous network of the nuclear matrix connects the residual nucleolus to the lamina and generally constitutes greater than 90% of the final matrix components (200). Deoxyribonucleic and ribonucleic acids together comprise about 2% of the nuclear matrix while phospholipids and neutral carbohydrates make up the rest (200).

The nuclear matrix seems to be an ubiquitous structure present in all eukaryotic cells (200). Though the most work has been done on the rat liver cell nuclear matrix, nuclear matrices have been isolated and characterized from a great variety of cell types including lymphocytes (200), erythrocytes (201), tumor cells (202), and from certain ciliated protozoans (203). Though the ratio of protein to nonprotein constituents varies considerably among cells, the chemical composition of proteins is remarkably consistent. Matrix proteins consist of a group of polypeptides with molecular weights of 62K, 66K and 69K daltons by SDS-PAGE, along with several other minor proteins in the range of 50K and 100-200K daltons (200). All these proteins are immunologically related and though chemically distinct from other major cytoskeletal proteins, bear a strikingly resemblance to nonmuscle actin, tubulin and clathrin in their amino acid composition (200). The significance of this observation has not yet been evaluated.

The proposed functions of the nuclear matrix are diverse. There is good evidence that the polynucleotide components of the nuclear matrix are not contaminants but that matrix DNA and RNA

is specifically associated with the nuclear matix protein network (200). Strong evidence exists that matirx RNA is exclusively ribosomal RNA that has been newly synthesized (204). The proteins of the nuclear matrix line nuclear-pore lamina and nucleoli (205). As these structures are known to function in transport of newly syntheisized RNA from the nucleus to the cytoplasm (206), it is quite possible that nuclear matrix function to channel synthesized nuclear components through nuclear pores during export to the cytoplasm. In addition, there is good evidence that that the proteinaceous component of the nuclear matrix contains specific steroid binding sites for a host of steroid hormones in many steroid-sensitive cells (200). As soluble cytoplasmic steroid hormone receptors are known to translocate into the nucleus and to specifically associate with chromatin there (200), the nuclear matrix may also serve as a structural scaffolding upon which to direct movement of steroid receptors into and out of the nucleus. Finally, in organisms where DNA replication is inititate and carried out in close association with membrane, as in bacteria which replicate DNA at membrane sites called replisomes (207), the nuclear matrix may spacially organize and thereby control DNA replication (200). Pulse-chase experiments in which nuclear matrices were isolated from labelled cells at different phases of DNA replication have shown that different amounts of DNA specifically associate with matrix proteins at different times during DNA replication (208). Isolated HeLa metaphase chromosomes stripped of histones retain loops of a residual protein structure in loops which coincide in size with S

phase replicon lengths found in vivo (209), further suggesting a structural role for nuclear matrix proteins in DNA replication.

Generally, when nuclear matrices are isolated, they retain the characteristic shape of the intact nuclei from which they are extracted (200). During differentiation of normal cells and upon neoplastic transformation of other cells, the nucleus undergoes dramatic changes in shape (200). At every stage of development, nuclear shape changes are reflected in changes in nuclear matrix shape. Changes in nuclear shape induced by drugs or by alterations in the ionic environment of cells in culture are paralleled by changes in nuclear matrix morphology (210). These observations have led to the hypothesis that the nuclear matrix defines nuclear shape by virtue of its function as a cytoskeletal framework upon which nuclear structures are spatially organized (200). One interesting observation perhaps pertinent to nuclear shape changes coincident with cellular transformation is that viral products such as T antigen (200) and E3 protein (211) are often found specifically associated with nuclear matrix components after infection. Perhaps virally-induced changes in matrix morphology are intimately involved in the functional changes in nuclear activity associated with viral transformation.

When cultured cells are fixed with glutaraldehyde and $OsO_4$, dehydrated, critical-point dried and examined in the high voltage electron microscope, an irregular three-dimensional lattice is observed which appears to connect and support the cytoskeletal matrices of microtubules, microfilaments and intermediate filaments. This lattice extends throughout the cytoplasm and

concentrates immediately beneath the plasma membrane (57). The elements of this matrix, known as "microtrabeculae", consitute a fifth and final cytoskeletal system known as the "microtabecular lattice" (57). Microtrabeculae are 3-6nm diameter filaments apparently not seen in embedded, sectioned samples in the electron microscope because the electron-scattering properties of the embedding resins are similar to those of the microtrabeculae (212). The whole field of the microtrabecular lattice is wide open for investigation: at this point in time, it is not known whether this lattice is composed unique proteins or whether it is composed of known cytoskeletal proteins such as actin and tubulin, what role(s) it may have in cellular function, or even whether the whole network is actually an artifactual agglutination of known cytoskeletal components produced during fixation and dehydration.

In the preceeding sections, I have described the major groups of cytoskeletal structures found in nature. Though the classification of these components is based on notable differences in structure and function, this is not to imply that these filaments are rigidly segregated within the cell, and that they do not interact with one another. On the contrary, there is much evidence, both anatomical and biochemical, which demonstrates extensive, intricate communications among filaments from all the cytoskeletal groups. One outstanding example with which to illustrate this point is the frog axon. When this structure is prepared for electron microscopy by the quick-

freeze, deep-etch, and rotary-shadowing method (213), elaborate physical associations of microtubules with intermediate filaments (neurofilaments), and actin filaments with microtubules and neurofilaments are found. Proteinaceous cross-bridges, much like those between microfilaments, link different filaments together. In the dense material associated with desmosomes, microfilaments have been found to interact indirectly with intermediate filaments through cross-links (214). Direct physical evidence for an interaction of microfilaments with microtubules has come from in vitro studies on actin gelation. Under conditions which favor gelation of purified actin filaments, mixtures of purified microfilaments and isolated microtubules form gels as a result of weak interactions between actin filaments and the MAPs found on the surface of the microtubules (215). These gels consist of random, three-dimensional networks in which a number of actin filaments run in parallel with microtubules over short distances. This observation has been further substantiated by a study in which a subset of MAPs (molecular weight 250,000 daltons) and two tau protein fractions purified from cells to homogeneity were found to individually cross-link actin filaments (173). However, these microtubule-associated proteins have not yet been shown to cross-link actin filaments with purified tubulin. Moreover, one of these MAP proteins (MAP-2) has been shown by immunofluorescence to specifically associate with intermediate filaments in cultured brain cells (216). Thus, microtubule-associated proteins and MAP-like proteins may serve as cross-linkers among filaments from different cytoskeletal classes.

The functional significance of such interconnections remains unclear; however, several models for subcellular movements involving surface receptor capping (57) and changes in cell shape accompanying cell attachment to artificial surfaces (19) enlist such interactions in regulating the spacial distribution of different types of filaments within the cell during these events. The details of these models will be discussed in later sections.

## Techniques By Which To Study The Cytoskeleton

Any scientific attempt to definitively prove that a particular cytoskeletal structure is involved in a specific cellular event must initially involve identification of its chemical constituents and determination of its structure and spatial distribution in the system under study. Only then can one confidently explore the question of whether the integrity of a particular cytoskeletal structure is requisite for biological function. To this end, a great variety of microscopic, biochemical and immunological techniques have been developed and employed in the study of the cytoskeleton. In this section, I will present an overview of these approaches and discuss the theory and supporting experimental evidence behind their use.

### Microscopy

Early phase contrast light microscopy was used to initially classify microtubules and microfilaments on the basis of their size. The development of electron microscopy and in particular, improvements in fixing and sectioning samples for microscopy,

have been invaluable in determining the precise spatial distributions of cytoskeletal arrays both in vitro and in vivo (19,57). More recently, Normarski and interference reflection microscopy have been extremely useful in determining the spatial arrangement of filaments in regions of close contact with the substratum (19,217). Most recently, stereoscopic electron microscopic techniques (218) in conjunction with deep-etch, rotary-shadowing of quick-frozen samples (213), have opened up the field of the cytoskeleton to a visualization of the three-dimensional distribution of cytoskeletal systems relative to one another.

By far the most important and extensively utilized advance in the visualization of the cytoskeleton has been an immunological one. Immunofluorescence microscopy can be used to identify and localize specific cytoskeletal proteins in fixed, demembranated cells (217). In this technique, antibodies raised to specific cytoskeletal proteins are chemically tagged with fluorophores, usually fluorescein (71) or rhodamine (217,219), to yield highly sensitive probes for cytoskeletal proteins. A popular variation on this theme is indirect immunofluorescence, in which untagged antibodies to cytoskeletal proteins are used in conjunction with fluorescently tagged secondary antibodies which will specifically recognize the immunoglobulin class of the primary antibody used (71,217). The distinct advantage of indirect immunofluorescence over direct immunofluorescence is the great increase in sensitivity achieved because several fluorescent antibodies can attach to each primary antibody bound.

However, this technique is fraught with its own particular problems arising from undesirable antibody crossreactivity and nonspecific antibody aggregation (71). Indirect immunofluorescent staining of cytoskeletons from cells solublized with detergents and fixed with methanol or glutaraldehyde has been extensively used to identify and localize actin (56,175), extracellular matrix proteins (220), myosin (71), tubulin (221), tropomyosin (75,175), alpha-actinin (71), dynein (10), intermediate filament proteins (222) and a host of actin-associated proteins (71) in fixed cells and in cytoskeletal preparations in vitro (57). With the advent of monoclonal antibody production, immunofluorescence has become extremely useful in double immunofluorescence studies in which two cytoskeletal proteins are localized by indirect immunofluorescence using two antibodies and two different fluorophores (217). In these studies, the specificity of the anitbodies used in recognizing particular cytoskeletal proteins is of the utmost importance, for high crossreactivity can easily obscure the evaluation of results. In fact, in all forms of immunofluorescence, proper controls are essential for meaningful analysis. Common controls in indirect immunofluorescence include substitution of preimmune sera for primary antibodies, and ommission of primary antiserum prior to staining with fluorescent second antibody (71,217).

Cytoskeletal proteins stained for immunofluorescence are visualized in an epifluorescence microscope fitted with interference filters specifically designed for the fluorophore(s) being used. Each fluorophore has its own unique spectro-

photometric properties which give it advantages and disadvantages relative to other probes available. One great disadvantage of flureseceinated probes is the rapid photobleaching of fluorescein (71,217); therefore, the signal-to-noise ration increases quickly with time of observation with fluoresceinated probes and observations must be done quickly. This particular problem has been successfully circumvented with the development of video-intensified imaging microscopy (217). Video imaging vastly increases the sensitivity of the recording apparatus and reduces the intensity of the fluorescence signal required for high resolution.

Immunological approaches to studying the cytoskeleton have taken other directions as well. Immunoenzyme assays, in which an antibody raised to the cyoskeletal protein of interest is coupled to an enzyme such as horseradish peroxidase, have been used to identify and localize specific cytoskeletal proteins (71). Ultra-structural distribution of cytoskeletal proteins in tissues has also been accomplished by staining sectioned samples with antibodies coupled to electron-dense ferritin (223) or to avidin in conjunction with biotin (217), then viewing samples in the electron microscope (71).

In studies of intact cytoskeletal filaments in vitro and in vivo, it is often desirable to know the polarity of filaments with respect to adjacent membranes and subcellular organelles. Establishing the polarity of filaments in situ can often help determine the feasiblity that particular cytoskeletal filaments are involved in producing directed movements associated with

specific intracellular events. In the case of microfilaments, binding of proteolytic cleavage fragments of myosin, i.e., heavy meromyosin (HMM) or myosin subfragment S-1, has become a widely used criterion for determining the polarity of actin filaments (10,19,57,71). These fragments bind to actin subunits in a very specific orientation, such that stained microfilaments appear decorated with proteinaceous arrowheads when viewed in the electron microscope (19,57). In all cases, arrowheads line up in the same direction along the entire length of each actin filament; furthermore, arrowheads always point in the direction of filament growth (19,57). Fluorescently labelled heavy meromyosin, in conjunction with the epifluorescence microscope, has been used to determine the polarity of actin microflaments in situ (57). The specificity of HMM and S-1 for actin-containing filaments has been confirmed by colocalization studies in which actin filaments were additionally stained with other actin-specific fluorescent probes such as FITC-phalloidin (262,388,392).

In contrast to microfilaments, there are no tubulin-specific agents available which might be used to determine the polarity of microtubules. However, electron microscopic studies analyzing the orientation of dynein side arms in cilia and flagella (225) have yielded information concerning microtubule polarity. Moreover, studies on the growth of microtubules from microtubule organizing centers in vitro have succeeded in determining the direction of tubulin polymerization (224).

Biochemical approaches to the study of cytoskeletal structures have grown in two directions: the development of techniques with which to isolate intact, spatially unaltered cytoskeletal structures, and the establishment of techniques with which to test cytoskeletal involvement in cellular events by the use of chemical and physical agents to specifically alter cytoskeletal structures in living cells (19). Isolation of intact cytoskeletons was pioneered by R. E. Stephens (226) in his work on the use of nonionic detergents as a means to prepare demembranated flagellar axonemes. Triton X-100 solubilization of isolated flagella led to isolation of an intact 9 + 2 axoneme; this accomplishment made possible investigations into the localization of the subunits of tubulin in the A and B tubules (226). Since then, detergent extraction using Triton X-100 (227,385) or Nonidet P-40 (228) has led to the successful isolation of intact cytoskeletons from a host of cell sources. Detergent solubilization has been a key step in the isolation and characterization of many nuclear matrix components (199,200). Solubilization of cells differentially releases membrane lipid and protein, and can be used to determine the asymmetrical distribution of membrane-bound proteins relative to cytoskeletal components (229). Triton X-100 solubilization, in conjunction with SDS-PAGE analysis, has been extensively used to identify which intracellular proteins are specifically associated with different cytoskeletal structures. In the case of ascites tumor cells, for example, Triton solubilization removes 60% of the membrane lipid and 70-80% of lactoperoxidase-labelled cell

surface proteins (230). Proteins still associated with the cell cytoskeleton after detergent solubilization were identified by SDS-PAGE, and included actin and alpha-actinin. NP-40 solublization of purified murine tumour and lymphoid plasma membranes has been used to demonstrate that the resulting cytoskeleton retained significant amounts of actin (231). Detergent preparations of other cytoskeletons have been used to identify non-structural proteins associated with the cytoskeleton. For example, Triton X-100 solubilization of Dictyostelium Discoideum cells has been used to identify cyclic AMP chemotaxis receptors specifically bound to cytoskeletal matrix proteins (392).

The use of physical and chemical agents has been especially useful in implicating cytoskeletal systems in the undelying mechanisms of movement involved in various cellular events. By far the most extensively used agents to study actin-containing filaments have been the cytochalasins - cytochalasins A,B,C,D,E, and F, and in particular cytochalasin B - a family of fungal metabolites isolated from different molds and related to one another by structure and biological activities (19,232). Cytochalasin B inhibits the formation of microfilament cables and networks in vivo (175,233); furthermore, it is known that cytochalasin B disrupts networks of actin filaments in vivo (175,235). This drug has been used to demonstrate microfilament involvement in a host of cell movements including phagocytosis (234), membrane ruffling (236), pinocytosis (175), intracellular substrate transport (237), microvillar extension (237) and changes in cell shape (238). Cytochalasin B can totally inhibit

cytoplasmic cleavage without interfering with divsion of the nucleus, resulting in large, multinucleate cells (238). Presumably, this effect results from dissolution of the bundle of actin filaments comprising the cleavage ring (10). Cytochalasin B inititates complete disassembly of stress cables in living cells with consequent cessation of cell locomotion (238). In vitro, cytochalasin B inhibits actin polymerization (57), and the formation of actin gels in crude cytoplasmic extracts (175), as well as gelation of purified actin filaments in the presence of macrophage actin-binding protein (175). The effects of the cytochalasins on actin filaments in vitro parallel those on microfilaments in vivo and give strength to the arguement that in vitro results reflect situations in living cells. Cytochalasin B and various analogs such as dihyrocytochalasin B (238) specifically interact with microfilaments and do not alter patterns of microtubules in treated cells (238). As a result, cytochalasin treatment has become a major criterion used in establishing specific involvement of microfilaments in many subcellular events.

Though the precise mechanism of action of cytochalasin B on actin filaments is not yet known, there is good evidence that this agent inhibits growth of microfilaments by preventing addition of actin monomers to the preferred growing end of the filament (175). There is electron microscopic evidence that cytochalasin B binds to F-actin filaments; there is no such evidence for binding to actin monomers (175). Work on filament growth in vitro (174) further suggests that the dramatic effects of cyto-

chalasin B on microfilament organization in vivo may be the result of an inhibitory effect on filament cross-linking in addition to inhibition of actin polymerization. Supportive evidence for this claim has come from studies of rabbit pulmonary macrophages and HeLa cells treated with cytochalasin B. Here, cytochalasin B was clearly shown to dissociate actin filaments from those actin-associated proteins responsible for gelation without affecting the integrity of the actin filaments themselves (239).

Cytochalasin B and the other cytochalasins have additional effects on cell function which may not be mediated through microfilament organization. Cytochalasin B induces extrusion of the nucleus within minutes after addition to a variety of cultured cells (240). Several of the cytochalasins inhibit uptake of exogenous sugars (240) and transport of thymidine (242) into cells, most probably by direct interaction with membrane lipids (10). Cytochalasin B inhibits initiation of DNA synthesis by serum growth factors in 3T3 mouse fibroblasts (243), and inhibits cell cycling through effects on DNA synthesis in many cells in culture, including adrenocortical cells (242). Cytochalasin B is known to inhibit such diverse biological activites as thyroid hormone secretion (244), growth hormone release (245), and platelet aggregation (246); in these cases, the possibility that this agent promotes "nonspecific" effects which do not involve the cytoskeleton makes it difficult to conclusively prove that disruption of microfilament integrity is the causitive factor in its mechanism of inhibitory action. Interestingly, cytochalasin

binding studies using labelled cytochalasin B analogs have suggested that cells have specific surface receptors for cytochalasin B (238). It may be that effects of cytochalasin B which are mediated through the cytoskeleton and those which seem to be microfilament-independent, e.g., sugar transport, are mediated by separate cytochalasin B receptors with different affinities for cytochalasin B (238).

In addition to the cytochalasins, other drugs have been used to specifically alter microfilament integrity. Phalloidin is a toxic, bicyclic peptide from the mushroom Amanita phalloides which stabilizes actin filaments, presumably by binding to actin subunits and lowering the critical concentration for polymerization (247). Phalloidin-actin filaments are very resistant to treatments, such as heat denaturation and 0.6M KI, which depolymerize actin filaments, and do not inhibit DNase I (19). Subtoxic doses of phalloidin or phalloidin analogs in isolated rat liver cells promote rapid microfilament formation from membrane-associated actin (248); this observation has been used to implicate actin filaments in phalloidin-induced endocytotic events in these cells (19).

In a similar vein, a host of microtubule-specific drugs have been used to correlate microtubular structure with cellular function. Colchicine inhibits polymerization of microtubules by specifically binding to the 6S tubulin heterodimer (19,57). This agent has been used on living cells to implicate microtubules in the regulation of cell shape (19,249), metaphase chromosome separation (10), intracellular transport (251,381), and ciliary

and flagellar movement (10). As with cytochalasin B, colchicine has been found to have effects on cells which do not seem to be mediated by changes in cytoskeletal organization. These include inhibition of nucleoside transport (251), inhibition of protein synthesis (252), and inhibition of fluid transport by membranes (250). In investigations of membrane-cytoskeleton associations, demonstration of colchicine binding has been used as evidence for specific membrane-tubulin interactions (57).

The Vinca alkaloids, vinblastine and vincristine, also cause reversible dissolution of microtubule arrays in living cells (10). For example, vinblastine and colchicine both disrupt microtubules of the mitotic spindle of cells in metaphase (253). The cytoplasm of treated cells often contain large, birefringent star-shaped crystals which are composed of tubulin complexed with vinblastine as well as large 34-38nm diameter microtubule matrices made of loose helices of microtubular proteins packed into cylinders (10). Though the significance of these stuctures is not yet understood, they testify to the dramatic effect these agents has on microtubule integrity.

It has long been known that cold and pressure alter the consistency of the cytoplasm, and that these changes effect the structure and function of subcellular organelles (57). It is known, for instance, that lowering the temperature of cells or of cilia or flagella to $0^{o}C$, or exposing them to pressure, will induce depolymerization of associated microtubules (19). In vitro studies on microtubule structure confirm this observation (19). Pressure treatment reduces the number of microvilli on the

surface of lymphocytes (254). Microfilaments contained in the microvilli of certain cells undergo dramatic changes in shape when exposed to pressure (255), and cold treatment destroys the microvilli and the terminal web filaments of embryonic intestinal cells (254). Actin-based gelation of cell extracts in vitro can be partially reversed by cold or pressure (19). All these changes involve specific disruptions of underlying microfilaments and/or microtubules, and have been used to support the arguement that affected cytoskeletal structures are intimately involved in producing the movements associated with these organelles.

In recent years, a new technique has come into prominence which promises to yield important information on cytoskeletal structure and function in vivo. Microinjection combines a number of the techniques discussed above, e.g., use of cytoskeletal-specific drugs with immunofluorescence microscopy, to explore the status of the cytoskeleton in intact, living cells. Microinjection involves the introduction of specific molecules into cells via membrane-bounded vesicles by endocytosis (257). Microinjection has the distinct advantage over other approaches involving chemical agents in that only minute quantities of any drug need to be introduced into the cell in order to produce effects on cellular function similar to those seen with larger, externally applied doses. Furthermore, microinjection allows introduction of agents, which because of their size (e.g., very large proteins) or chemical nature (e.g., hydrophilicity), are unable to penetrate the plasma membrane of their own accord.

To date, microinjection of fluoresceinated probes has been

successfully applied to reveal the in situ distribution of numerous cytoskeletal proteins. These include actin (219,258,259), tropomyosin (259), 130,000-molecular weight protein (260), tubulin (261), and calmodulin (219). Evidence suggests that such probes incorporate into cytoskeletal structures without disrupting the organization or function of the subcellular organelles in which these filaments are found. Microinjection has been used to follow the time-course of incorporation of fluorescently-labelled probes for specific cytoskeletal proteins into the cell. For example, this technique has been used to plot the movement of newly introduced rhodamine-labelled actin into cultured fibroblastic cells from membrane ruffles into actin stress cables (219), in much the same way as pulse-chase experiments using radioactive probes have been performed to chart the movement of many proteins through a cell. Microfilaments in situ have been visualized by fluorescently-tagged phalloidin (392,262,388) and by FITC-heavy meromyosin (57) microinjected into living cells. In these experiments, decoration of microfilament structures were correlated with disturbances in biological function as a function of time. A variation on the fluorescence theme has been microinjection of antibodies, including anti-actin antibodies (263), antimyosin antibodies (264), and intermediate filament antibodies (265), to localize the distribution of particular cytoskeletal proteins and to determine the involvement of specific cytoskeletal structures in specific cellular activities. Antibodies, by virtue of their potential specificity, are excellent probes in such experiments;

microinjection offers a reliable way of introducing these large molecules into cells in low doses.

Taken together, these techniques represent an arsenal of approaches to exploring the structure and function of the cytoskeleton. In the remaining sections of this review, I will focus on a number of cellular events whose functions are manifestations of changes in cytoskeletal organization. The techniques mentioned here have been instrumental in demonstrating cytoskeletal participation in and control of these activities.

## Cytoskeleton and the Plasma Membrane

An obvious consequence of any cytoskeletal role in cell locomotion, in cell attachment, or in cell shape, is that cytoskeletal structures must in some way interact with the plasma membrane of the cell, for the plasma membrane is intimately involved in all of these activities. Because of their asymmetrical distribution within the cytoplasm and their close physical association with the membrane, cytoskeletal filaments have long been presumed to influence movements of the plasma membrane. This expectation has been amply met by both direct and indirect experimental evidence which demonstrate precise interactions of cytoskeletal proteins with components of the plasma membrane. Abundant light and electron microscopic evidence exists to show that microfilaments, microtubules and intermediate filaments all contact the cytoplasmic face of the plasma membrane either directly or through peripheral or integral membrane proteins (19,57). In every cellular organelle examined, from

microvillus to pseudopodium, from desmosome to stress cable, there is anatomical evidence that the cytoskeketal filaments contained within these structures interact with overlying plasma membrane at discrete regions of the membrane. In the case of the intestinal microvillus, for example, the bundle of microfilaments within the core of the microvillus attaches to the closely adhering plasma membrane along the sides of the microvillus by proteinaceous side-arms which can be seen in the electron microscope (19,57). Attachments of microfilaments into electron-dense desmosomal material (19), and into plasma membrane-dense bodies in smooth muscle cells (19), offer other concrete examples of microfilament-membrane associations. Microtubules are frequently bridged to the plasma membrane of cilia and flagella (19,57). In the case of vertebrate photoreceptor cells (266), for example, the outer microtubule doublets are bridged to the membrane by champagne-shaped structures which are integral membrane components. Filamentous connections have been observed between cytoplasmic microtubules and the plasma membrane in the axons of cultured nerve cells (19,57), at the post-synaptic density (267), and between microtubules and the membranes of mitochondria in lamprey axons (268), and of synaptic vesicles in the frog motor endplate region (269). Microtubules have been observed ending in dense material at inpocketings of the plasma membrane in some cells (294), and within dense masses immediately under the plasma membrane in other cells (19). Evidence for direct attachment of actin filaments and microtubules to the plasma membrane, without intervention of apparent intermediary

proteins, also exists (19); in these cases, it has been proposed that cytoskeletal elements may interact directly with membrane lipids through hydrophobic interactions (271).

Convincing microscopic evidence for attachment of intermediate filaments to the plasma membrane exists in the case of rat myoneuronal junctions (272). Here, the post-synaptic membrane is paralleled by a 10-nm filament which is bridged to the adjacent membrane by side arms found every 25nm. In epidermal cells of the guppy, tonofilaments arranged in four different arrays - as filament bundles, as a desmosomal reticulum, as bundles which interconnect desmosomal networks, and as separate filaments near the plasma membrane - each closely associated with distinct regions of the plasma membrane (273). Intermediate filaments have also been seen inserting into dense material associated with the plasma membrane (218).

Electron microscopic analysis of these and other examples suggests that cytoskeletal-membrane associations may be very heterogeneous in molecular organization. Attachment without local differentiation has been reported for all cytoskeletal elements. Stereo electron microscopy of isolated Acanthamoeba plasma membrane, for example, has revealed actin filaments which contact the membrane at random sites. Similar studies have demonstrated diffuse attachment of microtubules (19) and intermediate filaments (274) with the plasma membrane. In many cases, on the other hand, contact of cytoskeletal structures with the plasma membrane is made at defined regions of the membrane. Such is the case with the intestinal microvillus, where actin filaments

insert into a plaque of electron dense material at the tip of the microvillus, most probably by interacting directly with the alpha-actinin there (275). Attachments of cytoskeletal elements into electron-dense material at discrete locations in cells have been clearly demonstrated in cases such as actin filaments inserting into desmosomal material (19) and microfilaments embedding into plasma membrane-dense bodies in smooth muscle cells (19). Other microscopic and biochemical studies have shown that bundles of cytoplasmic microfilaments (i.e., stress cables) insert into the plasma membrane at regions of close contact with the substratum (113). Many cell-cell junctions consist of specific regions of close membrane contact into which cytoskeletal filaments insert. For example, actin microfilaments attach directly to the filamentous mat of the fasciae adherentes in the cardiac intercalated discs of vertebrate cardiac muscle (19), and in patches of dense material called post-synaptic densities found on the cytoplasmic surface of many post-synaptic cell membranes (276). In some cardiac smooth muscle cells, cytoskeletal-membrane associations consist of complex structures in which a layer of filamentous material lies sandwiched between electron-dense material adjacent to the plasma membrane and cytoplasmic microfilaments (277).

Little is known about the molecular details of cytoskeletal filament-membrane attachment sites. In cases in which intermediary proteins are known to link filaments to the plasma membrane, the precise molecular interactions between cytoskeletal proteins, linker proteins and integral membrane proteins have not

been worked out. The one notable exception, however, is the human erythrocyte. In this cell, all of the cytoskeletal and membrane-bound proteins involved in attaching the cytoskeleton to the plasma membrane have been purified and assigned specific functions (see part D, section C for a detailed discussion).

One curious observation concerning cytoskeletal protein-plasma membrane attachment deserves mention here. Alpha-actinin, a protein first isolated in abundance from the Z-disk material of striated and cardiac muscle (278), has been found in a diversity of nonmuscle cells in regions of the cell where cytoskeletal filaments closely contact the plasma membrane (19). Immuno-fluorescent staining using antibodies to muscle alpha-actinin has localized cross-reactive proteins to intestinal epithelial desmosomes (279), fibroblast attachment plaques (280), and to the side arms attaching actin to the membrane of brush border cells (275). The presence of alpha-actinin or a family of closely related proteins in all these sites suggests that this protein may serve a common function in different cells, a role which most likely involves attaching of microfilaments to the plasma membrane. Future biochemical studies may reveal other ubiquitous cytoskeletal proteins involved in cytoskeleton-membrane interactions.

The classical biochemical approaches of isolation of plasma membrane and analysis of associated proteins have been used to identify particular cytoskeletal proteins which interact with the plasma membrane. Identification of membrane as plasma membrane commonly involves analysis by electron microscopy (19) or identi-

fication of plasma membrane-specific enzymes or lipid markers (19). Membrane preparations can be further stripped of cytoskeletal proteins by detergent solublization to determine the distribution of these proteins or to analyze their role in membrane functions (19). In conjunction with amino acid analysis and peptide mapping, these techniques have been used to identify several cytoskeletal proteins as specific plasma membrane-bound components. These include myosin (19), tubulin (19), dynein (281), alpha-actinin (282) and tropomyosin (75). Heavy meromyosin binding and in vitro activation of myosin ATPase have also been used in the identification of membrane-associated actin (283). Ultimate proof of membrane occupancy for actin has come from reconstitution experiments using purified cytoskeletal proteins (284) or purified membrane vesicles (185) in which specific binding sites for F-actin were identified.

Evidence for association of tubulin and microtubules with the plasma membrane has come from immunofluorescence staining of isolated plasma membrane using antibodies against tubulin (57). In these studies, microtubules were commonly seen terminating at the plasma membrane (57). In vitro studies using rhodamine-labeled tubulin and fluorescein-labeled plasma membranes from polymorphonuclear leukocytes have demonstrated resonance energy transfer between microtubules and the membranes themselves, indicating a close association of these cytoskeletal filaments with the membrane (286). Direct binding of purified microtubules to isolated secretory granules and to isolated granule membranes has been demonstrated. (187). A large number of binding studies

have shown that tubulin is specifically associated with toher cellular membranes as well. These membranes include synaptosomal preparations (57), synaptic vesicle membranes (288), and myelin membranes (289).

That membrane-associated tubulin is not simply a contamination of membrane preparations is supported by several experimental observations. Peptide maps of various presumptive membrane tubulins are similar, but not identical, to soluble (i.e., cytoplasmic) tubulin (57). In the case of ciliary membrane tubulin, analysis of membrane-associated tubulin has revealed significant differences in amino acid composition from axonemal tubulins, differences which would explain the observed increase in hydrophobicity of this molecule (290). Finally, there is some chemical evidence that in distinction to soluble tubulin, membrane-bound tubulins are glycosylated (57). All of these observations argue that membrane-associated tubulin is distinct from soluble tubulin and therefore does not represent a membrane contaminant.

Evidence for association of actin with membranes is equally impressive. Actin has been identified as a major component of membrane-associated filaments by chemical identification in conjuntion with electron microscopy in sections of whole cells from Acanthamoeba (291), Dictyostelium discoideum (292), CHO fibroblasts (293), lymphocytes (57), and macrophages (294). Similar analysis has identified actin in membrane-bound filaments in membrane preparations from 3T3 cells (295), synaptosomes (296), chick fibroblasts (297), in membrane "ghosts" from D.

discoideum (198), and in purified plasma membranes of ascites tumor cells (125).

Given that microtubules and microfilaments physically attach to the plasma membrane in cells, it might be concluded that such specific interactions function in those cellular events which involve the plasma membrane. To date, relatively little direct experimental evidence exists to support this hypothesis. For the most part, experimental approaches have been indirect, and have relied on showing that removal or disruption of a cytoskeletal component alters a particular motile event by specifically disturbing the integrity of the plasma membrane to which the affected cytoskeletal structure is attached. As with the cilium and the microvillus, the supposition here is that the cytoskeletal structure somehow defines the motile event, i.e., that structure and function cannot be dissociated. Implicit in this hypothesis is the assumption that any cytoskeletal structure involved in a particular motile event must directly or indirectly interact with the plasma membrane involved in the event. In the following section, I will present two well-studied examples which illustrate how complex associations of cytoskeletal elements with plasma membrane components function to control membrane organization and function. In the first case, that of receptor capping, the rearrangement of membrane-bound cytoskeletal filaments, which parallels the reorganization of surface membrane proteins, has been worked out; in the second case, that of the red blood cell, the precise molecular interactions between cytoskeletal and integral membrane proteins have been defined.

Capping

There is a good deal of evidence to suggest that the distribution of certain components of the cell surface is infuenced by interactions with elements of the cytoskeleton. The phenomenon of capping of ligands following binding to specific surface receptors has been extensively studied as a model system involving membrane-cytoskeleton interactions. By far the most well-studied examples are capping of surface receptors for the tetravalent lectin, concanavalin A (Con A), and for antibodies to surface immunoglobulins (Ig) on lymphocytes (19,57,249). These receptors in uncapped cells are distributed randomly over the surface of the plasma membrane; however, upon binding of Con A or anti-Ig antibodies, the distribution of these receptors shifts, from a uniform distribution over the entire cell surface through an intermediate stage involving patches or clusters of ligand-receptor complexes, to a single region located near the Golgi region of the cell (19). This resulting "cap" consists of surface receptors cross-linked together in some as yet undefined way (19). Cap formation is reversible; upon removal of ligand by washout, the cap disassembles and surface receptors return to a random surface distribution (57). Capping, but not patching, is an energy-dependent phenomenon; poisoning cells with oxidative uncouplers such as dinitrophenol inhibits the former but not the latter event (44). In most cases, capping is followed by internalization of the receptor-ligand complex (57).

Capping of Con A and immunoglobulin receptors are not the only surface proteins which cap on lymphocytes. Capping of other

surface components, including Fc receptors, histocompatibility antigen H-2, and theta antigen, have been reported in these cells (299). Moreover, capping is not restricted to lymphocytes and thymocytes; this event has been observed in a host of other cells including polymorphonuclear leukocytes (300), pulmonary alveolar macrophages (57), fibroblasts (301), Chinese hamster ovary cells (57) and rabbit ovarian granulosa cells (302), lymphoblastoid cells (303), and the slime mold Dictyostelium discoideum (292). The widespread occurrence of this phenomenon testifies to its generality and implies an underlying function(s) of great importance.

Several lines of evidence point to cytoskeletal involvement in the capping event. Treatment of certain cells with microfilament-disrupting drugs such as cytochalasin B (or D) will cause moderate to severe inhibition of Con A and immunoglobulin capping (19). This is true for these receptors on lymphocytes, CHO cells and on mouse fibroblasts (19). In other cases, such as with pulmonary alveolar macrophages (300), cytochalasin B promotes Con A capping, suggesting microfilament involvement in this process. On the other hand, in cells such as human peripheral blood neutrophils (300), cytochalasin B has no effect on Con A cap formation. Cytochalasin B can also cause disruption of pre-formed caps in some, but not all, cases of Con A capping (19). Most studies have concluded that colchicine treatment has little or no effect on lymphocyte immunoglobulin capping (19); however, colchicine causes moderate to substantial promotion of capping of concanavalin A in a variety of cells (19) including

lymphocytes. In these cases, the effect of colchicine is specific to microtubules as analogs of colchicine, such as lumicolchicine, which do not bind to tubulin (304) have no effect on capping. These studies suggest that capping may also involve microtubules and argue against the possibility that the effect of colchicine on capping is due to a direct effect on the membrane itself.

The observation that artificially-induced alterations in cytoskeletal organization coincide with disruption of the capping process implies that the cytoskeleton participates in capping. In light of the that drugs such as cytochalasin B and colchicine have other effects on cell function which may not involve the cytoskeleton, a final verdict on precisely which cytoskeletal structures are involved in each specific capping event awaits future corroborative investigation.

A second line of evidence suggesting involvement of the cytoskeleton in capping derives from frequent observations that cytoskeletal proteins often concentrate on the cytoplasmic face of the plasma membrane directly beneath capped surface receptors (305). In these cases, aggregation of cytoskeletal proteins strictly coincides with capping of surface receptors. Electron micrographs of isolated plasma membrane from Con A-capped ovarian granulosa cells (57) clearly show aggregation of microfilaments and microtubules into a dense mat adjacent to and underneath the caps. Co-capping of surface receptors with tubulin (306), myosin (57), actin (57) and alpha-actinin (249) has been observed in lymphocytes and HeLa cells using immunofluorescence microscopy to compare the distribution of fluorescently-labelled ligands and

antibodies to specific cytoskeletal proteins. In other studies on fibroblasts using double staining immunofluorescence microscopy, it was found that clustering of surface components induced by Con A binding produced a striking alignment of surface components with the cytoplasmic stress fibers within the cell (307). The formation of such physical associations of cytoskeletal filaments with membrane caps strongly implies some sort of functional relationship.

Addition support for a physical association of cytoskeletal proteins with capped surface receptors comes from work on isolated apparent complexes between these components. Capped regions of lymphocyte membranes show a much greater proportion of total cellular actin than similar regions from uncapped cells (308). In lymphocytes and P3 myeloma cells, more Ig receptor will coprecipitate with membrane-derived actin from capped membrane than from uncapped membrane (308). Capped regions of Dicyostelium plasma membrane are resistant to Triton X-100 solublization and have allowed the isolation of these membrane fragments (292). In these preparations, analysis by electron microscopy (for cytoskeletal filaments) and by SDS-PAGE (for cytoskeletal proteins) has demonstrated a significant increase in membrane-associated microfilaments and microtubules, as well as in total actin and myosin, in capped regions of the membrane over noncapped regions, again suggesting that filaments composed of actin and myosin specifically associate with the region of the membrane adjacent to the surface cap.

These results strongly suggest, but do not prove, that ligand receptors on the surface of the plasma membrane interact directly or indirectly with cytoskeletal proteins on the inner surface of the plasma membrane. However, without such a link it would be difficult to reconcile the coincident changes in the distribution of these elements observed.

Several models of molecular organization have been proposed to explain how cytoskeletal elements might be involved in the phenomenon of capping. The early model of Bretscher (309) suggests that capping is simply a consequence of membrane flow. This hypothesis postulates that plasma membrane lipids are in a constant state of motion such that there is a constant flow of membrane lipid from one region on the cell surface where it is added to the membrane to another area where it is removed. However, upon binding of ligands to their respective receptors, cross-linking of receptors and the formation of receptor patches results in a decrease in receptor mobility due to a change in its membrane diffusion coefficient. The consequence of decreased mobility is an aggregation of receptor patches as receptor-ligand patches are swept into the region of the membrane where lipid is being removed during membrane flow. The genesis of this model lies in experimental evidence that both membrane phospholipids and membrane-bound proteins are asymmetrically disposed within the lipid bilayer (229). This model can explain why capping of some surface proteins, such as lymphocyte theta and H-2 antigens (57), are not affected by cytoskeletal disrupting agents. However, this model disregards as unimportant any interactions of

cytoskeleton with membrane components, and does not offer any explanation for the observed specificity of capping, i.e., that each ligand will induce capping of its own receptors but not those of other ligands.

An alternative model proposes that membrane receptors are anchored to microtubules and that capping is a consequence of microtubule movement (310). In this model, all cell surface receptors interact with one class of membrane-bound microtubules. Binding of a ligand to its surface receptor initiates tubulin polymerization and promotes anchorage of receptors to newly formed microtubules. Capping results from a reorganization of microtubules beneath the plasma membrane: as membrane-associated microtubules cluster together, attached surface receptors are pulled together into one region of the membrane. Though this model is consistent with the observation from colchicine studies that the capping event is dependent on microtubule integrity, again it does not explain either the numerous cases in which microtubule-disrupting agents have no effect on capping or the specificity of capping. If all surface receptors are indeed linked to one set of microtubules, as this model implies, then one might expect that capping of one class of receptors would influence the surface distribution of other receptors. Though some experimental evidence exists to support this conslusion, e.g., the observation that capping of lymphocyte Con A receptors inhibits subsequent capping of surface Ig receptors (299), this model fails to offer an explanantion for the many cases in which such a receptor interaction cannot be demonstrated. Finally, this

model is complicated by the finding that in some cells, such as phagocytotic polymorphonuclear leukocytes (311), caps form in regions of the membrane devoid of microtubules. As this model hinges on a physical interaction of microtubules with the plasma membrane, this observation seems incompatible with the tenets of this model.

Based on observations that clusters of microfilaments are often seen in areas devoid of microtubules, such as in regions of the membrane involved in phagocytosis (311) and near the cleavage furrow in dividing cells (311), Oliver and Berlin (249) have proposed that microfilaments, not microtubules, physically link to surface receptors. In their model, capping results from the movement of ligand-receptor complexes by membrane flow to microfilament-rich regions of the membrane by virtue of some as yet undefined membrane property imposed by the presence of microfilaments. Microtubules serve only to regulate the distribution of microfilaments beneath the plasma membrane. Thus, microtubules play an important, but indirect, role in the capping event. This model implies that direct microtubule-receptor interactions are not necessary in the process of cap formation. Indirect support for this hypothesis comes from studies of Con A capping in polymorphonuclear leukocytes (312). In this system, capping can only be induced after treatment of cells with colchicine or other microtubule-disrupting agents. The authors have interpreted this observation to mean that the presence of membrane-associated microtubules somehow interferes with the clustering of microfilaments required for capping. Disruption of microtubules with

colchicine therefore allows capping by permitting aggregation of microfilaments.

If microfilaments and/or microtubules do regulate the distribution of surface receptors in the process of capping, how do they effect such movements? Several models have specifically addressed the molecular mechanism(s) by which cytoskeletal structures translocate during capping. Several of these propose a cytoskeletal contractile mechanism much like that found in skeletal muscle. Unanue and co-workers (313) have proposed that surface receptors are reversibly linked to underlying microfilaments, and that upon ligand binding, microfilaments are redistributed by an active process which involves interaction of microfilaments with myosin. In this model, surface receptors which are attached to underlying microfilaments would be dragged together into one region of the membrane as a consequence of attached microfilament redistribution. Singer et al. (95) have suggested a modified version of this model in which microfilaments interact with surface receptor via an unspecified membrane protein X. Again, capping is the result of aggregation of receptor-protein X-microfilament complexes due to sliding of microfilaments relative to one another by some mechanism which involves myosin. Numerous observations that actin, myosin and alpha-actinin are associated with patches of H-2 antigen (95), and that H-2 antigen receptors can form stable links with actin (315), offer some experimental support for such contractile models of receptor capping.

Many unanswered questions remain as to how the cytoskeleton is involved in the process of receptor cappping. For example, the precise nature of the link between membrane receptors and microfilaments and microtubules, as well as between actin filaments and myosin, remains to be elucidated. Because almost nothing is known about how microfilaments and microtubules interact, the details of such associations proposed in some models of capping remain undefined. Given the complexity of the cappping phenomenon, we cannot even be certain that all cases of capping involve the same molecular mechanism. Nonetheless, the bulk of the experimental evidence thus far strongly indicates that whatever the underlying mechanism(s) for capping is, it apparently involves the cytoskeleton.

## The Erythrocyte Plasma Membrane

The human erythrocyte plasma membrane represents an outstanding model system for the study of cytoskeletal-membrane interactions. More is known about the molecular organization, and in particular the interactions of cytoskeletal proteins with integral membrane proteins, of this membrane than any other membrane. Given the ease of obtaining high yields of purified plasmalemma "ghosts" which retain intact, unaltered attached cytoskeletons (316), study of this membrane has greatly contributed to our understanding of how the interaction of cytoskeleton with membrane influences cell shape and movement.

Triton X-100 solublization of erythrocyte ghost membranes reveals a complex array of membrane-associated cytoskeletal

structures. These include microfilaments (composed of actin), microtubules (composed of tubulin), intermediate filaments of vimentin and desmin, and a host of interfilament cross-linking proteins (316 ). The human erythrocyte cytoskeleton is composed primarily of three proteins: spectin (317), band 4.1 (316), nomenclature according to Steck (318), and erythrocyte actin (316). There are no microtubules (i.e., tubulin) or intermediate filaments (e.g., vimentin) found within this matrix of proteins. Spectrin accounts for approximately 75% of the total cytoskeletal protein mass (319). It is a large, asymmetric molecule composed of two nonidentical polypeptide chains, band 1 (alpha subunit, 240,000 daltons) and band 2 (beta subunit, 220,000 daltons), which are similar in chemistry and structure (318). Band 2 is multiply phosphorylated in vivo (320). Spectrin can self-associate to form a dimer of heterodimers, the $(alpha, beta)_2$ tetramer (920,000 daltons) which is probably the native state of the molecule (316). Electron micrographs of rotary-shadowed spectrin show the dimer as two loosely intertwined, flexible strands joined at their ends (316). Though spectrin is common to most mammalian erythrocytes, its distribution appears to be limited to the red blood cell (321).

The first clue to the function of spectrin came from freeze fracture electron micrographs of isolated erythrocyte plasma membranes which showed that the surface distribution and lateral mobility of integral membrane proteins band 3 and glycophorin were in some way dependent on an intact spectrin meshwork (322). Later binding studies of phosphate-labelled spectrin to plasma

membrane vesicles stripped of spectrin and actin (323) has clearly shown that the erythrocyte plasma membrane contains specific binding sites for spectrin. Spectrin binding is specific, saturable and of relatively high affinity ($K_d$=$10^{-7}$M). Other studies have identified band 2.1 and the group of sequence-related proteins, bands 2.2-2.6, as the integral membrane components which together constitute these spectrin-binding sites. These proteins were first named "syndeins" (324), but have become more commonly known as "ankyrin."

Ankyrin has now been purified to homogeneity in a biochemically active, water-soluble form (316). This ankyrin binds purified spectrin with a 1:1 stoichiometry (316). Electron micrographs reveal that ankyrin binds to a single strand of the spectrin heterodimer near the end of the molecule that participates in tetramer formation (315). Ankyrin in turn binds to the erythrocyte membrane via a direct interaction with another integral membrane protein, band 3 (316). Little is known about band 3 except that it exists in some 5-fold excess relative to ankyrin in the human erythrocyte (316), and also spontaneously forms tetramers in solution (326). Binding studies in solution using purified ankyrin and band 3 show that high-affinity band 3 binding sites for ankyrin saturate at approximately 4-7 molecules of band 3 per molecule of ankyrin.

The molecular interactions of spectrin, ankyrin and band 3 explain how spectrin is attached to the erythrocyte membrane but do not explain how Triton-extracted erythrocyte cytoskeletons maintain their integrity in the absence of band 3 (98). Inter-

actions of these proteins with other cytoskeletal proteins must be involved. Early work by Tilney and Detmers (327) and by Brenner and Korn (328) suggested that the shape of the erythrocyte membrane was due in large part to the network of cross-linked actin filaments, and that spectrin could act as a cross-linker of actin filaments. Subsequent binding studies using purified spectrin (329) have shown direct association of spectrin with F-actin in which spectrin dimers or tetramers in fact cross-link adjacent actin filaments by binding to the sides of the filaments. The actin-binding sites on the spectrin dimer are at the tail ends of the dimer (330). Moreover, spectrin as a dimer or tetramer can crosslink F-actin in solution in the presence of band 4.1 (98). The crosslinked complexes formed with band 4.1 have viscosities which closely resemble those found from actin gels extracted from intact erythrocytes (311), and show a sensitivity to calcium in terms of viscosity which parallels that found in vivo (329). These results suggest that band 4.1 may play a role in determining the deformability of the red cell cytoskeleton (316). Finally, both spectrin and actin can be removed from the erythrocyte membrane without disturbing the association of band 4.1 with membrane, suggesting that band 4.1 might serve as a secondary membrane site for spectrin binding (325).

The dense and convoluted matrix of actin filaments associated with the erythrocyte membrane have made it virtually impossibe to determine by microscopic evaluation whether or not microfilaments interact directly with the membrane. There is

experimental evidence that both purified G- and F-actin can bind to red cell plasma membrane. Though much of the evidence to demonstrate association of plasma membrane with monomer actin has been contradictory (316), recently it has been clearly shown that G-actin can bind to red cell ghosts or to the cytoplamic side of inside-out vesicles reconstituted with spectrin-actin-band 4.1 complexes (337). F-actin also binds to spectrin-actin-depleted inside-out vesicles (333) and is enhanced by addition of band 4.1 (330). Subsequent studies with purified cytoskeletal proteins have convincingly demonstrated that spectrin-actin-band 4.1 complexes can induce actin polymerization in vitro (334). This ability is a property of the complex itself and not of the individual proteins. Moreover, this complex can reassociate with erythrocyte membrane (332). These observations have prompted the hypothesis that spectrin, in physical conjuction with band 4.1 and actin, serves as a nucleation site for actin polymerization at the plasma membrane (385).

Though much is known about the interactions of actin, spectrin, ankyrin, and bands 3 and 4.1, nothing is at present known about the factors that control and modulate these associations in vivo. The observation that ankyrin, spectrin and band 4.1 are phosphoproteins (316) has prompted the intriguing suggestion that phosphorylation of these and other proteins might function to regulate their interactions. The fact that gelation of actin filaments with spectrin and band 4.1 are calcium-sensitive (329) might reflect another level of cytoskeletal control, perhaps at the level of a calcium-sensitive kinase or

directly at the level of cytoskeletal protein interactions.

One additional observation to emerge from the study of the red cell membrane deserves mention. Spectrin, although unique to the erythrocyte, is an actin-binding protein whose gross structure and interaction with actin filaments in solution are remarkably similar to those of other cytoskeletal dimeric, actin-binding proteins found in nonerythroid cells, including filamen and macrophage actin-binding protein (316,317). Furthermore, all of these proteins share a common size and immunoreactivity (316). Immunoreactive forms of ankyrin have been detected by radio-immunoassay in other cell types (336). Ankyrin immunoreactivity has been localized by immunofluorescence to the filamentous meshwork surrounding the nucleus and matches the distribution of anti-tubulin reactivity in the cytoplasm (317). Ankyrin-immunoreactive staining of metaphase cells matches the pattern of microtubules in the region of the mitotic spindle (337). Moreover, antibodies to the microtubule-associated protein MAP-I crossreact with ankyrin (317) and with a host of endogenous proteins which do not interact with microtubules (317). Ankyrin specifically binds to pure brain tubulin at the same binding site as for brain MAP-I (317). Taken together, these observations strongly suggest that ankyrin in erythrocytes has evolved from a microtubule-associated protein. The exciting implication here is that ankyrin-like proteins in nonerythroid cells might function to bind microtubules to membranes in a manner similar to the role of ankyrin in linking spectrin to the plasma membrane.

Out of these and other studies has emerged a detailed picture of the molecular organization of the erythrocyte membrane. In this schema, cytoskeletal proteins specifically interact with membrane-associated proteins which in turn link to integral membrane proteins. With the exception of some of the glycoproteins that require more study, the set of associations characterized by binding studies and electron microscopic analyses have led to a view of the erythrocyte membrane as an organelle fraught with specific interactions among all of the major membrane proteins. Evidence also exists to demonstrate a physical interaction of cytoskeletal proteins with the erythrocyte plasma membrane. It is now recognized that the transmembrane protein band 3 is the key intermediate between the lipid bilayer and the erythrocyte cytoskeleton. Recent electron micrographs of deeptly etched, fast-frozen erythrocyte cell membranes (318) reveal the three-dimensional relation of cytoskeletal elements to the cytoplasmic surface of the membrane, and for the most part support these conclusions. A detailed presentation of the experimental evidence supporting a role of the erythrocyte cytoskeleton in the maintainence of cell shape will be presented in the next chapter of this dissertation.

## Cell Attachment

Most eukaryotic cells are capable of making contact with a variety of extracellular surfaces. Cells in tissues contact one another and attach to intercellular basement membranes; cells in culture make firm contacts with glass and plastic tissue-culture

dishes. In cultured cells, contact with artificial substrata is mediated through specialized regions of the ventral surface of the cell, called "focal contacts", or "focal-adhesion plaques" (10,19,44,57,339). A variety of observations indicate that attachment through adhesion plaques involves both intracellular and extracellular structures. Membrane-associated cytoskeletal elements, in particular actin-containing microfilaments, concentrate at these regions and interact with other membrane-bound proteins which in turn link to proteins of the extracellular matrix. In this section, I will present some of the evidence supporting this hypothesis, with particular emphasis on the role of actin stress cables in the regulation of cell attachment.

It is now well established both by electron microscopy by and immunofluorescence microscopy that focal contacts are the termini of actin-containing microfilament bundles, or "stress cables," which run throughout the cytoplasm near the ventral surface of the cell (56,175,340). Judging from examination of living cells by interference reflection microscopy, which can distinguish among membrane-surface contacts of varying thickness, cytoplasmic stress cables appear to associate much closer to the substrate at focal contacts that at other areas of the plasma membrane (341). Studies using reflection interference microscopy in conjunction with high voltage electron microscopy of whole cells (342) have clearly shown that every focal contact in a migrating cell is associated with the end of a microfilament bundle that extends back through the cell towards the nucleus.

Several lines of evidence indicate that stress cables are related to adhesion of cells to the substratum. First, there is the microscopic evidence that stress cables concentrate in the regions of focal contact (19,57). Corroborative studies have shown that the tightness of adhesion can be correlated with the presence of these cables (57). Second, agents which disrupt specific cytoskeletal structures can alter cell adhesion. The overwhelming consensus from studies using drugs which disrupt microfilaments (343) is that the presence of intact stress cables is essential for the maintainence of firm contact with artificial substrata. For example, administration of cytochalasin B, at doses which do not affect sugar transport or cell division, disrupts actin stress cables in a host of cells without any effect on microtubules (344). Concomittant with loss of stress cables, cytochalasin-treated cells lose firm attachment (19), and in some cases, and can be removed from the surface with mild shaking (19). The effect of cytochalasin B on stress fibers can also be demonstrated in cells in intact tissues (345), though this is more difficult than in isolated cells in culture. Moreover, agents which promote adhesion in some cells, such as cyclic AMP (346), also promote the appearance of stress cables. Finally, much evidence exists to show that viral transformation of many cells leads to a loss of actin stress cables concomittant with rounding of asymmetric cells both in culture (347,348) and in vivo (57). Of the many functional changes characteristic of the transformed state is a marked decrease in the ability of affected cells to adhere tightly to artificial surfaces

(339,389), again suggesting a possible cause-and-effect relationship between stress cable integrity and cell attachment. As will be discussed in detail later (see chapter 6), such alterations in cytoskeletal organization may be manifested in more profound changes in cell function, in particular in the control of cell growth. These results together strongly suggest that stress cables are intimate components of contact plaques, and implicate these cytoskeletal structures in the process of cell attachment.

In addition to the actin filaments which terminate there, two actin-related proteins have been identified close to or within focal adhesion plaques. Vinculin, a 130,000 dalton intracellular protein, is closely associated with adhesion plaque membrane (339). Using immunoferritin electron microscopic staining for vinculin in frozen cell sections of a series of nonmuscle and muscle cells, Geiger et al. (340) have demonstrated that vinculin is sharply localized close to the membrane at focal contact sites. On the basis of other studies on vinculin-actin filament interactions in vitro, these authors proposed that vinculin might anchor stress cables to the membrane in these regions of the membrane by serving to promote actin polymerization from these sites (348). Avnur et al. (348) have recently tested this hypothesis directly by showing that vinculin can bind to intact focal adhesion sites depleted of actin, and can promote actin bundle formation outward from these sites. Moreover, vinculin has been detected by immunofluorescence in contact sites in newly plated cells prior to the appearance of

plaque actin (349). These observations suggest that adhesion plaque vinculin may serve as nucleating sites for the formation and growth of actin stress cables in attaching cells.

The second actin-associated protein found in cell attachment sites is alpha-actinin. In addition to decorating stress cables along their lengths (57,348), this protein is concentrated at their insertion termini into the plasma membrane (348,350). To date, there is no experimental evidence to support any function of this protein in cell attachment; however, in light of its known role in myofilament attachment to the Z-disk (44), a similar role in stress cable insertion into the adhesion plaque is quite likely. Until recently, it has been impossible to biochemically analyze adhesion plaque sites because of difficulties in isolating these regions of the plasma membrane. Bornstein et al. (351) have partially succeeded in this regard by developing a method to prepare isolated, intact contact sites from plated cells by ripping off the rest of the cell with a stream of liquid. They have shown by immunofluorescence microscopy that the remaining membrane and cytoskeleton contains actin and alpha-actinin; furthermore, they have presented evidence that in addition to these two proteins, myosin is present. Whether myosin is present at focal contact regions in other cell types, and its role in cell attachment, remains to be tested.

The extracellular matrix is the other structural component thought to be involved in cell attachment. The extracellular matrix (ECM) is a highly complex, heterogeneous filament matrix,

composed primarily of fibrous proteins embedded in a hydrated polysaccharide gel, which cells in culture lay down upon plating onto artificial surfaces (44). In chemical composition and three-dimensional organization, the ECM of cultured cells strongly resembles the extracellular basement membranes of cells in vivo (44). In addition to crosslinked collagens and a number of proteoglycans and hyaluronic acid, the extracellular matrix is composed of the fiber-forming glycoprotein fibronectin. There has been much interest in fibronectin since it was discovered to be present in greatly reduced amounts on the surface of transformed cells in culture (339). As transformed cells adhere poorly to surfaces to which their nontransformed counterparts firmly attach, fibronectin has been implicated in the molecular mechanism of cell attachment (339).

Several lines of evidence support the hypothesis that extracellular fibronectin is involved in linking the cell membrane to the substratum. Upon secretion from the cell, fibronectin specifically binds to another component of the ECM, collagen Type I, which is a major component of the fibrous network of filaments in this matrix (389). Electron and immunofluorescence microscopy have demonstrated that fibronectin is present in abundance on the external surface of the plasma membrane (339). That fibronectin may be specifically associated with cytoplasmic microfilaments has been suggested from immunofluorescence studies which have shown overlapping patterns of surface fibronectin with underlying actin stress cables (339,383). Whether fibronectin interacts directly with actin is at present not known; however, the fact

that in most nontransformed cells, surface labelling generally has failed to reveal the presence of actin or other intracellular cytoskeletal proteins on the external aspect of the plasma membrane (382) argues against a direct interaction. Nevertheless, the observation that cytochalasin B leads to the release of cell-surface fibronectin (352) does support the evidence for some sort of interaction, direct or otherwise, between actin microfilaments and fibronectin. Perhaps the stongest indirect evidence for a functional role of fibronectin in cell attachement is the observation that addition of fibronectin to transformed cells restores their ability to attach to artificial surfaces (353,384), and addition of this protein to normal cells promotes stronger adhesion (354).

The question of whether or not fibronectin is specifically associated with focal contact regions is still unsettled. Electron microscopic evidence supports both arguements for (356) and against (355) localization of fibronectin at adhesion plaques. Several immunological studies suggest that surface fibronectin spatially corresponds to vinculin, an intracellular cytoskeletal protein that is greatly enriched in regions of actin-membrane interaction (339). Moreover, studies of the distribution of fibronectin and focal contact proteins indicate that well-spread nonmotile cells show more correspondences among the patterns of these proteins than do cells early in spreading (357). These observations have led Hynes and Destress (220) to propose that there are two classes of focal cell-substratum contacts: one of which contains little or no fibronectin, and one

of which contains fibronectin. They propose that it is the latter class which mediates cell adhesion and spreading of a cell through alignment of actin filaments and surface fibronectin. Avnur and Geiger (359) have expanded this hypothesis. They have proposed that fibronectin-negative focal plaques represent transient contacts made upon initial contact with the surface. In their scheme of cell attachment, fibronectin initially promotes cell adhesion at contact regions of the attaching cell. As the cell spreads and flattens onto the surface, fibronectin is cleared from adhesion plaques and vinculin becomes associated with these regions. With time, cytoskeletal actin fibers elongate centripetally from contact regions to form stress fibers. Cell flattening is the final result of stress cable expansion into the cytoplasm. Though this model of cell attachment is still quite speculative, it does explain the observed relationships between actin, fibronectin, vinculin and focal contacts. Furthermore, the sequence of interactions proposed in this model matches the the formation and distribution of stress cable relative to contact sites seen in living cells as a function of time after plating (339).

Major questions still remain as to the precise nature of cytoskeletal-extracellular matrix interactions involved in cell adherence. In contrast to the erythrocyte membrane, virtually nothing is known about the integral membrane proteins and glycoproteins which most likely serve to link intracellular cytoskeletal proteins to extracellular matrix components. The significance of alpha-actinin and myosin in the region of

adhesion plaques, and the intriguing possibility that these proteins are involved in the underlying molecular mechanism(s) of movement during cell attachment, remains unresolved. Neverthelss, the experimental evidence so far strongly implicates both intracellular and extracellular cytoskeletal structures in the process of cell adhesion. As we will see in the final section of this review, cytoskeletal-membrane associations involved in contact with the substratum in turn play a crucial role in the process of cell locomotion.

## Cell Locomotion

In addition to many, diverse subcellular motile events involving the plasma membrane - ciliary and flagellar movement, surface receptor capping, pinocytosis and phagocytosis, cytokinesis - the ability of some cells in culture to migrate across artificial surfaces depends upon movements of the plasma membrane which seem to be under cytoskeletal control. In contrast to the rapid motions in such activities as cytoplasmic streaming and ciliary beating, cell locomotion presents as a series of relatively slow movements of the plasma membrane accompanying changes in the shape of the cell body in the process of forward advancement. When fibroblasts are allowed to settle onto a suitable substratum, they rapidly attach, flatten and begin to migrate. Locomotion begins with extension of regions of the plasma membrane called "lamellipodia." Each lamellipodium independently detaches from the substratum, lifts off the surface, waves about in the medium, and finally reattaches to the

surface in a new orientation. This scenario is repeated until one lamellipodium eventually dominates. This lamellipodium becomes what is termed the "leading edge" of the locomoting cell, and determines the direction of cell migration. Upon establishment of the leading lamellipodium, the cell body rapidly contracts in the direction of this lamellipodium. Eventually, the trailing edge of the cell detachs from the substratum and retracts in the direction of the leading edge of the dominant lamellipodium. In some cases, detachment is not complete and thin, stretched strands of cytoplasm extending from the attachment site to the advancing cell are formed (360). These strands eventually snap leaving small bits of the cell behind. The leading edge of a lamellipodium often shows characteristic undulations of its membrane or "ruffles" (361) as well as finger-like projections called "filopodia" or "microspikes" (362). These extend and adhere to the substratum, acting as guides for the advance of the lamellipodium.

Fibroblast locomotion has been analyzed using interference reflection microscopy (57). These studies show that locomoting cells contact the substratum at discrete points on their ventral surfaces. Three catagories of contact can be identified; small, discrete focal contacts separated from the substratum by 10-15 nm which correspond to the adhesion plaques found in nonmotile cells (363); broader areas of contact (30nm separation); and regions of greater (100-140nm) separation (364). From light microscopic analyses of locomoting fibroblasts (237), it is clear that the adhesion plaques are the only class of contacts which are stable

throughout the process of locomotion. These focal contacts are found concentrated at the peripheral edges of extending lamellipodia and filopodia, and in small areas at the posterior of the cell (342). Focal contacts are stationary relative to the cell body as the cell migrates over the area of these contacts; however, net movement is accompanied by formation of new areas of contact at the leading edge of the cell and disappearance of old contacts at the trailing edge (364).

Detailed analysis of the leading edge of migrating cells has been difficult due to the transient nature of this region of the cell membrane. Recently, procedures have been developed to prepare specimens of the leading edge which retain the characteristic cytoskeletal matrix associated with the leading edge membrane (365). These protocols utilize Triton X-100 extraction, glutaraldehyde fixation and negative staining to remove the overlying plasma membrane to reveal an underlying detergent-insoluble meshwork of microfilaments. This network is clearly distinct from the inner cytoskeleton when viewed both in electron micrographs of sectioned cells (237) and in immunofluorescent images of cells stained for actin, alpha-actinin and filamin (237,363). Within the leading edge, actin filaments predominate; intermediate filaments are totally excluded from this region (237). Microtubules, although absent from the anterior portions of the leading edge, are often seen emanating from the interior of the cell body and penetrating into the posterior aspect of the leading edge (237). Actin here is found in two organizations: actin-filament bundles within the filopodia

seen in the light microscope (362), and an actin meshwork adjacent to the plasma membrane (237). This meshwork extends throughout the body of the leading edge as a random array, but at the anterior border of the leading edge, meshwork filaments converge at specific foci that exhibit extra particulate material attached to the filaments (237). This same material is found at the tips of the microspikes (237).

Chemical characterization of leading edge proteins has identified many of the cytoskeletal components found in other motile organelles. In conjunction with immunofluorescence studies using antibodies to specific proteins, the leading edge and membrane ruffles of cultured cells have been shown to contain alpha-actinin (366), filamin (367), and fimbrin-crossreactive proteins (368). Other immunological studies have reported an absence of trpomyosin from membrane ruffles and filopodia, and the presence of vinculin-immunoreactive protein within discrete regions of the leading edge which correspond to the focal contact points observed by interference reflection microscopy (366). Myosin, although present throughout the cytoplasm decorating actin stress fibers at the ventral surface of the cell (57), is notably absent from the actively motile membrane areas of filopodia and membrane ruffles (369). There has been much speculation that these actin-associated proteins comprise the particulate material found associated with actin filaments at the anterior aspect of the leading edge (237).

The presence of cytoskeletal structures in the advancing edge of the migrating cell immediately suggests their involvement

in the underlying molecular mechanism(s) by which the cell locomotes. The observation that microtubules do not invade filopodia and lamellipodia (367) suggests that microtubules do not have a direct role in generating the forces that move the cell. Numerous studies with colchicine and other microtbule-disrupting drugs have shown that destruction of microtubules does not prevent membrane ruffling or cell extension; however, treatment causes these processes to beome randomized and as a result, directed migration is lost (370). The general consensus is that if microtubules are involved in cell locomotion, they function to define the direction of application of forces produced by other cytoskeletal structures (57).

Though much is known about the composition and structural organization of those parts of the cell engaged in locomotion, it has been difficult to define the underlying mechanism(s) which results in cell movement. Functionally, locomotion has been divided into several processes - filopodial extension, lamellipodial advance, and retraction of the trailing edge of the cell - each of which possibly involves different mechanisms for force generation. Several models for cell locomotion have emerged, each based on a different mechanism found in other motile systems. These schemes include involve membrane insertion models, gel contraction models, shearing and sliding filament models, and actin filament polymerization models. Though distinct, these models are by no means mutually exclusive; in fact, models for particular motile events involved in cell locomotion often combine several of these mechanisms to explain

the membrane movements observed.

Membrane insertion models propose that filopodial extension occurs as a result of insertion of new membrane components into the leading edge of the cell (371). Membrane ruffling in turn occurs from insertion of excess membrane into the advancing lamellipodia. These models are based on known fluid properties of the membrane lipid bilayer in which membrane components move freely within the membrane (229). In these models, membrane components are inserted into the leading edge membrane, then flow back over the cell surface to a "sink" at the posterior of the cell where they are removed and recycled (372). In one variation on this theme (373), membrane components are transported to the leading edge by actin filaments in conjunction with myosin, which are are oriented to direct transport to the advancing edge. Though these models address the mode of leading edge extension, most all but ignore the obvious presence of cytoskeletal structures.

Gel-sol transformation or contraction models implicate networks of actin filaments in the molecular modus operandi of cell movement. In these models, extension of lamellipodia and filopodia in the leading edge of an advancing cell results from the creation and subsequent contraction of actin gels within these regions which in effect "squeeze" the cytoplasm forward (374). Membrane ruffling would result from internal pressure of moving cytoplasm against the plasma membrane facing away from the substratum. This mechanism has been proposed in many diverse model systems for locomotion, most notably Mast's model for

locomotion in giant amoebas (375). Experimental support for these models comes from observations that cell regions such as lamellipodia which are actively engaged in motility are packed with actin filament networks (19,57). As such networks have been shown to produce gels which can contract under appropriate conditions in vitro (63), these models assume cytoplasmic movement is initiated by directed contraction of these networks in vivo. These models take into account both the presence of actin filaments and actin-associated proteins in the leading edge of the cell. Moreover, models based on actin filament contraction can explain the disappearance of actin filament bundles and the appeareance of actin filament networks in the trailing region of locomoting cells prior to contraction of the posterior of the cell (237). However, the observation that myosin or myosin-like molecules are absent from this region of the cell weakens any arguement for a myosin-based contractile mechanism. Though actin-filamin gels have been shown to shrink in the absence of myosin (376), whether or not such contraction occurs in vivo is still unsettled.

The possibility exists that actin networks in motile regions of the plasma membrane do in fact contain nonmuscle myosin, albeit in trace amounts which are below the level of detection of available procedures. In other motile organelles which contain actin filaments, myosin is commonly found in greatly reduced amounts relative to the actin (398). Actually, models based on actomyosin-catalyzed microfilament contraction are very attractive, for they would explain several observations

concerning in vitro gelation. For example, gels formed with either cytoplasmic extracts or purified F-actin and gelation factors anatomically resemble networks of microfilaments frequently identified in other motile structures, such as microvilli, which do contain nonmuscle myosin (57). Furthermore, most cytoplasmic extracts induced to gel are found to contain traces of myosin (377). Moreover, mixtures of pure actin and actin-binding protein or fluid cell extracts will contract when purified myosin is added (162). Pure muscle actin and myosin can be induced to form large networks of filaments in vitro (379). Upon appropriate stimulation, these networks can be further induced to contract to produce thin filaments which will then actively locomote across an artificial surface in a manner similar to the rotation of microfilament bundles from Nitella cells in vitro. Though such observations strongly suggest that an actomyosin-based contractile mechanism could function in vivo to produce cytoplasmic gelation, the final word on whether this is in fact the case awaits further study.

The presence of abundant actin filaments and actin-associated proteins in the motile areas of the cell leaves little doubt that microfilaments are in some way involved in the motile events during locomotion. This conclusion is strongly supported by functional studies using drugs such as cytochalasin B and phalloidin (63) to show that alterations in actin filament organization coincide with disruptions in cell locomotion. For example, administration of cytochalasin B to locomoting fibroblasts in culture inhibits locomotion and membrane ruffling

(49,236). This inhibition is completely reversed upon washout of the drug. The fact that cytochalasin B treatment specifically disrupts actin stress cables and inhibits actin polymerization in such cells led Small et al. (365) to propose that controlled polymerization of actin might be the underlying mechanism in forward extension of the leading membrane of locomoting cells. In their model for lamellipodial extension, forward growth of the leading edge of the migrating cell involves inward polymerization of actin filaments, initiated at the focal contact regions found on the ventral surface of the leading edge. Concomitant with filament growth is cross-linking of actin filaments by actin-associated proteins (e.g., filamin and alpha-actinin) to form a temporarily stable network. Uplifting of the leading edge off the substratum and forward lamellipodial extension then results from traction of actin filaments against immobilized focal contact points. Several observations support this hypothesis. First, controlled polymerization has been shown in other systems to be responsible for specific organelle movement, such as the formation and extension of the acrosomal process in Limulus sperm (275). Second, in addition to its effect on cell locomotion, cytochalasin B is known to specifically disrupt cytoplasmic stress cables, the same actin structures which insert into focal adhesion plaques (408), suggesting that inhibition of locomotion by cytochalasin B involves these cables. Furthermore, decoration of actin filaments within the leading edge of locomoting cells with heavy meromyosin clearly demonstrates a uniform polarity of these cables in one direction relative to the contact sites into

which they insert (365). The direction of these actin filaments in the leading edge predicts that filament growth at the "barbed" ends of the filaments would lead to addition of actin subunits to those tips which contact the anterior edges of the lamellipodium. Therefore, growth of actin filaments should be in the same direction as that seen for lamellipodial extension. In this case, the prediction in the direction of membrane movement matches that actually found.

Based on the structure and function of cytoskeletal elements in the muscle sarcomere, Huxley et al. (380) have proposed still another model for cell locomotion. They suggest that a shearing force between anchored actin bundles and myosin in the cytoplasm might be responsible for filopodial and lamellopodial membrane movements. They suggest that the polarity of actin filaments in the leading edge is such that filament shearing would shift microfilaments anchored to the trailing edge membrane forward relative to leading edge filaments, and that this motion would essentially pull the cytoplasm in the rear of the cell forward toward the leading edge. Variations on this theme suggest that sliding of actin filaments anchored at focal contact plaques in the posterior region of the advancing cell slide past stress cables anchored in the leading edge of the cell in such a way that trailing edge membrane is gathered up in the direction of the leading edge (363). There is some evidence for focal contact regions in the rear of the migrating cell into which a second set of microfilaments might insert (342). Such models take into account the presice orientation of actin filament bundles

relative to one another. However, they fail to explain how filament sliding takes place, especially in motile regions of the cell where actin-associated proteins such as myosin and tropomyosin are either absent or in very small amounts. There has been some suggestion that sliding of actin filaments past one another might be mediated by non-myosin proteins of undefined nature (57). Alternatively, the motive force for filament sliding may be via actomyosin associations occurring at a distance from the site of membrane motion. This has been proposed for amoeboid psedopodia which lack detectable myosin internally but which are rich in myosin (as well as actin) at their bases (63).

Each of the models for cell locomotion presented here has both strong and weak points. Models based on actin filament sliding can only explain pulling of the rear of the cell forward, but cannot explain forward membrane extension, for these filaments cannot exert force ahead of their attachment points (57). Moreover, the polarity of actin filaments near the plasma membrane is such that any models based on microfilament sliding can only account for retraction, but not extension, of membrane. Models for lamellipodial extension based on polymerization of actin filaments run into problems explaining how actin monomers can be added to filament ends that are apparently attached to the membrane. Finally, none of the models put forth to date attempts to explain the control of cell locomotion.

Cell locomotion is an excellent topic upon which to end this review of the cytoskeleton, for the process by which a cell migrates through its environment encompasses many of the other motile events discussed earlier. Like the other cell processes mentioned throughout this review - cell attachment, receptor capping, ciliary motion, cytokinesis, et cetera - cell locomotion represents a complex interplay of cytoskeleton and plasma membrane. In every instance, cytoskeletal filaments interact with one another to produce motions which are manifested in movements of the attached cell membrane. The study of the molecular mechanisms underlying these motile events has just begun, and it is now exploding in every direction. Yet what is already emerging from this work is a deeper appreciation for the cytoskeleton, not as a static structure, as the name originally implied, but as a dynamic system of interconnected, interacting components which defines and controls these diverse motile activities of the cell.

# CHAPTER 2

# INTRODUCTION TO ACTH AND THE RAT ADRENOCORTICAL CELL

## The Hormone: Structure and Function of ACTH

Corticotropin, or $ACTH_{1-39}$, is a polypeptide hormone produced in and secreted from the anterior pituitary. The naturally isolated hormones from ovine, porcine, bovine and human sources are all composed of thirty-nine amino acids with identical $NH_2$- and COOH-terminal amino acid residues (406). Differences in amino acid sequence reside exclusively within the region of the molecule between residues 24 and 33. The COOH-terminal of the portein contains all of the acidic residues of the molecule, and the highly basic core of residues 15-18 is responsible for the highly basic isoionic point of the protein. By a variety of physicochemical parameters, ACTH appears to exist in solution as a random coil.

By far the most well-characterized action of ACTH on the adrenal is the rapid stimulation of adrenocortical steroidogenesis. Structure-function studies using a variety of natural and synthetic ACTH analogs have revealed several notable chemical characteristics concerning this activity. The amino acid residues near the carboxy terminus (positions 24-34) do not appear to be necessary for full biological activity, an observation which is consistent with the notable heterogeneity of residues within this region observed among ACTHs from different sources (406). Segments of the native molecule lacking residues in this region are full agonists of ACTH in terms of

steroidogenesis in cultured adrenocortical cells and in vivo, but must be used at much higher concentrations to produce stimulation comparable to the native protein (407). It has been suggested that the carboxyl region of ACTH is important in vivo in conferring stability of the protein to degradative enzymes perhaps by promoting the binding of hormone to plasma carrier proteins (408). Experimental analysis of in vivo half-lifes of ACTH analogs supports this view (408). The COOH-terminal region of the molecule is thought not to be involved in the interaction of hormone with its adrenocortical receptor as $ACTH_{1-19}$ is equipotent with the native molecule in stimulating steroidogenesis in isolated adrenocortical cells (408).

The structure of the basic core of residues 15-18 has been extensively analyzed in relation to steroidogenic activity (406). Studies using ACTH analogs which lack this region entirely have demonstrated that this region is not essential for activation of steroidogenesis. Studies in which the net charge of the protein was varied by specific residue substitutions in the 15-18 region have clarified that it is the basic nature of this region, and not the particular amino acids, which is important in conferring full biological activity. Subsequent binding studies using radiolabelled ACTH analogs as ligands have suggested that the lysyl-arginyl-arginyl-arginyl sequence is important for binding of the hormone to adrenal receptor. It appears that this region is important in an electrostatic interaction of hormone with receptor. As this region alone has no intrinsic biological activity, the binding of this hormone to its target receptor must

be distinguished from the transduction of information leading to the biological response, and other regions of the protein have been analyzed in search of that specific region containing the "signal transducing" information.

Alteration of the $NH_2$-terminal serine residue drastically reduces the biological activity of ACTH (408). Synthetic peptides containing residue substitutions in positions 1,2,3,4, and 5 have been used to show that although the $NH_2$-terminal pentapeptide is not essential for biological activity, it is important for maintaining full biological activity within the physiologically relevant range of hormone concentrations. Though all of these analogs are able to maximally stimulate steroidogenesis to the same extent as the native hormone, the affinities for the receptor are altered in parallel to the rank order of potency, as assayed by the concentration of analog required for half-maximal stimulation ($ED_{50}$). Substitution experiments in which the serine residue in position 1 was replaced with a variety of L- and D-amino acids have suggested that the alpha-amino group of this residue is involved in attachment of ACTH to its receptor.

A similar approach has been taken to analyze other regions of the molecule for intrinsic biological activity. Alterations in any of the residues between 6 and 14 significantly lowers the $ED_{50}$ value without changing the maximal biological response achievable with higher doses of the analog. As alterations in hormone affinity would explain reduction in biological activity without alteration in maximal response achievable, these results again suggest that this region is involved in binding of hormone

to receptor. Early binding studies (410) of radiolabelled ACTH analogs with subsitutions in this region to bovine adrenal homogenate fractions have supported this conclusion by showing that substitutions in this region alter the apparent affinity of the peptide to its putative receptor. Segment 6-9 appears to be more important than segment 11-13 in conferring biological activity to the hormone since segments 11-19 or 11-24 alone are unable to stimulate steroidogenesis, whereas segment 5-10 alone can. These results, taken together, support the conclusion that although no single amino acid in the native molecule is essential for biological activity, the residues 6-13 are very important in conferring full biological activity to the molecule.

The structure-function relationships have been the basis for a model for the molecular mechanism of ACTH-receptor interaction (406). In this model, the initial interaction of hormone with its adrenal receptor involves an electrostatic interaction of residues 15-18 with some region of the receptor, followed by augmentation of attachment involving residues 1-5. Binding of these regions then allows proper alignment of 6-13 region with the receptor such that transduction of the information resulting in stimulation of steroidogenesis can occur. The function, then, of the basic core of 15-18 is viewed as the means by which the 6-9 segment is targeted to the proper receptor site for transmission of the biological signal.

## The System: The Rat Adrenocortical Cell in Culture

Primary cultures of isolated adrenocortical cells from rat, bovine, and human sources, as well as a cell line of mouse adrenal tumor cells, have been established in culture for the study of the mechanism of action of ACTH on this principle target cell (411). Cultures of ovine and rabbit adrenocortical cells have also been grown (our laboratory, unpublished results). Protocols for the isolation and maintainence of adrenocortical cells from different sources are all slightly different but essentially involve isolation of intact adrenals, separation of capsular and cortical glomerulosa cells from medullary and cortical fasciculata and reticularis cells, and dispersal of the latter cells by enzymatic digestion. In the case of rat adrenocortical cells, the inner two zones of the adrenal cortex cleanly separate from the zona glomerulosa and capsular cells with mechanical scraping. Cultures of rat adrenocortical cells can be maintained for weeks in plastic culture dishes or on extracellular matrix-coated dishes, provided that the complex medium used (Medium M199 containing 10% fetal bovine serum) has been adjusted to retard fibroblast growth. The details of culturing conditions have been reported elsewhere (411).

Rat adrenocortical cells in culture retain the morphological and functional characteristics of fully differentiated fasciculata/reticularis cells for up to one week. Soon after plating, cells attach to the substratum, assume a polygonal shape, and continue to grow slowly until a highly confluent monolayer of cells is reached. At the electron microscopic level,

these cells exhibit the complex Golgi and smooth endoplasmic reticulum, and numerous lipid inclusions and mitochondria, characteristic of steroid-secreting cells (412). Throughout the first week in culture, these cells continue to produce and secrete corticosterone at a basal rate, and respond to physiological doses of ACTH by a 50 to 150-fold stimulation of steroid production and an equivalent increase in cyclic AMP production (413). Steroidogenic sensitivity to ACTH persists undiminished for the first 4-7 days in culture, then decays progressively over the next week; at the end of the second week in culture, the cells have lost all sensitivity to ACTH (413). Coincident with loss of steroidogenic sensitivity is a parallel decay of enzymatic activity of several of the enzymes involved in steroid biosynthesis (413). Full hormonal sensitivity can be regained by incubation of cells with physiological doses of ACTH; reappearance of sensitivity again is coincident with resumption of steroid biosynthetic enzymatic activity, most probably due to hormonal induction of synthesis of biosynthetic enzymes (414).

In addition to the marked sensitivity to ACTH in terms of steroidogenesis and cAMP production, isolated rat adrenocortical cells exhibit other ACTH-dependent cellular activities. These include inhibition of DNA synthesis and cell growth, morphological transformations which include changes in mitochondrial and endoplasmic reticulum ultrastructure, and alterations in cell shape (412). These cells also display hormone-induced desensitization of the steroidogenic response upon incubation of cells with ACTH for several hours (415). Many

of these effects are also produced in vivo when animals are administered ACTH (412). The observation that hormone-specific effects in vivo can be reproduced in isolated adrenal cells strengthens the arguement that these cells in culture retain and reflect the state of mature, fully differentiated adrenocortical cells in culture.

## The Biological Actions of ACTH

The principle actions of ACTH on the cells of the adrenal cortex involve stimulation of steroid and cAMP production. In both the intact adrenal gland and with isolated adrenocortical cells, these events occur very soon, i.e., within minutes, after hormone administration, and have been termed "tropic" actions of the hormone. In contrast, the other effects of ACTH mentioned, including hormonal induction of biosynthetic enzymes, maintainence of the differentiated state in culture, and effects of adrenocortical growth, are responses to ACTH which require much longer times, i.e., hours to days, of hormone treatment to be manifest. These are the so-called "trophic" actions of ACTH.

## Steroidogenesis, Adenylate Cyclase, and ACTH

It has been known for many years that ACTH can stimulate the rate of corticosterone production to more than one hundred times that of the basal rate in both the intact rat adrenal and in isolated rat adrenocortical cells (411,416). A similar stimulation can be produced by administration of exogenous cAMP in the absence of ACTH (417). Detectable increases in steroid

synthesis can be demonstrated within three minutes after hormone administration, and rates of steroid production reach maximal levels within the first 15 minutes of treatment (417). Coincident with increased steroidogenesis is activation of adenylate cyclase, and an increase in cAMP production. Studies using adrenal plasma membrane preparations have demonstrated that binding of ACTH to its presumptive receptor in these preparations directly stimulates membrane-associated adenylate cyclase activity (418).

The precise relationship between hormone stimulation of cAMP production and stimulation of steroidogenesis has not been resolved; however, since the early demonstration that cAMP can stimulate adrenal steroidogenesis in the absence of ACTH (406), considerable evidence has accumulated in favor of the concept that cAMP mediates the effects of ACTH on steroidogenesis (420). More recent studies using the o-nitrophenyl sulfenyl derivative of ACTH (NPS-ACTH) have stimulated re-evaluation of this hypothesis. This analog, in which the single tryptophan residue in the molecule has been modified, is a full agonist of ACTH in stimulating steroidogenesis both in hypophysectomized rats and in isolated rat adrenocortical cells, yet is only able to maximally stimulate cAMP production to only 3% that of the native hormone. The finding that NPS-ACTH is able to maximally stimulate adrenal steroidogenesis while only marginally increasing cAMP levels suggests that if cAMP is the intracellular mediator of ACTH action on steroidogenesis, then only a fraction of that cAMP potentially available to the cell is necessary for this function.

This leaves the rest of the cAMP produced in response to ACTH unaccounted for in terms of function.

Recent binding studies (419) have shown that although binding of ACTH is tightly coupled to stimulation of adenylate cyclase, there is a marked dissociation of hormone binding from stimulation of steroidogenesis, and again, only a fraction of available ACTH receptors need be occupied to achieve maximal rates of corticosterone production. For this reason, at this time it is premature to conclude that cAMP serves as the intracellular "second messenger" of this ACTH action.

The underlying molecular mechanism of action of ACTH stimulation of adrenal steroidogenesis has been the goal of many investigations. To date, the details remain elusive; however, numerous reports have uncovered major pieces of this puzzle. Several studies have demonstrated that ACTH acts to increase the availability of substrate for steroid biosynthesis to the intracellular biosynthetic factory (421). Garren (417) has obtained evidence that ACTH stimulates the formation of free cholesterol from lipid within cytoplasmic lipid droplets, and promotes the movement of de-esterified cholesterol from these lipid depots into mitochondria where steroid biosynthesis begins. Release of cholesterol from the lipid droplet involves hormonal activation of the cholesterol esterase resopnsible for the de-esterification of stored cholesterol. ACTH has also been found to control the activity of a cholesterol transporting protein (417). Furthermore, ACTH has been shown to effect both acute and long-term stimulation of mitochondrial side-chain-cleavage cytochrome

P-450 activity (422), possibly by regulating the interaction of adrenodoxin reductase, adrenodoxin, and cytochrome P-450 components of this enzyme system (423). As this enzyme complex is responsible for the first and rate-limiting step in steroid biosynthesis, i.e., the cleavage of cholesterol to pregnenolone, ACTH may regulate the level of steroidogenesis at this level. ACTH also affects the rate of several of the hydroxylations involved in steroid biosynthesis by altering the availability of NADPH to these enzymes (417). This suggests another locus for hormonal control of steroidogenesis.

The finding that inhibitors of protein synthesis can block ACTH stimulation of steroidogenesis (412) has led to the hypothesis that hormonal stimulation of adrenal steroidogenesis is mediated by some protein(s) whose synthesis is induced by ACTH (417). Administration of cycloheximide to ACTH-treated rats at concentrations which completely block protein synthesis results in rapid decay of the steroidogenic response with a $t_{1/2}$ of 10 minutes, suggesting that if such a protein regulator exists, it must turn over extremely rapidly. Other inhibitor studies have clarified that ACTH stimulation of steroidogenesis requires protein but not RNA synthesis (424).

Though the identity of this proposed mediator remains elusive, at present several candidate adrenocortical proteins have been identified whose sythesis is stimulated by ACTH (425,426,427). In addition to several of the biosynthetic enzymes including the 3-beta-hydroxysteroid dehydrogenase and several of the mitochondrial enzymes, ACTH has been reported to induce the

synthesis of several cytosolic adrenal proteins (426). Using perfused cat adrenal glands, Rubin et al. (428) and Laychock and Rubin (429) have shown that ACTH specifically stimulates the production of two proteins (MW of 12,500 and 70,000). Unfortunately, the intraadrenal source of these proteins was not determined. Using a dual labelling technique to catalogued proteins whose rates of synthesis change with ACTH treatment, Dazord et al. (425) have shown that ACTH markedly stimulates a 30,000 dalton protein in isolated intact rat adrenals within four hours of hormone administration. However, as ACTH had no evident effect on the synthesis of this protein earlier than 2 hours after hormone administration, it is unlikely that this protein directly mediates ACTH stimulation of steroidogenesis, at least at early times of stimulation. Dazord et al. (426) have reported that $ACTH_{1-24}$ stimulates the synthesis of a small peptide (MW 3500). Finally, Nakamura et al. (430) have presented evidence that ACTH specifically promotes the synthesis of two cytoplasmic proteins (MW 27,000 and 13,000) in Y-1 mouse adrenal tumor cells which become associated with adrenocortical mitochondria soon after synthesis, and which are in some way involved in ACTH-induced increases in pregnenolone production within the mitochondria.

If such a putative protein mediator of hormone-induced steroidogenesis does exist, its mechanism of action also remains elusive. One observation which suggests one possible mode of action is that cycloheximide inhibition of protein synthesis blocks the conversion of cholesterol to pregnenolone without

affecting other steps in the steroid biosynthetic pathway downstream from this event (412). In light of this observation, one locus of action for this protein might be the regulation of cholesterol transport to the site of the side-chain-cleavage enzyme in the mitochondria.

The observation that exogenous cAMP can stimulate adrenal steroidogenesis in the absence of ACTH, and that hormonal stimulation of cAMP production parallels stimulation of steroidogenesis, suggests that cAMP is in some way involved in the mechanism of ACTH action. The identification and characterization of cyclic AMP-dependent protein kinases in the adrenal cortex (431) has led to a search for ACTH-dependent phosphorylation products as possible mediators of hormone action. Cyclic AMP binding protein has been isolated from the adrenal which stimulates the activity of endogenous protein kinase(s) (417). This kinase can also phosphorylate exogenous proteins in vitro (450). Protein kinase activity has been found in adrenal microsomal fractions rich in smooth endoplasmic reticulum (Garren review). This reticulum is the site of several of the steroid biosynthetic enzymes, suggesting that kinase activity resides with these enzymes. There is some evidence, in fact, that some of the biosynthetic enzymes can be phosphorylated, and that phosphorylation leads to stimulation of enzymatic activities (451). Endogenous phosphorylation of the colesterol-ester hydrolase with a consequent increase in hydrolase activity has also been demonstrated (432). Other studies have reported phosporylation of subcellular proteins in broken cells of

adrenocortical tissue (433). Podesta et al. (427) have reported that ACTH induces phosphorylation of a cytoplasmic protein of 150,000 daltons in isolated rat adrenocortical cells. The time course of phosphorylation of this protein parallels both the stimulation of steroidogenesis and the increased binding of cAMP to endogenous protein kinase produced in response to ACTH. These authors have proposed that phosphorylation of this protein mediates the effect of ACTH on adrenal steroidogenesis. The effect of ACTH on phosphorylation of this protein could be mimicked by treatment of the cells with exogenous cAMP in the absence of ACTH, further supporting a role of cAMP in mediating the effect of ACTH on steroid production. Conclusive proof for mediation of protein phosphorylation in the action of ACTH on adrenal steroidogenesis awaits further investigation.

In addition to its ability to mimick ACTH stimulation of steroidogenesis, cAMP is also able to produce many of the trophic actions of ACTH in the absence of the hormone. Kahri (412,424) has provided electron microscopic evidence that cAMP can produce many, but not all, of the morphological transformations observed in adrenocortical cells upon treatment of the intact adrenal with ACTH. Cyclic AMP can also maintain adrenal weight, and DNA, RNA, and protein contents in the absence of ACTH when administered to hypophysectomized rats (416). Cyclic AMP has been reported to induce rat and human adrenocortical cell rounding similar to that observed with ACTH (434). Masui et al. (477) have shown that mutants of Y-1 mouse adrenal tumor cells are resisitent to the toxic effects of high concentrations of dibutyryl cyclic AMP seen

in the parent cell line. These mutants appear to have lost the normal Y-1 cell response to cAMP-dependent phosphorylation as well, suggesting that protein phosphorylation, mediated by cAMP, might be important in the resistance of these cells to the deleterious effects of this nucleotide on cell growth and survival. Taken together, these observations implicate cAMP in the regulation of these trophic actions of ACTH.

## The ACTH Receptor

Though many attempts to demonstrate specific ACTH binding to adrenal tissue and to isolated adrenocortical cells have been made, only recently has the characterization of ACTH-receptor interactions been successful. Early attempts to demonstrate binding, and to correlate binding to biological activity, i.e., to stimulation of steroidogenesis and to activation of adenylate cyclase, were hampered by difficulties in producing labelled ACTH probes which retained full biological activity (435). Binding studies using these labelled ACTH probes yielded varying estimates of receptor capapcity and affinity for hormone. Recently, an ACTH analog was developed in our laboratory which retains full biological activity, relative to the native molecule, when iodinated (449). With this probe, Buckely and Ramachandran (419) have thoroughly characterized ACTH receptors on isolated rat adrenocortical cells. Their results clearly demonstrate one class of high affinity ($K_d$ = 1.4nM) receptors of low capacity. Binding is hormone-specific, rapid, and completely reversible. Moreover, though binding of labelled analog directly

correlates with stimulation of cAMP production, maximal stimulation of steroidogenesis is achieved at less than 3% receptor occupancy. This obvious dissociation between the binding of ACTH and stimulation of cAMP production, and activation of steroidogenesis, once again demands a re-evaluation of the hypothesis that cAMP mediates the effect of ACTH on steroidogenesis. The precise interaction of ACTH with its receptor, and the interaction of this receptor with membrane-associated adenylate cyclase, awaits further study.

## ACTH Trophic Actions

In addition to stimulation of steroidogenesis and activation of adenylate cyclase, ACTH plays an important role in maintaining the differentiated state of the adrenocortical cell. These trophic actions of ACTH include the maintainence of steroidogenic function through regulation of steroidogenic enzymes, and effects on adrenal growth through control of DNA and protein synthesis.

## ACTH and Effects on Adrenal Growth

It is well documented that ACTH is able to prevent the extensive atrophy of the cells of the adrenal cortex which occurs in animals after hypophysectomy (452). Prolonged administration of ACTH to animals increases adrenal weight (436). Chronic administration of ACTH promotes an increase in adrenal DNA content relative to untreated glands after 4-5 days of treatment, demonstrating that ACTH stimulates cell proliferation in this gland (486). Moreover, ACTH increases incorporation of $^{3}H$-

thymidine into nuclear DNA of quartered adrenal glands (417), confirming a stimulatory effect of this hormone on the adrenal cell cycle as well. Stimulation of DNA synthesis is seen after 16 hours of ACTH adminsitration, and continues to rise through 48 hours of treatment after which no more stimulation occurs. In the case of compensatory adrenal growth in unilaterally adrenalectomized animals, it has been shown that administration of specific ACTH antiserum has no effect on the increase in DNA content and cell proliferation of the remaining adrenal, suggesting that the increase in adrenal size in this case is mediated by some factor(s) other than ACTH (437).

Paradoxically, the effect of ACTH on adrenocortical growth in isolated cells seems to be opposite in effect. ACTH has been shown to inhibit DNA synthesis in normal rat adrenocortical cells in culture (438) and in Y-1 mouse adrenal tumor cells (439). Direct measurement of cell number has confirmed that ACTH inhibits the proliferation of adrenocortical cells in culture (440). Interestingly, inhibition of cell proliferation parallels the decrease in $^{3}$H-thymidine incorporation in response to ACTH, and occurs over the same range of hormone concentrations as that required for stimulation of steroidogenesis (438).

The explanation for the antagonistic effects of ACTH on adrenal growth in vivo and on isolated adrenocortical cells in culture have not been fully resolved, but several observations may have some bearing on this issue. Though ACTH increases adrenal DNA content upon chronic administration in vivo, the effect of ACTH on adrenal weight may be indirect as other

evidence (441) suggests that ACTH causes hypertrophy rather than hyperplasia of the gland. Furthermore, those studies using isolated adrenocortical cells deal with the effects of ACTH on cell growth and division of a relatively pure population of adrenocortical cells, i.e., fasciculata/reticularis cells, whereas studies of the intact adrenal consider the effects of this hormone on a more heterogeneous population of adrenal cells. A differential effect of ACTH on other cell types in the intact adrenal gland might explain the observed increase in adrenal growth and DNA content, for the presence of non-responsive cells might mask the inhibitory effects of the hormone on the cells of the inner adrenal cortex.

ACTH Effects on Protein Synthesis

The maintainence of steroidogenic capacity during chronic exposure to ACTH also seems to be under hormonal control. Hypophysectomy results in rapid loss of ACTH sensitivity in parallel with decay of steroid biosynthetic enzyme activities (414). Studies with normal rat adrenocortical cells in culture have clearly shown that ACTH is necessary for the continued maintainence and synthesis of specific enzymes involved in steroid biosynthesis, including the 11-beta-hydroxylase, and the 18- and 21-hydroxylases (414). In the case of the 3-beta-hydroxy-steroid dehydrogenase isomerase (3-beta-HSD), decay of enzymatic activity in cultured rat adrenocortical cells progresses linearly over a period of ten days after isolation (413). However, full recovery of enzymatic activity is achieved by treating cells with

ACTH for more than 12 hours. Using an enzymatic stain to quantitate 3-beta-HSD activity, Liles and Ramachandran (413) have found that ACTH induces the activity of this enzyme. Induction of 3-beta-HSD activity was dependent on protein and RNA synthesis; moreover, the effect of ACTH could not be attributed to synthesis of some protein intermediate. Cycloheximide had no effect on the rate of decay of 3-beta-HSD activity in the presence or absence of ACTH, suggesting that the effect of ACTH was not due to a change in the rate of enzyme degradation. Pulse-labelling experiments of cultured rat adrenocortical cells treated with or without ACTH further suggested that ACTH stimulation of 3-beta-HSD activity was instead due to induction of synthesis of the enzyme.

In addition to the steroidogenic enzymes, ACTH is known to increase the activity of several of the enzymes involved in DNA synthesis. Chronic administration of ACTH to guinea pigs for 48 hours results in a four-fold increase over untreated animals in DNA polymerase I activity in crude adrenal extracts (417). ACTH also stimulates thymidine kinase activity in these same preparations. The increase in activity in these enzymes may be involved in the increase in DNA content of adrenals in vivo seen with ACTH treatment.

It is well established that ACTH in vivo stimulates total adrenal protein synthesis (436). In addition to its effects on adrenal DNA content, hypophysectomy results in inhibition of total protein synthesis in vivo; this inhibition can be overcome by administration of ACTH (417). The effect of ACTH on protein

synthesis in isolated adrenocortical cells is less clear-cut. Some studies have demonstrated that ACTH inhibits total protein synthesis (442), while others have claimed that ACTH stimulates total protein synthesis (443). Nevertheless, the involvement of ACTH in the maintainence of protein synthesis, and the importance of protein synthesis in adrenal function, is implied in the observation that hypophysectomy promotes both inhibition of total protein synthesis and adrenal atrophy coincident with loss of steroidogenic capacity .

Trophic Action of ACTH on Adrenocortical Morphology

The maintainence of steroidogenic capacity involves not only the continued synthesis of biosynthetic enzymes, but also the precise anatomical arrangement of subcellular organelles involved in steroid synthesis. As the enzymes involved in steroid biosynthesis are distributed between the mitochondria, the smooth endoplasmic reticulum, and the cytoplasm, it is not surprising to discover that ACTH has profound effects on the ultrastructural of all these organelles. As with ACTH effects on cell growth and protein synthesis, its effects on cell morphology are trophic actions, and require hours of hormone treatment to be manifest.

Kahri (412,424) has thoroughly studied the long-term effects of ACTH treatment on adrenal morphology. Using fetal rat adrenals as a model system, and the electron microscopy as a means of analysis, he has presented extensive evidence that ACTH promotes significant ultrastructural changes in the mitochondria and in the endoplasmic reticulum of adrenal cells in the zona

fasciculata. Micrographs of cells from untreated adrenals display the relatively undifferentiated morphology typical of zona glomerulosa cells of the adult rat adrenal cortex. In the absence of hormonal stimulation, these cells are homogeneous in size and shape. They contain numerous small mitochondria which display an internal membraneous structure consisting primarily of lamellar cristae. Some of these mitochondria contain tubular cristae; others display both types of membrane structures. The cytoplasm of unstimulated cells contains numerous free ribosomes and a few lipid droplets. The Golgi complex is well developed, and consists of an assemblage of both flat, saccular elements and small vesicles.

Upon chronic ACTH administration, however, cells of the zona fasciculata transform within days into cells more typical of fully differentiated adult fasciculata cells. This metamorphosis involves an increase in total cell volume, a marked increase in the number of lipid droplets in the cytoplasm, and hypertrophy of the Golgi apparatus. Though rough surfaced endoplasmic reticulum does not appear to have changed in appearance, smooth surfaced endoplasmic reticutlum wildly proliferates and is now found bundled into numerous tightly packed tubular stacks. The most prominent feature of change in cellular morphology, however, is a transformation of inner mitochondrial membranes from tubular or tubulo-vesicular cristae into numerous large (600Å) vesicles free in the mitochondrial matrix, and contained within protrusions of the inner mitochondrial membrane. Mitochondria are greatly swollen in size and rounded in appearance. Several other changes

in cellular morphology are noted in these ACTH-treated cells, including an increase in nuclear size, proliferation of plasma membrane microvilli, and an increase in the number of polysomes concomittant with a disappearance of single ribosomes.

Studies using cycloheximide to inhibit protein synthesis have demonstrated that some, but not all, of these ACTH-induced alterations in adrenocortical cell morphology are dependent on protein synthesis (412). Alone, this agent has no effect on adrenocortical ultrastructure; however, it completely supresses the ACTH-induced development of smooth surfaced endoplasmic reticulum, hypertrophy of the Golgi apparatus, and accumulation of lipids without any effect on hormonal promotion of polysome formation. On the other hand, chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, blocks ACTH-induced changes in mitochondrial morphology without affecting extra-mitochondrial changes, implying that regulation of mitochondrial responses to ACTH may involve specific mitochondrial synthetic machinery independent of that located in the cytoplasm. Actinomycin D completely blocks all ACTH-induced changes in morphology. This result implies that although the trophic action of ACTH on adrenocortical cell morphology requires protein synthesis, the regulation of morphological changes may ultimately be controlled at higher levels, i.e., at the level of the nucleus.

## Extra Adrenal Actions

In addition to the effects of ACTH on the adrenal, ACTH has

known actions on other target organs. ACTH causes darkening of melanophores in isolated amphibian skin by inducing dispersion of pigment granules (444). The fact that ACTH contains intrinsic melatocyte stimulating activity is not surprising in light of the fact that ACTH and the melanocyte stimulating hormones (alpha- and beta-MSH) share significant sequence homologies (445). The segment of the MSH molecules corresponding to segment 4-10 is shared between these hormones and ACTH, and has been shown to confer pigment dispersing activity to the melanophores (445). As with ACTH and adrenal steroidogenesis, cAMP alone can mimick the effect of ACTH and the melanotropins on melanophore dispersion, suggesting that this response may be mediated through cAMP.

ACTH exerts a pronounced lipolytic effect on adipose tissue. This lipolytic effect has been demonstrated in isolated adipose tissue from rat and rabbit, and more recently has been confirmed with isolated rat and rabbit fat cells (446). The adipokinetic effect of ACTH can be produced with the melanotropins on adipose tissue from the rabbit, but not from the rat (447). As with the melanotropic and steroidogenic actions of ACTH, stimulation of lipolysis by ACTH can also be mimicked by exogenous administration of cAMP. In contrast to the case of the adrenocortical cell and steroidogenesis, in the rat fat cell there is a direct correlation between binding of ACTH to its adipose receptor, and ACTH stimulation of cAMP production and activation of lipolysis (448), strongly suggesting that cAMP mediates the biological activity of ACTH in this system.

# CHAPTER 3

# EVALUATION OF ACTH-INDUCED CHANGES IN ADRENOCORTICAL CELL SHAPE

## Statement of Purpose

The genesis of this dissertation dates to an observation made by a colleague several years ago in the Hormone Research Laboratory that ACTH induces rounding of isolated rat adrenocortical cells in culture upon chronic exposure to the hormone. At the time, the intriguing aspect of this finding was that this cell retraction seemed to be hormone-specific and occurred concurrently with other known trophic actions of the hormone, namely inhibition of DNA synthesis and induction of steroidogenic enzymes. ACTH-induced adrenocortical cell rounding had been reported in several publications prior to this time, yet no further investigation of this phenomenon had been undertaken. When SDS-PAGE gels of cell lysates from ACTH-treated and untreated rat adrenocortical cells were analyzed for protein content by densitometric scanning, it became apparent that cells chronically treated with ACTH contained significantly less protein(s) comigrating at a position corresponding to the cytoskeletal protein actin. As nonmuscle actin is known to participate in many motile events in cells, and in particular plays a key role in defining cell shape, the coincidence of a change in cell shape and the loss of putative actin in response to ACTH suggested the exciting possibility that this protein might be under hormonal control. For this reason, I began a more thorough investigation of the effect of ACTH on adrenocortical

actin organization and content to establish whether such a control indeed existed, and if so, to probe the underlying mechanism of action of ACTH on cell shape and cytoskeletal organization.

The approach I have taken to studying the action of ACTH on adrenocortical shape and cytoskeleton has been a three-step logical progression of investigations. First, I have firmly established that ACTH indeed alters cell shape, actin organization and actin content in cultured rat adrenocortical cells. Second, I have quantitated the hormone-induced change in actin content by using several distinct experimental techniques to confirm the original results from SDS gel scans, and to more accurately quantitate the change in actin content in response to ACTH. Finally, I have attempted to uncover the underlying molecular mechanism of action of ACTH on adrenocortical actin content by designing experiments which I feel support one model for ACTH action. In the following three chapters, I will present the course of this experimental investigation, discuss the motives for the directions taken, and explain the reasoning behind the conclusions drawn.

## Abbreviations

| | |
|---|---|
| PBS | Phosphate buffered saline (10mM sodium phosphate, pH 7.6, 150mM NaCl) |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SDS | Sodium dodecyl sulfate |
| GdnHCl | Guanidine hydrochloride |
| NP40 | Nonidet NP40 |
| LTI | Lima bean trypsin inhibitor |
| DNase I | Deoxyribonuclease I |
| BSA | Bovine serum albumin |
| 2-MeOH | 2-Mercaptoethanol |
| DOC | Deoxycholate |
| TEMED | Tetraethylmethylethylenediamine |
| ECM | Extracellular matrix |
| PMSF | Phenylmethylsulfonylfluoride |
| ATP | Adenosine 5'-triphosphate |
| cAMP | Cyclic AMP, Adenosine 3',5'-cyclic monophosphate |
| 8-Br-cAMP | 8-Bromo-cyclic AMP |
| Tris-HCl | Tris(hydroxymethyl)aminomethane |
| EGTA | [Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid |
| Triton X-100 | Alkylanyl polyether alcohol |
| Tween 20 | Polyoxyethylene sorbitan monolaurate |
| $But_2cAMP$ | $N^6,O^{2'}$-dibutyryl-adenosine 3',5'-cyclic monophosphate |
| DTT | Dithiothreitol |

## Reagents

| Material | Source |
|---|---|
| Insulin | Sigma |
| Tween-20 | " |
| $Na_2ATP$ (disodium salt) | " |
| Deoxyribonuclease I (beef pancreas) | " |
| Ribonuclease A | " |
| Deoxycholate (disodium salt) | " |
| PMSF | " |
| Protein A | " |
| Goat anti-rabbit IgG | Cappel Labs |
| Diethylaminoethyl cellulose (DE25) | Whatmann |
| GdnHCl | Whittaker |
| Protein A-Sepharose (CL-4B) | Sigma |
| Deoxribonuclease I-Sepharose | Millipore |
| NP40 | Particle Data Labs |
| $Na^{125}I$ | New England Nuclear |
| $^{35}S$-methionine | Amersham |
| Collagen (Type II) | R. Stern, UCSF |
| Rabbit muscle myosin | R. Cooke, UCSF |
| Beta-tubulin | M. Kirschner, UCSF |
| Laminin | D. Gospadarowicz |
| Fibronectin | " |
| Tris-HCl | Sigma |
| Sodium dodecyl sulfate | BioRad Labs |
| Acrylamide | " |
| N,N'-Methylene-bis-acrylamide | " |
| Ammonium persulfate | " |
| Coomassie Brilliant Blue R-250 | " |
| TEMED | " |
| Glycine | Sigma |
| Urea, ultra pure | Schwartz/Mann |
| Chloroquine (disodium salt) | Sigma |
| Colchicine | " |
| Glycerol | Merck |
| Cytochalasin B | Sigma |
| Cycloheximide | " |
| Actinomycin D | " |
| cAMP | " |
| 8-Br-cAMP | " |
| Gelatin | Allied Chemicals |
| Leupeptin | Penninsula Labs |
| Pepstatin | " |
| DNA (calf thymus, type I) | Sigma |
| EGTA | J.T.Baker Chem. Co. |
| Triton X-100 | " |
| DTT | Sigma |

## Methods

### Isolation and Cell Culture of Rat Adrenocortical Cells

Adrenocortical cells were isolated from the decapsulated adrenals of adult (400-450gm), male Sprague-Dawley rats as described by Ramachandran and Suyama (453), and plated onto tissue culture plates in medium M199-D-valine (Cell Culture Facility, UCSF) containing 10% dialyzed fetal bovine serum and garamycin (80ug/ml) as described by Liles and Ramachandran (413). Medium was changed every other day and cultures were used within 5-14 days after plating. Functional viability was assessed by ACTH stimulation of steroidogenesis and/or by ACTH stimulation of cyclic AMP production. Both corticosterone and cAMP production was assayed by specific radioimmunassays as described elsewhere (452,454).

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS-PAGE gels were either 7 or 10% acrylamide (1.5mm thickness) with 3% stacking gels, and prepared according to the method of Laemmli (455). All samples for electrophoresis were solublized by heating for 2.5 mins to 90°C in 58mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.4% DTT or 2% 2-MeOH, and 0.1% bromphenol blue (SDS solublizing buffer). Gels were stacked at 18mA for 15 min. and run at 35mA for 4 hr. Gels were stained with 0.025% Coomassie brilliant blue (R-250) overnight and destained first in 25% isopropyl alcohol, 10% acetic acid, 0.0025% Coomassie blue, and then in 10% acetic acid. Gels were

dried onto filter paper for documentation or between dialysis membrane for densitometric scanning.

Indirect Immunofluorescence of Rat Adrenocortical Cells

Cells were grown at low density for 4-8 days on glass coverslips. After drug treatments, cells were washed twice in microtubule stabilizing buffer (MSB = 100mM PIPES, pH 6.9, 1mM EGTA, 4M glycerol) and incubated in MSB containing 0.5% (w/v) Triton X-100 for 5 mins. at 37°C to permeablize cells. Cell cytoskeletons were washed twice for 1 min. in MSB at 37°C, then fixed in absolute methanol at -20°C for 5 mins. Cytoskeletons were rehydrated in PBS for 1 min. and incubated with guinea pig anti-actin at a 1:50 dilution in PBS for 1 hr. in a humidified box at 22°C. After two 3 min. washes in PBS, cytoskeletons were incubated with FITC-labelled rabbit anti-guinea pig IgG (heavy and light chains, Cappel) preadsorbed with rabbit liver acetone powder for 30 mins. at 22°C at a 1:20 dilution in PBS. After two rinses in PBS, cytoskeletons were sealed under glass coverslips in 90% glycerol in PBS and stored at 4°C. All samples were visualized within 4 days using a Zeiss III photomicroscope fitted with a Planapo 63 lens and 528nm interference filter and photographed with Kodak Tri-X Pan 400 black and white film pushed to 1600.

DNase I Inhibition Assay for Actin

The assay was performed essentially as described by Blikstad et al. (456). Cells were treated with hormone or other agents,

and at specified times, cells were washed 3 times with ice-cold PBS and lysed mechnically by scraping in 150ul of DNase lysis buffer (5mM phosphate, pH 7.6, containing 150mM NaCl, 2mM $MgCl_2$, 0.2mM $Na_2ATP$, 0.2mM DTT, 0.5% (v/v) Triton X-100, 10uM PMSF, and 0.001% LTI). A 50ul aliquot was immediately removed, mixed with 50ul of dissociating buffer (20mM Tris-HCl, pH 7.6, 1M sodium acetate, 1mM $Na_2ATP$, 1.5M GdnHCl), and incubated on ice for 15 min. Twenty microliters of DNase I (0.1mg/ml in 50mM Tris-HCl, pH 7.5, 0.5mM $CaCl_2$, 10uM PMSF) were then added. Sixty microliters of this mixture were removed and added to 3mls of DNA at a concentration of 50ug/ml in 100mM Tris-HCl, pH 7.5, 4mM $MgSO_4$, 1.8mM $CaCl_2$. The change in absorbance at 260nm was recorded for at least 5 min. to determine the $V_{max}$ of the enzymatic activity. Standard curves were generated in exactly the same way using purified rabbit skeletal muscle actin as a standard.

Identification of Rat Adrenocortical Actin by DNase-Sepharose Affinity Chromatography

The procedure used was essentially that described by Williard et al. (457). Cultured rat adrenocortical cells were washed thrice with ice-cold PBS, then scraped into 500ul of 10mM Tris-HCl, pH 7.5, 1mM $CaCl_2$ and sonicated for 45 seconds at a 30 setting using a Bronwill Biosonik ultrasonicator. After centrifugation at 100,000 x g for 1 hr., the supernatant was passed twice through a 0.7ml DNase-Sepharose column (Biorad) equilibrated with above sonication buffer. The column was then washed sequentially with 5.5mls each of 10mM Tris-HCl, pH 7.5,

5mM $CaCl_2$, and 0.5M sodium acetate, pH 6.5, 0.75M GdnHCl, 1mM $CaCl_2$, 30% (v/v) glycerol, and 0.5M sodium acetate, pH 6.5, 4M GdnHCl, 1mM $CaCl_2$, 30% glycerol. All washes were dialyzed for 40 hrs. against 10mM Tris-HCl, pH 7.5, 1mM $CaCl_2$, then lyophilized to dryness and resuspended in 100ul of SDS lysis buffer for electrophoresis by SDS-PAGE. Fifty microliters of samples were applied to each lane of the gel.

Production of Anti-Actin and Anti-DNase Antisera

Purified rabbit skeletal muscle actin was used as immunogen. Anti-actin antisera were generated in four out of six guinea pigs by repeated subcutaneous, multiple site injections of increasing amounts (20, 80, 220ug) of actin over a period of five months. Antisera were collected monthly by cardiac puncture. Upon collection, sera were immediately lyophilized to dryness and stored at -70°C. When needed, antisera were reconstituted to original concentrations and stored for up to one month at 4°C.

Antibodies to bovine pancreatic deoxyribonuclease I were generated in six rabbits by subcutaneous injections of 500ug of protein per rabbit followed by repeated injections of 200ug over a six-month period. Blood was collected from the ear by venous puncture, and blood lyophilized and stored at -70°C. Anti-DNase antibodies of varying titres were generated in all six rabbits.

Enzyme-Linked Immunoassay (ELISA) for the Detection of Anti-Actin and Anti-DNase Antibodies

ELISAs for the detection of anti-actin antibodies were

performed in polyvinyl chloride multiwells (Flow Labs) as follows: 200ng of purified rat adrenal actin was plated in 220ul PBS overnight at 4°C. After twice washing with 0.9% NaCl and 0.05% Tween-20, guinea pig anti-rabbit muscle actin antiserum was added at various dilutions in 200ul PBS containing 0.1% BSA, 0.1% $NaN_3$, 0.05% Tween-20 (PBS/BSA) for 3 hrs. at 37°C. Wells were washed 3X with NaCl/Tween-20, then 200ul of rabbit anti-guinea pig coupled to horse radish peroxidase (HRP) diluted 1:150 in PBS/BSA was added for 1 hr. at 22°C. After a final 3X wash, wells were incubated with 250ul of 2,2-azino-di-(3-ethylbenthiazoline sulfonic acid) (Sigma) at 1mg/ml in 0.1M potassium phosphate, pH 5.0 containing 10ul/30mls of 30% $H_2O_2$ for 1 hr. at 22 °C. Color development was quantitated using a Titertech spectrophotometer. The ELISA for the detection of anti-DNase antibodies was conducted in an identical manner except that pancreatic DNase I was used as antigen and sheep anti-rabbit IgG coupled to HRP used as conjugate.

Figures 1 and 2 show two steps in the production of second antibody-enzyme conjugates. Covalent coupling of second antibody to horse radish peroxidase was performed according to the protocol of Nakane and Kawaoi (458). After coupling of HRP to second antibody (sheep anti-rabbit IgG or rabbit anti-guinea pig), enzyme-linked antibody was separated from unreacted reagents by gel filtration over a sephacryl S-200 as shown in Figure 1. Fractions were monitored for protein by absorbance at $OD_{280nm}$ and for HRP at $OD_{403nm}$. Those fractions containing material with a high enzyme:protein absorbance ratio were pooled

and stored at 4°C. This procedure yields conjugates with an average of 2-3 enzyme molecules per antibody molecule which retain full immunoreactivity (458). Dilution curves were generated for each conjugate prepared to determine the amount of conjugate to use for linear color development over the period of one hour. One such curve is shown in Figure 2. Here, 200 ng per well of guinea pig IgG was reacted with increasing dilutions of rabbit anti-guinea pig-HRP conjugate and color developed for 1 hour. Color development, monitored at $OD_{414nm}$, is plotted as a function of conjugate dilution. The relationship between color development and conjugate dose demonstrates that such conjugates retain high immunoreactivity for IgG; the lack of color development in the absence of plated antigen confirms that this reactivity specific involves interaction of conjugate with immunoglobulin and is not the result of nonspecific binding of conjugate to the well.

Use of the ELISA technique to detect antisera generated against DNase I is shown in Figure 3. Here, dilutions of two rabbit anti-DNase antisera were tested in an ELISA in which purified DNase I was used as antigen and sheep anti-rabbit IgG-HRP conjugate used as second antibody. Both antisera reacted strongly in this assay; rabbit preimmune serum, on the other hand, could not replace anti-DNase antisera for it did not cross-react with conjugate to any significant degree.

DNase Immunoprecipitation Assay

Essentially that developed by Snabes et al. (459) with modifications described in the text (see Chapter 4, page 195). Cells for analysis were solublized in lysis buffer (25mM Tris-HCl, pH 7.4, containing 0.4M NaCl, 0.1% SDS, 0.1% DOC, 1% NP40, 2% 2-MeOH, 0.001% LTI, 0.01% bacitracin) on ice, spun for 15 mins. at 8700 x g, and supernatants immediately diluted at least ten-fold in DNase buffer (PBS, pH 7.4, containing 0.1% gelatin, 0.2mM $CaCl_2$, 0.2mM $Na_2ATP$, 0.02% $NaN_3$). Purified rabbit muscle and rat adrenal actin standards were treated in an identical fashion. One hundred ul of actin standard or cell lysate were added to 500ul of DNase buffer containing 10ng DNase I in 12 x 75mm glass tubes. After addition of 100ul of $^{125}I$-rabbit skeletal muscle actin (10,000 cpm), tubes were incubated overnight at 4°C. At this time, 100ul of rabbit anti-DNase antiserum at 1:50 dilution in DNase buffer was added, and the incubation continued for 4 hrs. at 4°C. After addition of 100ul of sheep anti-rabbit gamma globulin precipitating antibody (Cappel Labs) at 1:1 dilution in DNase buffer, and a 6 hr. incubation at 4°C, 2.0mls of ice-cold PBS were added and the tubes spun at 1500 x g for 20 mins. Supernatants were decanted and the pellets counted for radioactivity in a Beckman 4000 gamma counter. Blank values were determined in the absence of DNase I, or by using preimmune rabbit serum in lieu of anti-DNase antiserum in the assay. All data has been corrected for blank values. Determination of half-maximal displacement values ($ED_{50}$) from displacement curves was accomplished using a nonlinear least squares analysis computer

program developed in our laboratory by L. Nadasi (Eotvos University, Budapest, Hungary).

Iodination of Protein A

Iodination conditions are essentially that described for iodination of ACTH (449). Protein A (5ug in 20ul 0.2M phosphate buffer, pH 7.6) was placed in a polypropylene microfuge tube, then 0.3mCi $Na^{125}I$ (2ul) added, and iodination initiated by the addition of 0.35% chloramine T (6ul) to the reaction vessel. After mixing for 30 seconds, 20ul of 10% 2-MeOH was added to stop the reaction, and the reaction mixture was immediately chromatographed on an LH-20 column (0.5 x 11cm) in PBS. Fractions containing radioactivity which eluted at the void volume of the column were pooled and stored in aliquots at -4°C.

Miscellaneous

Bovine serum albumin, fraction V, was purchased from Miles Corp. and further purified by extensive dialysis against charcoal and $H_2O$ as described previously (460).

Highly purified porcine $ACTH_{1-39}$ (461), synthetic corticotropin-inhibiting peptide (462), and synthetic beta-endorphin (463) were prepared in our laboratory by the published procedures.

Protein was determined by the method of Lowry et al. ( ) or by the Bradford assay (465).

DNA was determined by the method of Ceriotti (460) using the modification of Short et al. (467).

Corticosterone production (452) and cyclic AMP production (454) were quantitated by specific radioimmunoassays developed in our laboratory.

All protein stocks were analyzed specrophotometrically using absorbance at $OD_{280nm}$ and published extinction coefficients to determine concentrations.

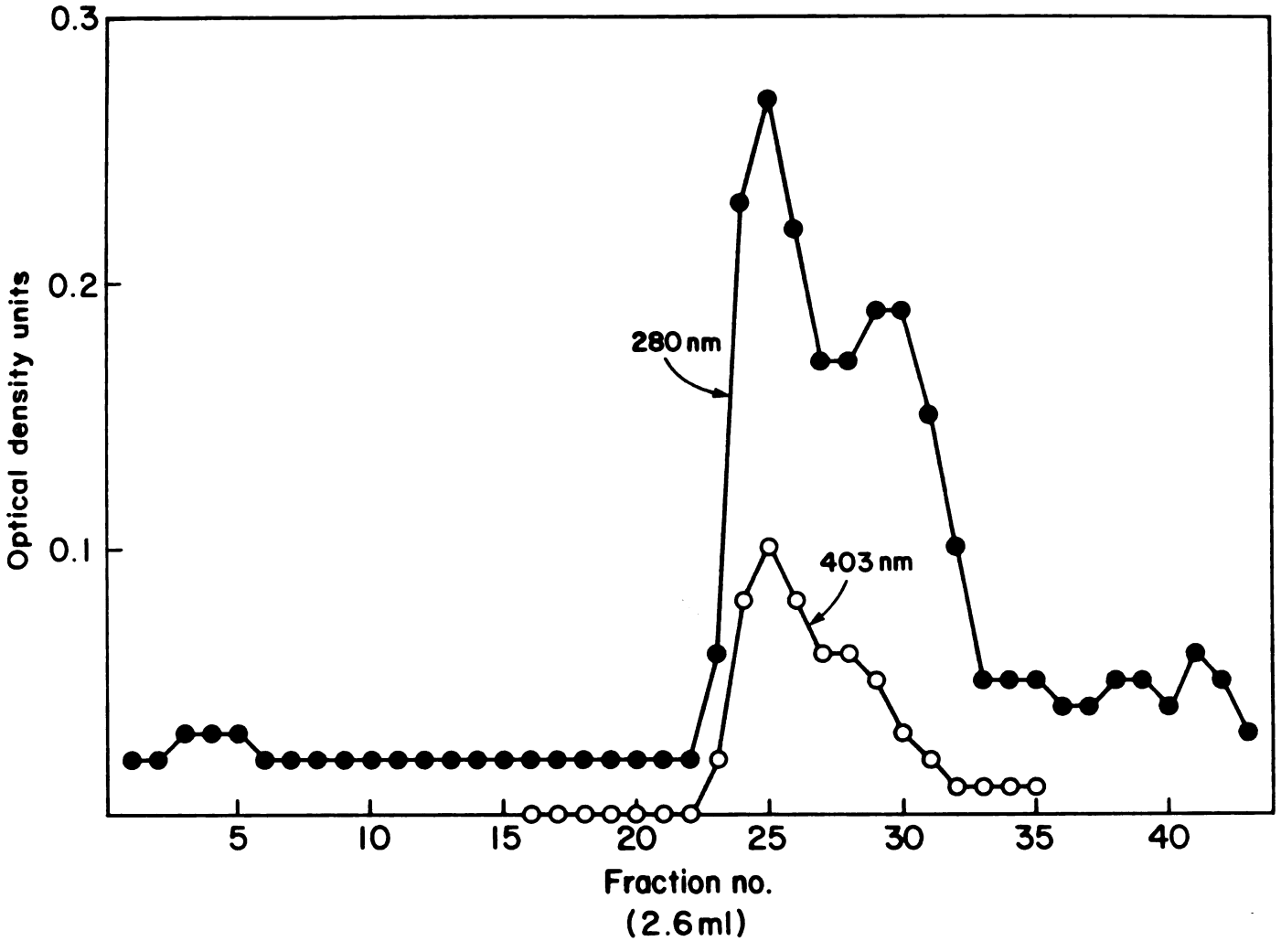



Figure 1. Gel Filtration of Horse-Radish Peroxidase-IgG Conjugates. After chemical coupling of HRP to IgG immunoglobulins according to the prodedure of Nakane and Kawaoi (458), the reaction mixture was chromatographed on a S-200 column (1.5 x 48cm) in PBS at 6mls/hr. Protein was monitored by absorbance at $OD_{280nm}$; HRP was monitored at $OD_{403nm}$. Fractions #23-26, containing the highest ratios of HRP:protein, were pooled and stored at 4°C.

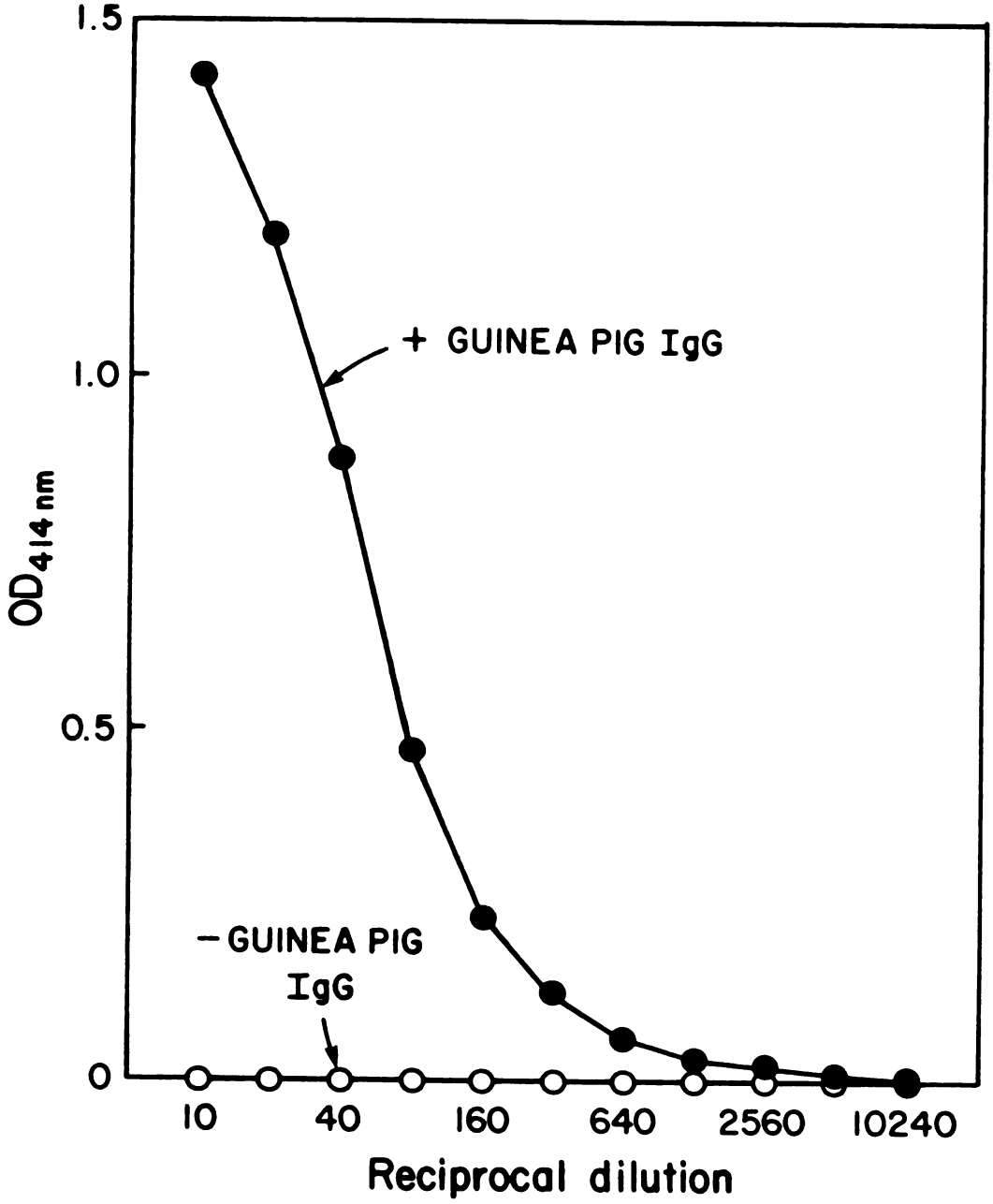



Figure 2. Dilution Curve of HRP-Guinea Pig IgG Conjugate. The procedure for the ELISA is described in detail in the "Methods" section of Chapter 3. Guinea pig IgG (200ng) in each well was used as antigen; HRP-IgG conjugate was tested at the dilutions indicated. Reaction with substrate was allowed for one hour, and a dilution of conjugate yielding good color development (1:80) was chosen for subsequent experiments.

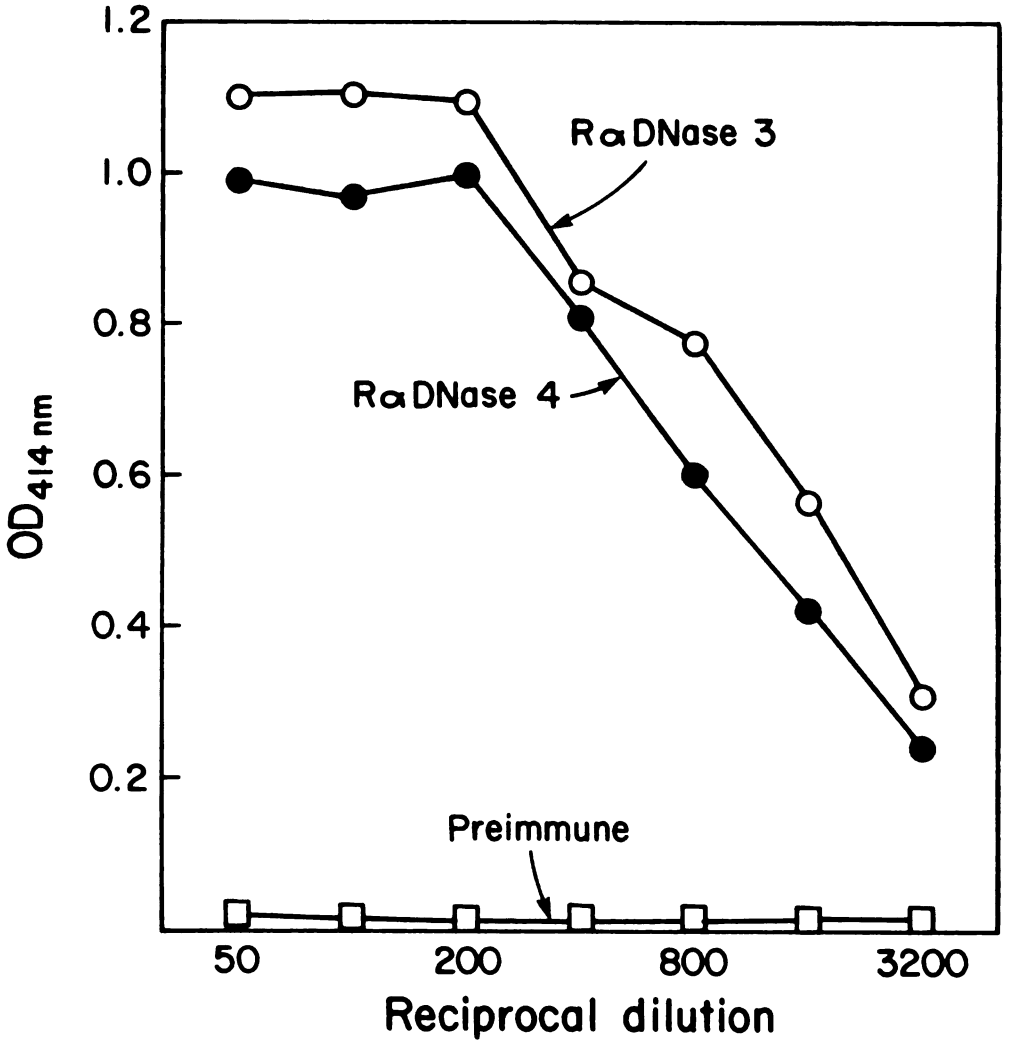



Figure 3. Identification of Rabbit Anti-DNase Antibodies by ELISA. Bovine pancreatic DNase I (200ng) was plated as antigen, and rabbit anti-DNase antisera and rabbit preimmune serum were tested at the dilutions indicated. Color development after one hour was quantitated by absorbance at $OD_{414nm}$ in a Titretech multiscan spectrophotometer.

## The Initial Observation: Cell Retraction at the Light Microscopic Level

The photographs displayed in Figure 4 document the effect of ACTH on the shape of cultured rat adrenocortical cells. Isolated adult rat adrenocortical cells, seven days after plating onto plastic tissue culture dishes, are shown in Figure 4A. Previous studies have confirmed that these cells represent relatively pure populations of adrenocortical cells of the zona fasciculata/ reticularis type. These cells have flattened onto the substratum, appear polygonal in outline and homogeneous in size, and have made extensive contact with one another such that at confluency they form an uninterrupted monolayer of cells. Earlier studies have characterized these cells in terms of their steroidogenic potential (411). They are exquisitely sensitive to ACTH, and respond to physiological doses of the hormone by greatly increasing both corticosterone and cAMP production. The cells are firmly attached to the culture wells as it requires trypsinization or calcium chelation to remove them.

Figure 4B is a light micrograph of these same cells after 30 hours of ACTH treatment. At the concentration of 2.2nM, a dose of ACTH which results in maximal stimulation of steroidogenesis, their shape has changed dramatically: they have lifted off the surface of the well, have rounded up, and have retracted away from one another. Many of these cells have assumed an markedly fusiform shape rarely seen in unstimulated cells. This pronounced cell rounding can be observed as early as six hours after ACTH administration; however, maximal retraction requires at least 12

hours to be manifest. Though doses of ACTH greater than 1nM are required to produce maximal cell retraction, this effect appears to be qualitatively dose dependent. Concentrations of ACTH below 0.5nM do not affect cell shape at the light microscopic level. Moreover, the effect of ACTH on adrenocortical cell shape is hormone-specific as other basic polypeptide hormones such as insulin, glucagon, and B-endorphin have no effect on gross cell shape. However, the cyclic AMP analog 8-Br-cAMP, which is alone able to stimulate adrenocortical steroidogenesis to levels achieved by ACTH (415), can effect a similar cell retraction in the absence of hormone (Figure 4C).

The effect of ACTH on adrenocrotical cell shape is completely reversible. Figure 4D shows fully retracted cells which have been returned to fresh medium lacking hormone for 24 hours. These cells have regained the polygonal appearance of cells never treated with hormone. The return toward an unstimulated shape can be seen as early as 10 hours after hormone removal. Moreover, both the retraction due to ACTH and reflattening upon homone removal are independent of protein and RNA synthesis as both events take place in the presence of cytloheximide or actinomycin D at concentrations which completely inhibit translation and transcription, respectively, in these cells. At the concentration of cycloheximide used in the experiments reported here (100ng/ml), this agent inhibited adrenocortical protein synthesis to 81-88% (see Figure 43).

As cytoskeletal structures, and in particular microfilaments and microtubules, have been implicated in defining and regulating

cell shape in many cells, the effects of drugs which specifically disrupt the organization and distribution of these filaments in situ were tested for their effects on adrenocortical cell shape. As shown in Figure 4E, cytochalasin B, a mold metabolite which binds to and depolymerizes actin-containing microfilaments, produced cell retraction which visually resembled that change induced by ACTH. Moreover, other cytochalasins tested, cytochalasin D and E, produced similar cell rounding (not shown). The effect of these agents on cell shape could not be attributed to any toxic effect on the cells, for these cells were fully responsive to ACTH when placed in fresh medium lacking these drugs. Furthermore, the effect of the cytochalasins appeared to be specific to microfilament-disrupting agents, for colchicine, a drug known to disrupt microtubules in living cells without affecting the integrity of microfilaments, had no effect on adrenocortical cell shape (Figure 4F). These results point to a specific involvement of microfilaments in maintaining adrenocortical cell shape.

Thus, it appears that ACTH induces a profound change in cell shape in cultured adrenocortical cells. This affect is produced only with chronic exposure of cells to ACTH, and is reversible, hormone-specific, and qualitatively dose-dependent. As this effect requires hours of hormone treatment to be manifest at the light microscopic level, ACTH-induced cell retraction must be considered another trophic action of the hormone. The ability to mimick the effect of ACTH with microfilament-disrupting agents not only implicates microfilaments in adrenocortical cell shape,

A.

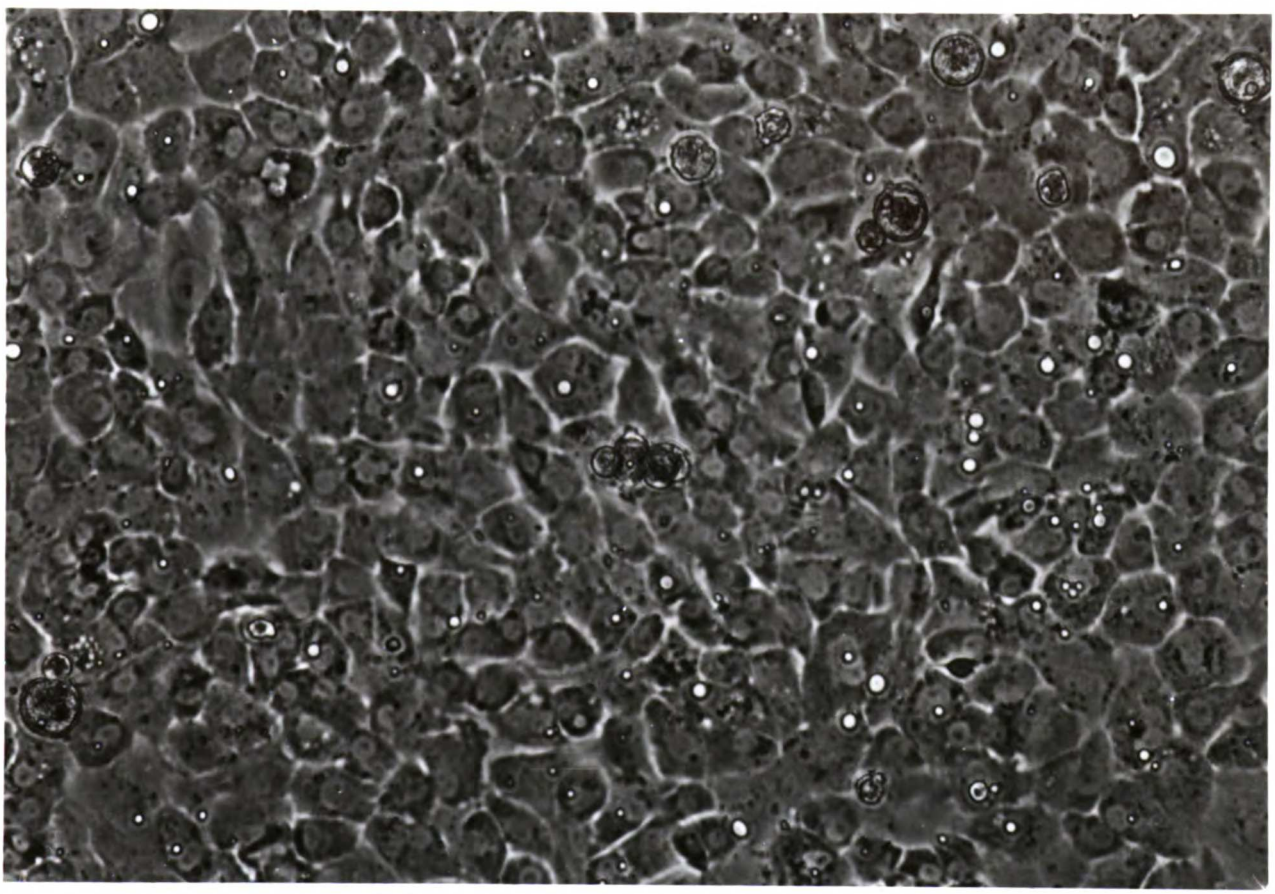
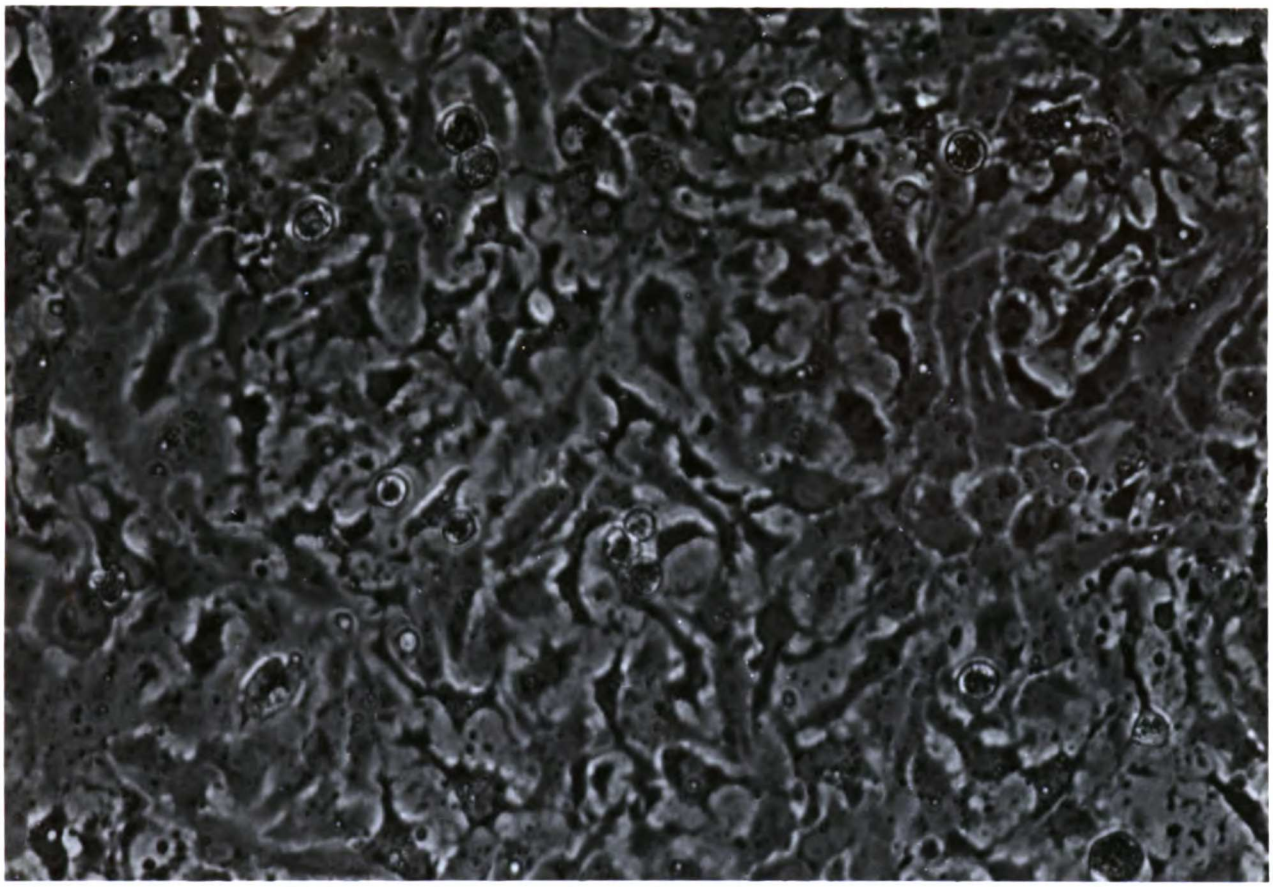
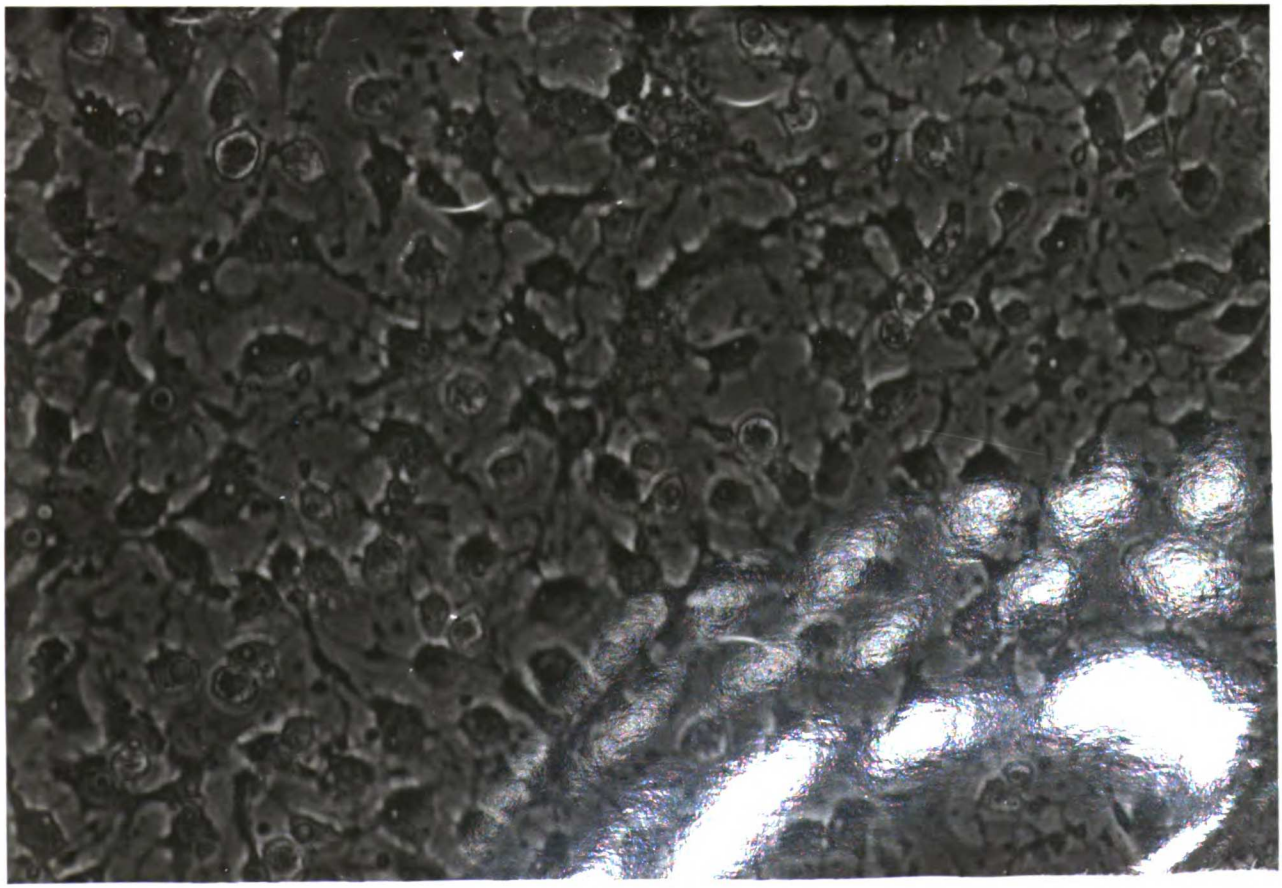


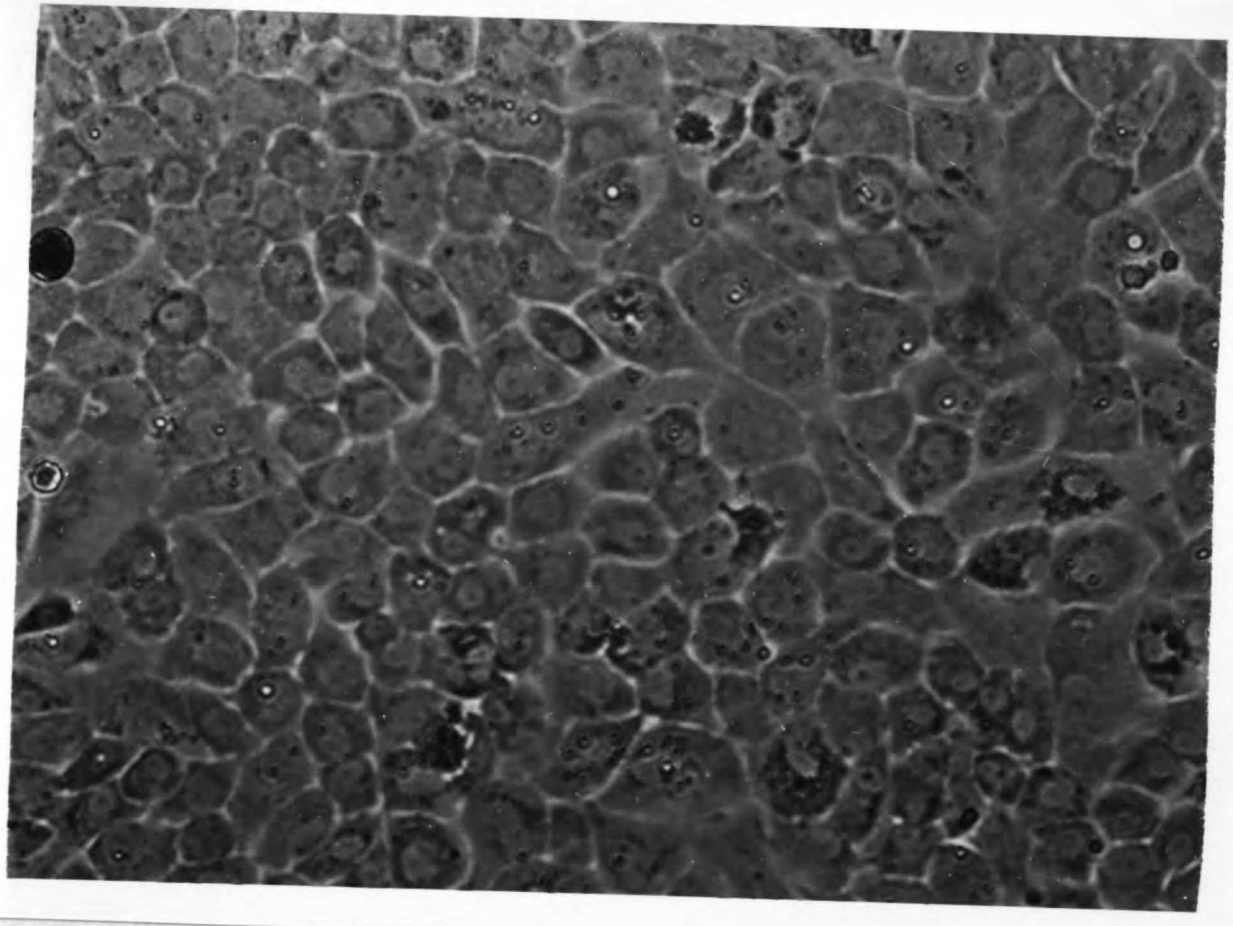
Figure 4. Phase Contrast Light Microscopy of Cultured Rat Adrenocortical Cells. All treatments were for 24 hours unless otherwise noted. A) control; B) 2.2nM ACTH; C) 2mM 8-Br-cAMP; D) 2.2nM ACTH followed by 24 hrs. incubation without hormone; E) 10uM cytochalasin B; F) 50uM colchicine.



B.



C.



D.



E.

F.



but also suggests the intriguing possibility that ACTH might effect cell retraction by altering actin filament organization, i.e., that microfilament integrity within this cell might be under hormonal control. For this reason, a more detailed analysis of microfilament organization in the adrenocortical cell was initiated in hopes of further elucidating the mechanism of action of ACTH on cell shape.

To this end, the technique of indirect immunofluorescence was chosen as a means to investigate whether ACTH treatment has any effect on the integrity and/or spatial arrangement of these cytoskeletal structures. As this technique requires the use of immunoreagents which specifically recognize and react with the protein(s) of interest, it was necessary for this purpose to generate anti-actin antibodies which would specifically cross-react with actin contained within adrenocortical microfilaments.

## Generation of Anti-Actin Antibodies

Past attempts to generate anti-actin antibodies have been relatively unsuccessful. Actins from muscle and nonmuscle sources are notably poor immunogens. There is a strong prejudice that this lack of immunogenicity is due to the high degree of sequence homology among actins from different sources, i.e., that actins throughout evolution have minimally diverged (468). In fact, comparison of amino acid sequence data from muscle and nonmuscle actins reveals extensive sequence homology, supporting this belief.

Within the past decade, however, several laboratories have

succeeded in generating anti-actin antibodies (469,470,471,474). Unfortunately, most of these antisera have been of very low titre and low immunoreactivity, and therefore of minimal use in techniques, such as indirect immunofluorescent staining, which require large amounts of high-affinity antibody. Naturally occurring autoimmune anti-actin antibodies have been found in patients with active chronic hepatitis (472), but again show low titre and are rare and difficult to procure. Attempts to boost actin immunogenicity by chemical modification in order to produce higher titres of high affinity anti-actin anitbodies, either by covalently coupling actin to inert particles (473), by creating alum precipitates of actin (474), or by denaturing actins with SDS (475), have been marginally successful. The use of SDS to increase immunogenicity, and in conjunction with SDS-PAGE, to ensure chemical purity of the immunogen (476), has been somewhat promising. Though treatment of actins with SDS seems to boost their immunogenicity, this procedure also tends to promote nonspecificity, especially in immunofluorescent staining techniques. Furthermore, actin denaturation destroys antigenic determinants which are conformationally dependent, and exposes determinants buried within the molecule which are not available in the native state. This is a distinctly undesirable effect when attempting to identify actin in its native conformation, as would be the case with actin in microfilaments in situ.

Another outstanding feature of anti-actin antisera is that though they are specific to actin, they tend to be species nonspecific, e.g., antibodies raised to muscle actins generally

cross-react with nonmuscle actins, and vice-versa (468). This suggests that some antigenic determinant(s) on the molecule is common to actins from different sources, and has been faithfully conserved through evolution.

In light of these observations, and to avoid the possible complications of using denatured actins purified by SDS-PAGE, attempts were made to generate anti-actin antibodies using native rabbit muscle actin. As purified adrenal actin was at this time not available, and because rabbit muscle actin is easy to obtain in highly pure form in large quantities, muscle actin was chosen as immunogen to avoid the task of purifying adrenal actin.

Purification of Rabbit Skeletal Muscle Actin

The purification of rabbit skeletal muscle actin closely followed the protocol of Feuer et al. (478) for the preparation of muscle acetone powder, and that of Spudich and Pardee (479) for the subsequent extraction of muscle actin. Dorsal lateral skeletal and hind leg muscles excised from two freshly killed rabbits were the starting material for the acetone powder. Acetone powder was prepared by homogenization of the excised muscles in a Waring blender, and sequential extraction with 0.1M KCl, 0.05M $NaHCO_3$, and 1mM EDTA buffers. Residual material was washed with 1 liter of acetone at -20°C, then dried over desiccant and stored at -20°C. Actin was extracted from acetone powder by stirring 10 grams of this material in 200mls of depolymerization buffer (2mM Tris-Cl, pH 8.0, 0.2mM $Na_2ATP$, 0.5mM 2-MeOH, 0.2mM $CaCl_2$ and 0.005% $NaN_3$) for 2 hrs. at 4°C. Bulk

solids were removed by filtration through cheesecloth followed by centrifugation at 10,000 x g for 20 mins. Actin in the supernatant was polymerized by addition of KCl to 50mM, $MgCl_2$ to 2mM, and ATP to 1mM, and the solution was allowed to stand at 4°C for 2 hours to ensure full polymerization. A visible increase in the viscosity of the solution was evident after 30 mins., a clear indication of polymerization (479). To eliminate contaminating traces of tropomyosin, solid KCl was slowly added to polymerized actin to a final concentration of 0.6M, and the resulting actin filaments were separated from soluble tropomyosin by pelleting F-actin at 80,000 x g for 3 hrs. Contaminants trapped in the actin pellet were released by resuspension of the pellet in fresh depolymerization buffer containing 0.6M KCl and recentrifugation. The pellet of F-actin was finally resuspended in depolymerization buffer and dialyzed against several changes of this buffer for 3 days at 4°C. This procedure has been reported to effect greater than 90% depolymerization of actin (479). Residual F-actin was removed by another high speed centrifugation, and actin repolymerized as before.

Electrophoretic analysis of actin at this time demonstrated that the protein was greater than 95% homogeneous; however, to ensure the highest purity, actin was subjected to three more cycles of polymerization/depolymerization. Actin preparations so prepared were consistently greater than 97% homogeneous as judged by SDS-PAGE. Purified muscle actin was stored as aliquots of unpolymerized actin at -70°C. Though it has been reported that freezing and thawing of actin can destroy its ability to

polymerize and to activate myosin ATPase (479), storage at -70°C did not seem to affect the performance of actin in the enzymatic assays and immunoassays employed in our studies.

To ensure removal of trace contaminants which might co-purify with actin, and to achieve the highest purity possible, purified muscle actin was subjected to a final gel filtration according to the procedure of Spudich and Pardee (479). Actin-associated contaminants in these preparations have been found to affect the stability of actin and to alter its ability to polymerize under proper ionic conditions (480).

Purified rabbit muscle actin was identified as actin by co-migration in SDS gels with purified rabbit muscle actin standard (Sigma), and by amino acid analysis after HCl hydrolysis as described by Moore (481).

To demonstrate that the purification procedure itself had not denatured the rabbit muscle actin, this actin was analyzed for its ability to polymerize under a variety of ionic conditions. It is well known that the ability of actin to polymerize is dependent on tertiary conformation of the molecule, and that denaturation with chaotropic agents such as potassium iodide, or treatment with guanidine hydrochloride or urea, can destroy its ability to polymerize (482). Actin polymerization was assessed by gel filtration of actin. For these experiments, chromatography was performed on a TSK3000SW gel filtration column (Toyo Soda, Japan) under 350psi of pressure. This column yields excellent resolution of proteins over a wide range of molecular sizes, and greatly reduces running time without sacrificing

resolution (511). Figure 5 shows two calibration curves using a series of protein standards. The column buffer was 2mM Tris-Cl, pH 8.0, containing 150mM Nacl, 0.2mMMg$Cl_2$ and 0.2mM$CaC_2$, and the flow rate was 0.5ml/min. Proteins eluted from the column at times predicted from their known molecular dimensions. When data from these runs was analyzed by the method of Fish (483), a plot of effective radus as a function of erf value yielded a straight line, indicating that the proteins behaved during filtration as theoretically predicted. The table below the chromatographs in Figure 5 gives some physicochemical data pertinent to the use of these proteins as standards.

Figures 6 and 7 are composites of chromatographs of muscle actin gel filtrated under various conditions. Under standard conditions of filtration (150mM NaCl in the presence of Mg++ and Ca++ ions), polymerized actin (filamentous, or F-actin) elutes at the void volume (at t = 16 mins.) of the column as expected (Figure 6, panel A), whereas monomeric (globular, or G-actin) elutes at a time predicted for a globular protein of about 45,000 daltons (at the arrow, see Figure 6, panel C). Howver, when G-actin is polymerized by the addition of ATP and KCl, as shown in Figure 6, panel D, the majority of protein now elutes at the void volume, indicating that polymerization has occurred. Furthermore, when F-actin is treated with 0.6M KCl (Figure 6, panel B) or 4M urea (Figure 7) prior to filtration, a significant peak of protein co-elutes with monomeric actin (arrow), suggesting that these treatments have promoted actin dissasembly. Pretreatment of F-actin with 0.75M GdnHCl, a condition know to reversibly

depolymerize actin (456), effects a similar shift in peak positions from F- to G-actin (Figure 7), again suggesting that the effects of KI and urea result in F-actin disassembly. The observation that G-actin is able to polymerize in conditions which favor polymerization, and that actin behaves as predicted during gel filtration when denatured or depolymerized, supports the conclusion that this purified protein is chemically intact and functionally viable, and thus has retained at least some degree of native conformation.

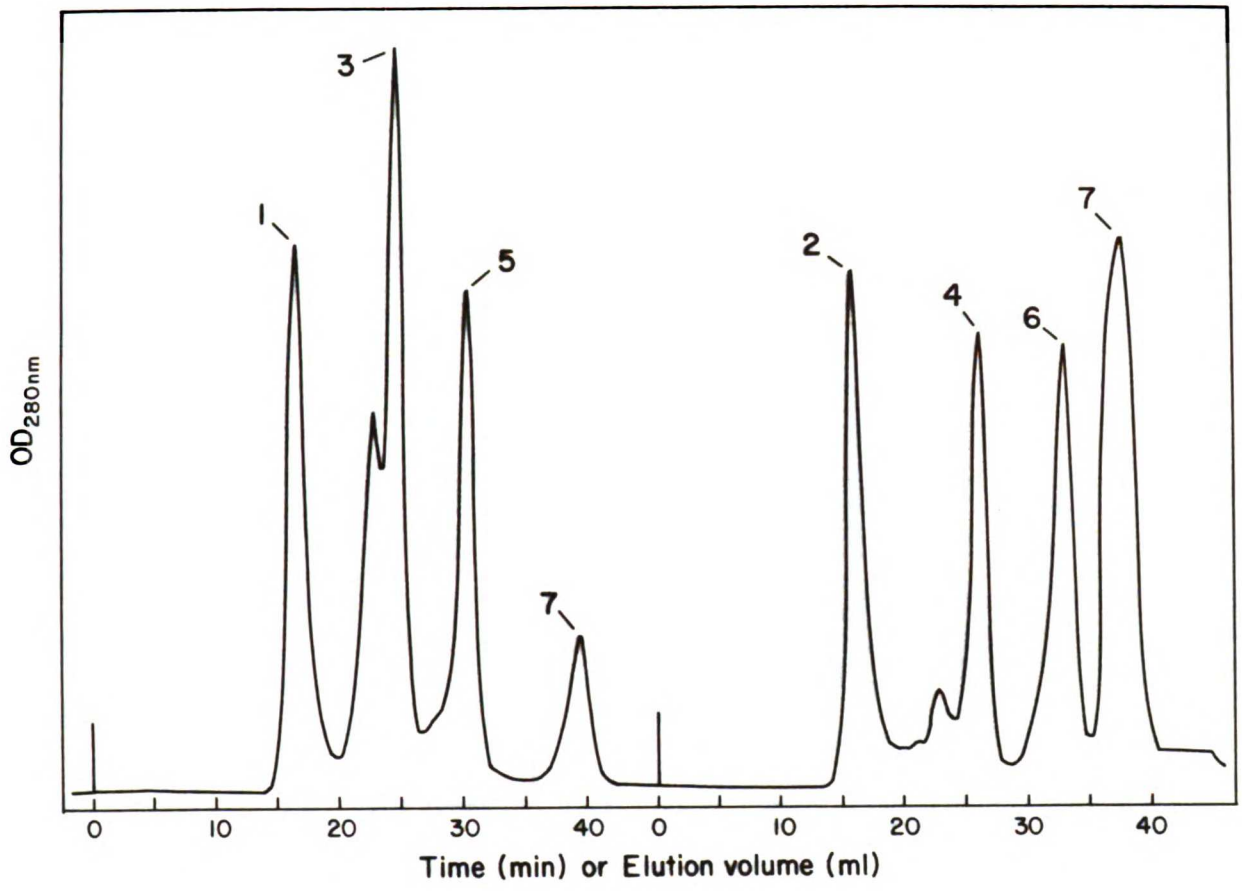
Identification and Characterization of Anti-Actin Antibodies

Any conclusions drawn concerning the state of rat adrenocortical actin in studies using anti-actin antisera are premised on the assumption that such antibodies specifically recognize actins, and in this case, adrenal actin. As proof that these antisera do in fact recognize actin, antisera were tested for their ability to specifically immunoprecipitate iodinated rabbit muscle actin. Figure 8 shows the results of one such experiment in which guinea pig anti-actin antiserum and guinea pig preimmune serum at various dilutions were tested for their ability to react with $^{125}$I-actin. Antibody-bound label was separated from free label by immunoprecipitation using Staphylococcus aureus protein A (BRL). It is evident that the anti-actin antiserum, but not the preimmune serum, is capable of selectively precipitating labelled actin.

As a second way of confirming that the anti-actin antisera generated against rabbit muscle actin did cross-react with

adrenocortical actin, antisera were tested for their ability to react specifically with actin extracted from adrenocortical cell lysates run on SDS gels. For this purpose, the method of Burridge et al. (484) was employed. According to this protocol, adrenocortical cells were lysed in SDS lysis buffer, and proteins separated on 10% polyacrylamide SDS gels. After electrophoresis, gel lanes were either stained for protein or incubated with guinea pig anti-actin antiserum or with guinea pig preimmune serum. After an appropriate time of incubation and extensive washing to remove unbound antibodies, positive reactivity was visualized by reacting remaining antibodies with iodinated protein A, followed by autoradiography.

The autoradiograms shown in Figure 9 document the results of one such experiment. The far left lane entitled "Cell Lysate" represents the coommassie-stained total protein pattern in the adrenocortical cell lysate. Note the prominent band located at 43,000 daltons; this band comigrates with purified muscle actin, and represents adrenocortical actin. The middle lane of Figure 9 shows the autoradiograph of the same cell lyste incubated with one of the guinea pig ani-actin antisera and overlaid with $^{125}$I-protein A. The location of the one prominent band of radioactivity matches exactly the position of purified actin at 43,000 daltons. This band was not evident in autoradiographs of lysates in which guinea pig preimmune serum replaced anti-actin antiserum, as shown in the far right lane of Figure 9. The smear of radioactivity seen in the anti-actin sample in the 120-200K dalton range is an artifact, and was not reproducible in repeated




| Protein | MW x $10^{-3}$ | R(Å) | Elution Vol (ml) |
|---|---|---|---|
| 1. Thyroglobulin | 660 | 86 | 16.0 |
| 2. Catalase | 244-250 | 52.2 | 16.0 |
| 3. BSA | 68 | 36 | 24.3 |
| 4. Ovalbumin | 43 | 27.6 | 26.2 |
| 5. oProlactin | 22.6 | 24.9 | 30.3 |
| 6. RNase A | 13.7 | 18 | 33.0 |
| 7. Tyr-Lys | 0.309 | — | 39.5 |

Figure 5. Calibration of TSK3000SW of Purified Protein Standards. The column buffer was PBS and the flow rate was 1.0ml/min.; the perfusion pressure was maintained at 350psi. Two runs with different proteins are shown. The table contains pertinent physicochemical data and the observed elution volumes of the proteins used.

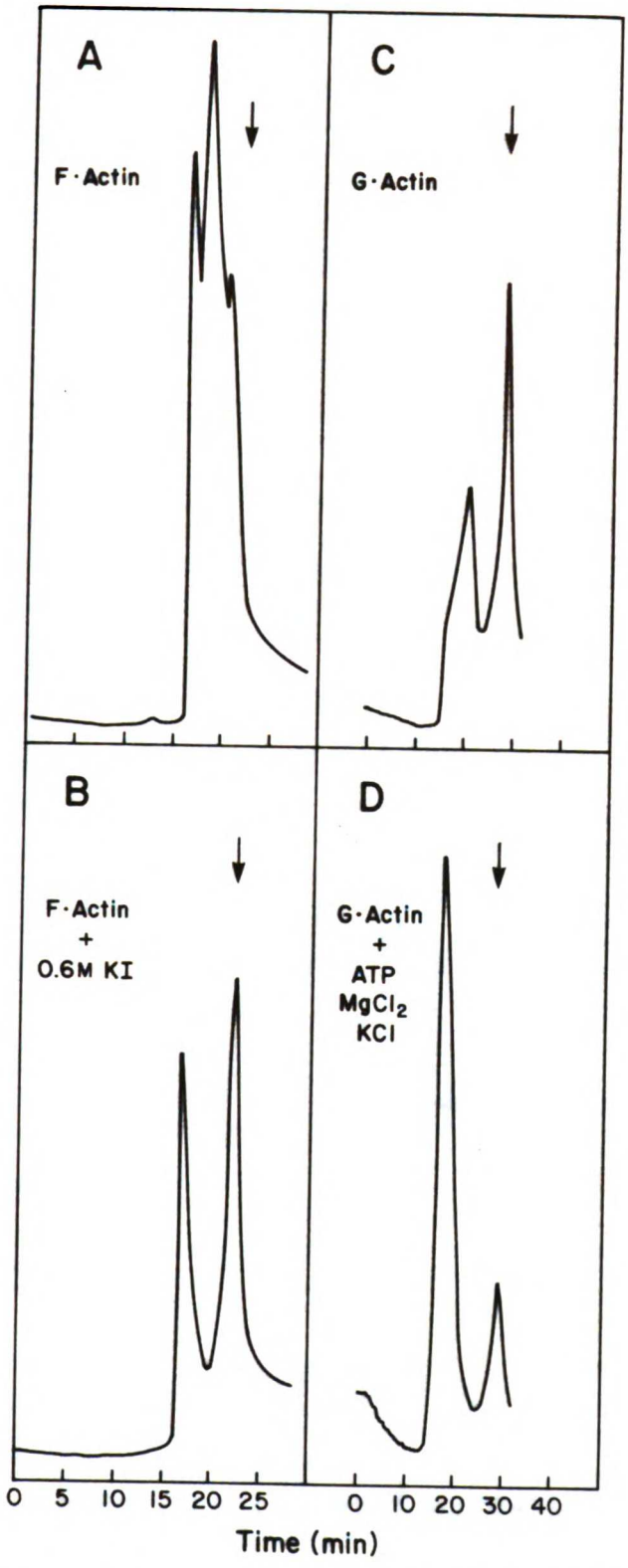



Figure 6. Gel Filtration on TSK column of Rabbit Skeletal Muscle Actin under Different Ionic Conditions. Column buffer was PBS. A) F-actin; B) F-actin pretreated with 0.6M KI; C) G-actin; D) G-actin polymerized with 0.5mM $Na_2ATP$, 0.2mM $CaCl_2$, and 50mM KCl prior to filtration. Arrow indicates the position expected for a globular protein of 43,000 daltons, i.e., G-actin.

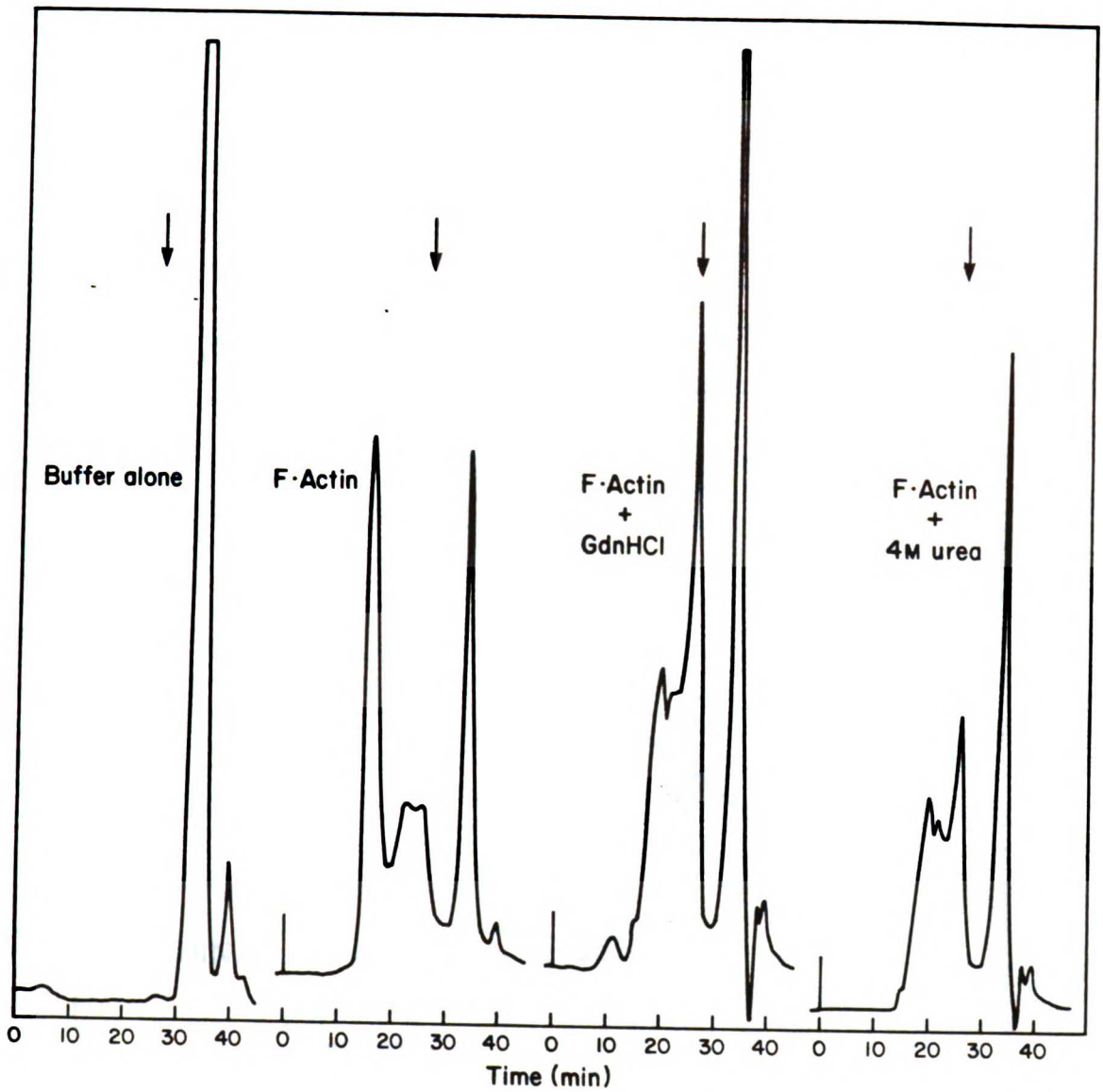



Figure 7. TSK Chromatography of Purified Rabbit Skeletal Muscle Actin in Various States of Polymerization. Column buffer for all runs was PBS; flow rate was 0.5ml/min. A) Sample buffer alone. Note the presence of ATP in the same solution eluting at 34 mins.; B) F-actin; C) F-actin pretreated with 0.75M guanidine-HCl; D) F-actin pretreated with 4M urea. Arrow indicates the elution position expected for monomer actin (G-actin).

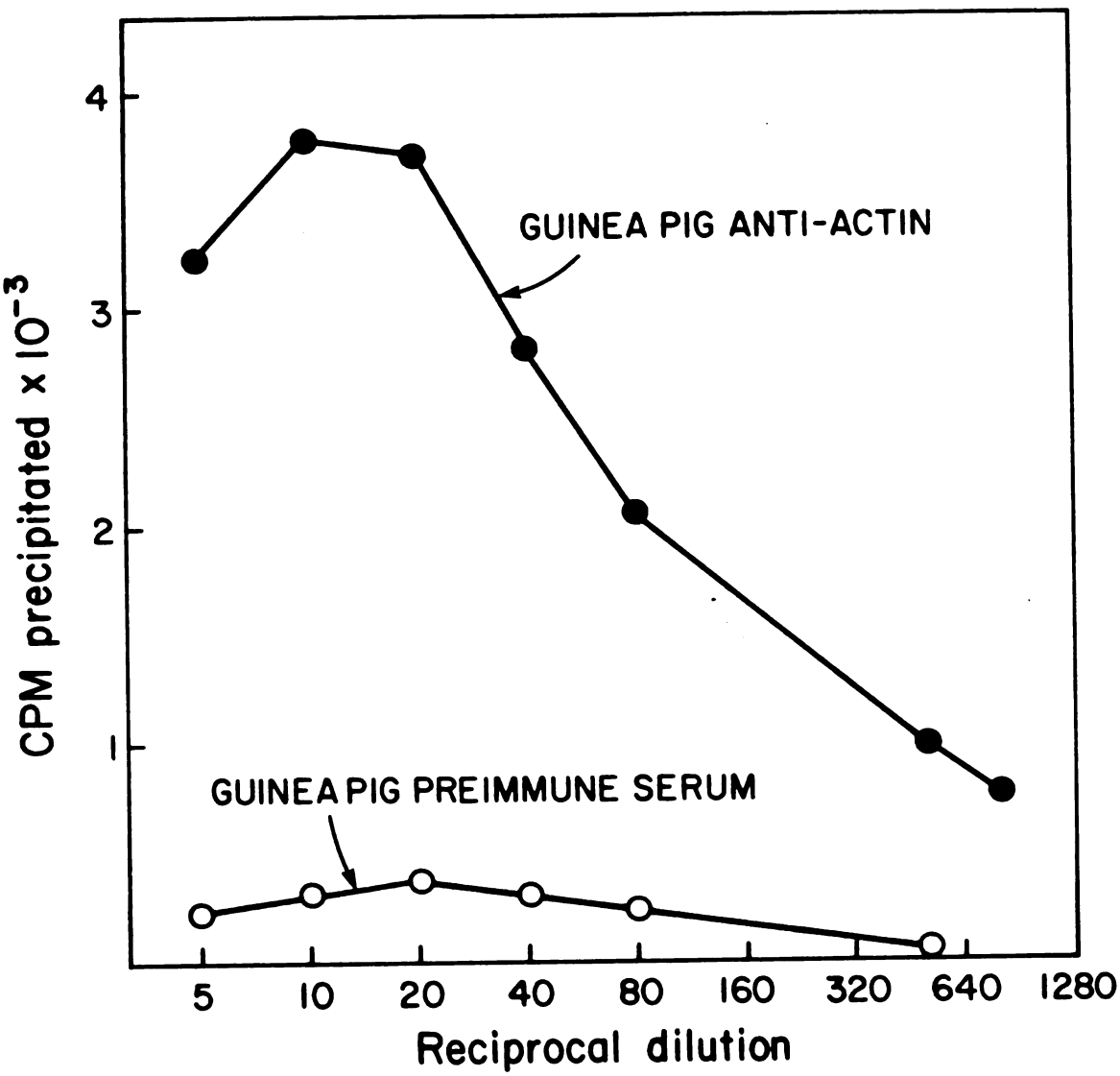



Figure 8. Detection of Anti-actin Antibodies by Immunoprecipitation of $^{125}$I-Actin. One hundred ul of 10mM sodium phosphate, pH 7.4, containing 0.5% BSA and $^{125}$I-labelled rabbit skeletal muscle actin (10,000cpm), were added to 100ul of guinea pig anti-skeletal muscle actin antiserum or guinea pig preimmune serum at the dilutions indicated. After an overnight incubation at 4°C, bound label was precipitated by addition of 2mg of formalin-fixed Staphylococcus aureus (Staph A) cells (BRL), tubes were centrifuged, and tips counted for radioactivity.

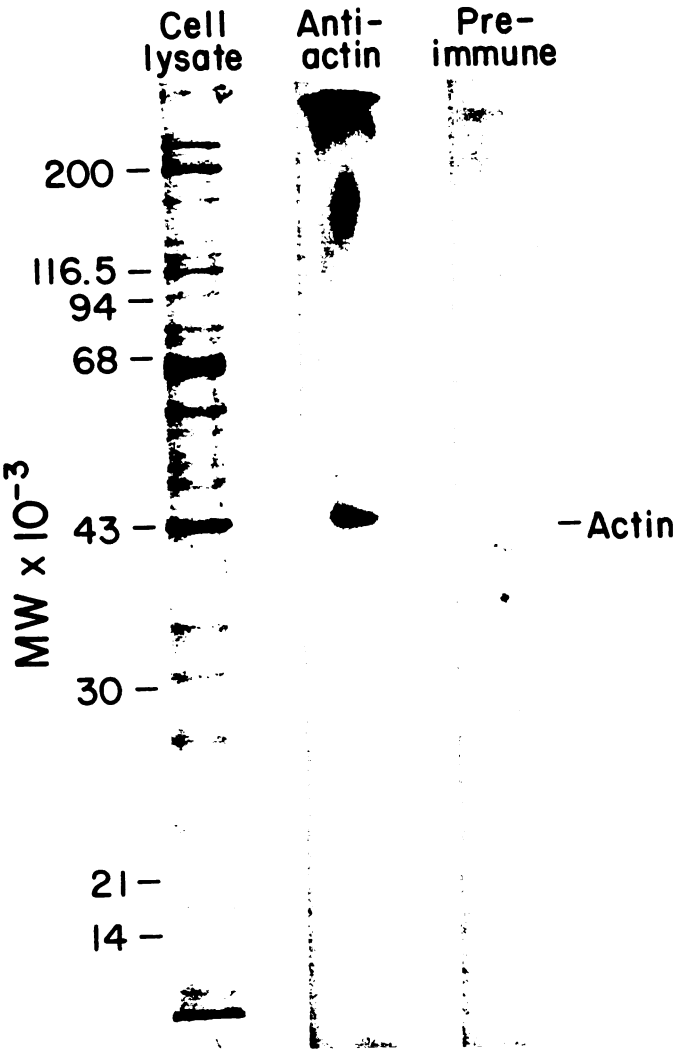



Figure 9. Identification of Adrenocortical Actin in SDS Gels by the Gel Overlay Procedure of Burridge et al. (484). Autoradiographs of SDS gels of solublized rat adrenocortical cells. After electrophoresis, lysates cell were either stained for protein (lane labelled "Cell Lysate") or incubated with guinea pig anti-muscle actin ("Anti-actin") or guinea pig preimmune serum ("Preimmune") followed by incubation with $^{125}$I-protein A. Gels were dried onto filter paper and autoradiographed using Kodak XAR-5 film for 120 hrs. at -70°C.

experiments. Taken together, the immunoprecipitation and Burridge gel experiments strongly support the conclusion that the anti-actin antisera generated does recognize adrenocortical actin.

Immunofluorescent Staining of Rat Adrenocortical Cytoskeletons: Evaluation of Cell Rounding at the Immunofluorescence Microscopic Level

Having established that the anti-actin antisera produced do cross-react with adrenal actin, adrenocortical cell cytoskeletons were prepared from cultured cells and actin-containing microfilaments visualized in situ by indirect immunofluorescence using these antisera. Cytoskeletons were prepared from cultured adrenocortical cells by solublization of cells in Triton X-100. Briefly, cultured rat adrenocortical cells were solublized by incubation in a Triton X-100 buffer at low detergent concentration for a short time to extract all soluble proteins, then fixed with absolute methanol to precipitate remaining cytoskeletal proteins. It is well known that cytoskeletal filaments such as microfilaments and microtubules are insoluble in this nonionic detergent; this protocol has been extensively used to isolate intact cytoskeletons from other cells (485). Moreover, treatment of cells with Triton X-100 does not alter the spatial arrangement of microfilaments in situ. Glycerol and EGTA were included in the lysis buffer to ensure preservation of adrenocortical microtubules, for these agents are known to stabilize microtubules (10).

Immunofluorescent staining of adrenocortical cytoskeletons

with anti-actin antisera reveals that in addition to a profound effect on cell shape, ACTH promotes a striking change in microfilament organization which is coincident with the change in cell shape. The effect of ACTH on the adrenocortical cytoskeleton is catalogued in the photographs of Figure 10. Unstimulated adrenocortical cytoskeletons are shown in Figures 10A and 10B. The brightly fluorescent concatenations highlighted by the anti-actin antiserum, especially prominent in the cell at high magnifaction in Figure 10B, correspond to the bundles of microfilaments, the so-called actin "stress cables", prominent throughout the cytoplasm of cultured cells. These cables converge at numerous points within the cell which, in the phase contrast microscope, correspond to the attachment sites of the cell to the surface of the culture plate. These sites, or "adhesion plaques", are thought to be involved in the interaction of the plasma membrane with the substratum during the proceses of cell attachment and cell locomotion (44,57). In addition, these attachment sites are thought to be intimately involved in the molecular mechanism by which some cells assume characteristic, asymmetrical shapes in culture (113). Fluorescence is particularly intense along the edges of the cell border, regions of the plasma membrane rich in dense microfilament networks. Note also the many stellar points of fluorescence throughout the cell; these most likely correspond to remnants of stress cables in focal attachment sites which have been sheared in cross-section as they converge near the plasma membrane on the ventral surface of the cell.

In contrast to unstimulated cells, adrenocortical cells incubated with ACTH show evidence of a major reorganization of actin microfilaments. Figure 10C depicts immunofluorescent staining of fully, retracted cells which have been treated with 2.2nM ACTH for 24 hours. Cytoskeletons of these cells reflect the dramatic retraction of cell shape seen in the light microscope. Most striking is the complete absence of all stress cables in these cells. Patches of fluorescence can be seen scattered throughout the cell, concentrated in regions around the nucleus and in thin cellular extensions outlining the boundaries of the cells prior to rounding. These extensions are remnants of the plasma membrane which have torn away from the cell body as the cell retracted from the surface of the plate. Figure 10D shows a higher magnification of one such retracted cell. This cell shows no evidence of stress cables. Residual fluorescence is punctate in appearance throughout the cytoplasm, and most likely represents depolymerized, monomeric actin, or actin residing as short oligomers. It is apparent that ACTH has induced a complete loss of actin stress cables in this cell.

A thorough examination of the dose dependency and time course of ACTH effect on microfilament organization was not established; however, there seems to be a strict correspondence between the effects of ACTH on cell shape and on stress cable integrity. As with cell rounding, ACTH-induced microfilament disassembly is hormone-specific; insulin and glucagon again have no effect on stress cables. As with cell rounding, administration of exogenous cAMP can mimick the effect of ACTH on stress cables

in the absence of hormone. At the earliest time of treatment tested, twelve hours after ACTH administration, a time at which cell rounding is first evident in the light microscope, actin stress cables are still evident but less clearly defined, and their spatial organization is in the process of changing along with the change in cell shape (Figure 10E). Punctate fluorescence is also beginning to appear at this time. At doses of ACTH less than 0.5nM, no evidence of either cell retraction or stress cable disassembly is evident. These results argue for a specific effect of ACTH on microfilament organization which parallels the ACTH-induced change in cell shape.

As with cell shape, the effect of ACTH on microfilament integrity is reversible. Figure 10F shows microfilament staining of fully retracted, ACTH-treated cells which have been returned to fresh medium lacking hormone for 30 hours. These cells have resumed the appearance of unstimulated cells. It is evident that the stress cables missing in the stimulated cells have reappeared, and the patches of fluorescence seen in treated cells have disappeared. Thus, ACTH-induced microfilament disassembly, as with cell rounding, is reversible upon hormone removal.

As with cell retraction, the effect of ACTH on the cytoskeleton appears to involve microfilaments as cytochalasin B can mimick the effect of ACTH on microfilament organization (Figure 10G). As with ACTH, cytochalasin B promotes complete disassembly of stress cables, resulting in depolymerized actin visualized as fluorescent patches throughout the cell. Moreover, the effect of ACTH on cytoskeletal architecture seems to be

specific to microfilaments, for colchicine has no effect on stress cable integrity. Figure 10H shows one such colchicine-treated cell. A profusion of stress cables is evident in this cell. Subsequent studies using an affinity-purified anti-tubulin antibody (gift of M. Kirschner, UCSF) to stain for adrenocortical cell microtubules have confirmed that ACTH has no effect on microtubule integrity (not shown).

As with the change in cell shape, ACTH-induced changes in microfilament assembly, and reversal of this effect upon hormone removal, are not dependent on synthesis of new protein or RNA; both effects occur in the presence of cycloheximide or actinomycin D.

The observation that cytochalasin B, but not colchicine, produces microfilament disassembly itself testifies to the specificity of the anti-actin antiserum used, for the cytochalasins are known to depolymerize actin filaments in vitro and in living cells (486). Further proof of the specificity of this staining procedure for actin-containing structures is shown in Figure 10J. Here, guinea pig anti-actin antiserum was saturated with purified rabbit muscle actin prior to staining. It is evident that this treatment prevents staining of stress cables. The diffuse background fluorescence is artifactual, as it is also present in controls in which guinea pig preimmune serum is used in lieu of anti-actin antiserum (Figure 10I).

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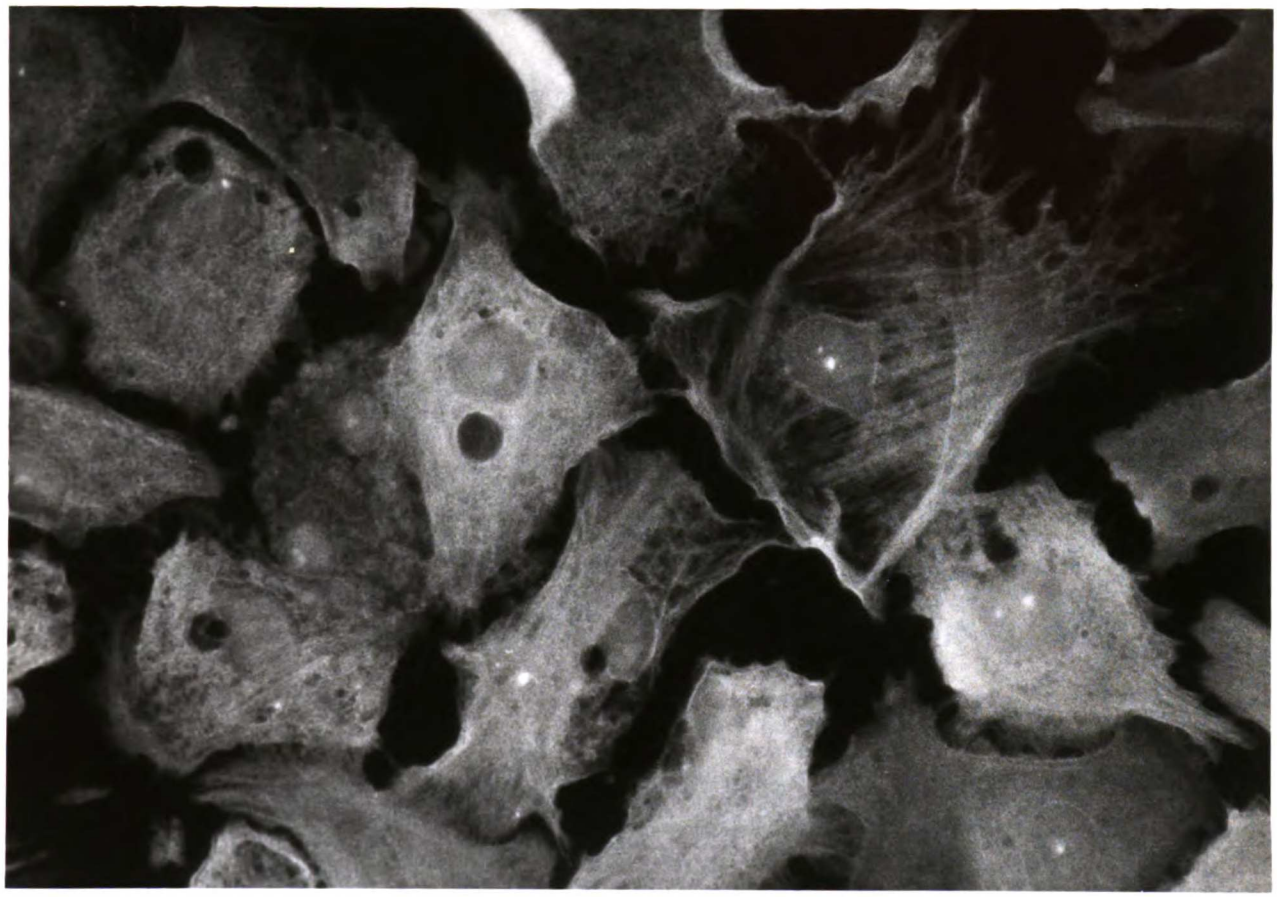
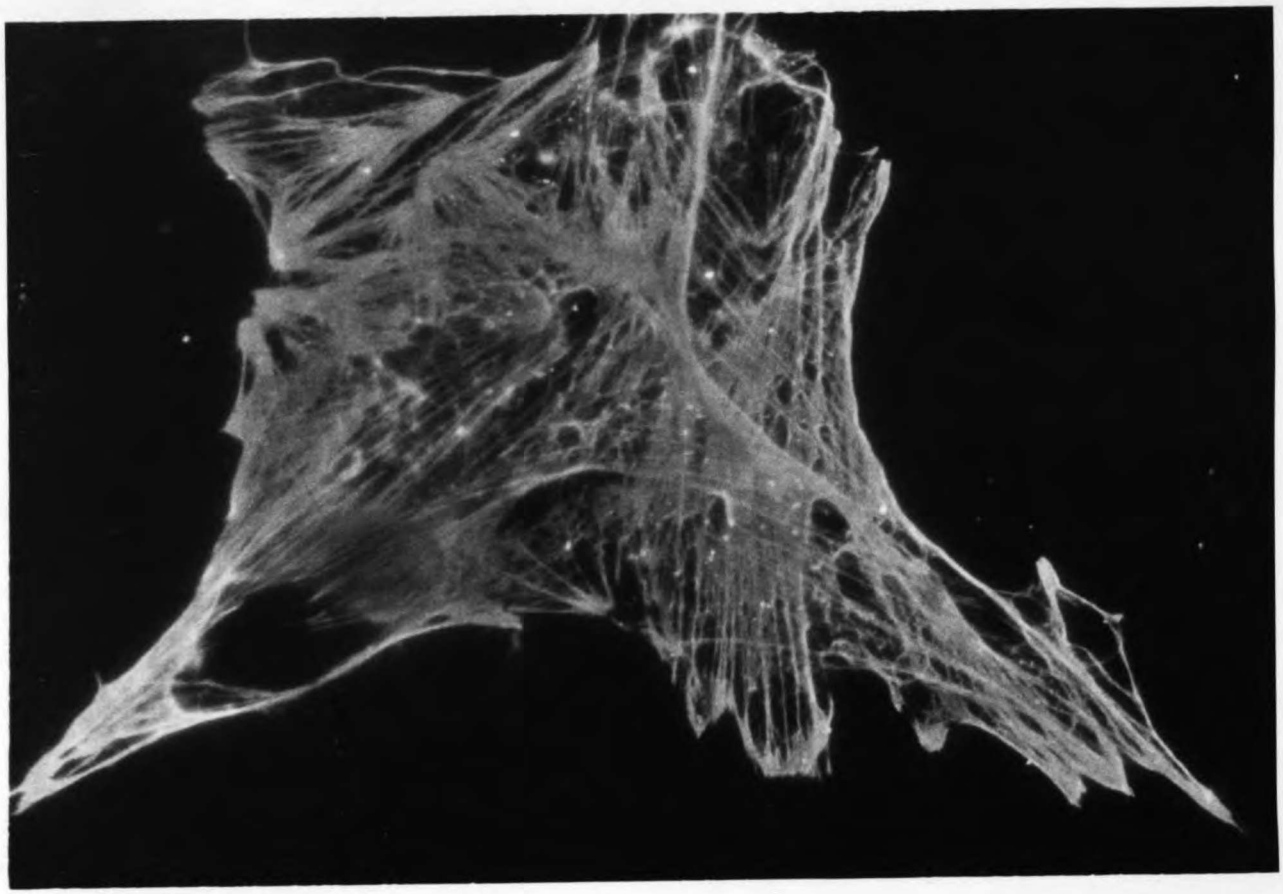
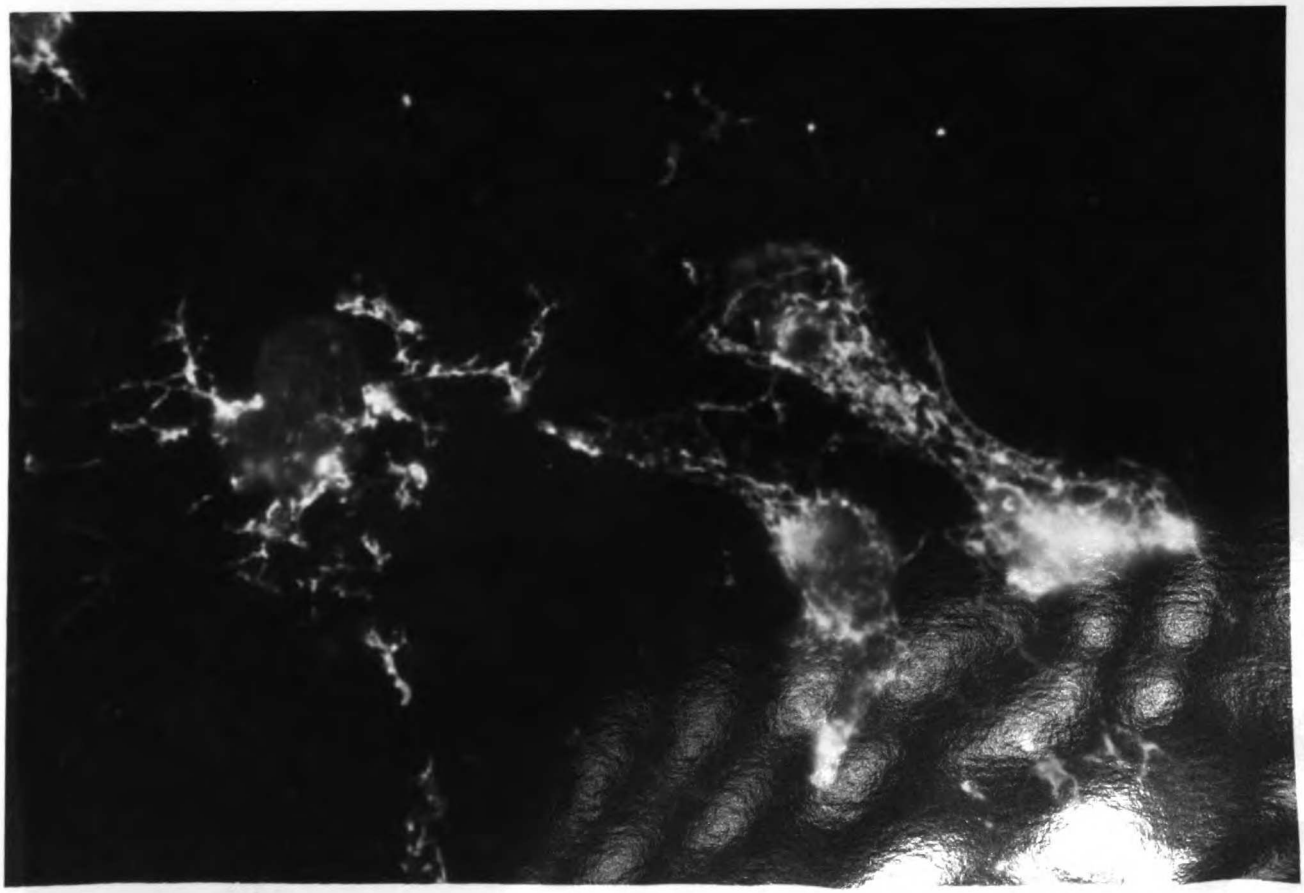


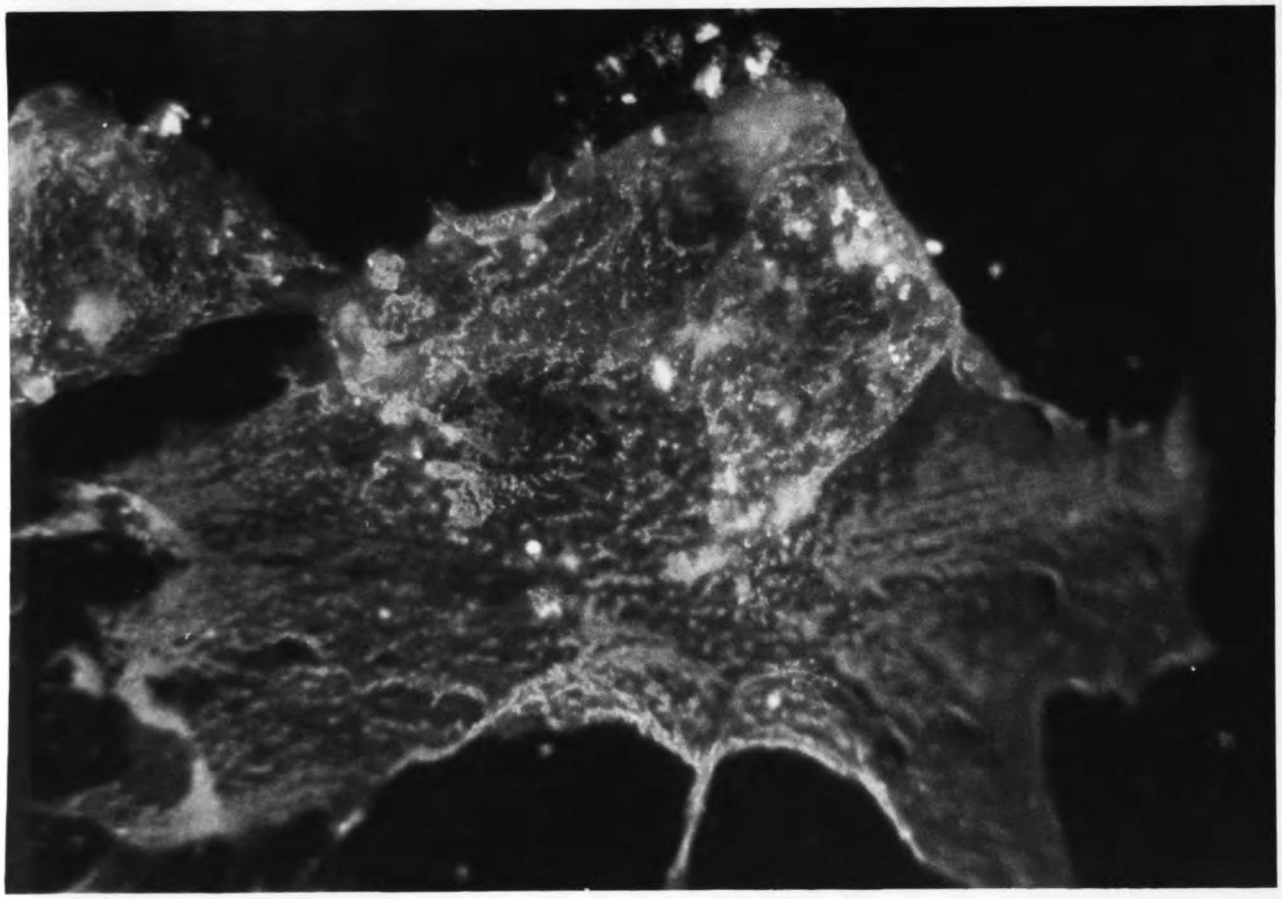
Figure 10. Indirect Immunofluorescence of Rat Adrenocortical Cells. Cells were grown at low density on glass coverslides, treated with ACTH or drugs as indicated, then processed for immunofluorescent staining as described in "Methods" section, Chapter 3. Guinea pig anti-actin was used at 1:20 dilution; FITC-labelled rabbit anti-guinea pig IgG was used at 1:25. A) control cells; B) high magnification of one control cell; C) 2.2nM ACTH for 24 hrs.; D) high magnification of one ACTH-treated cell; E) one ACTH-treated cell after 12 hrs. of treatment; F) 2.2nM ACTH for 24 hrs. followed by 30 hrs. without hormone; G) 10uM cytochalasin B; H) 50uM colchicine; I) control cell staining with guinea pig preimmune serum in lieu of anti-actin antiserum; J) anti-actin antiserum saturated with muscle actin prior to staining.



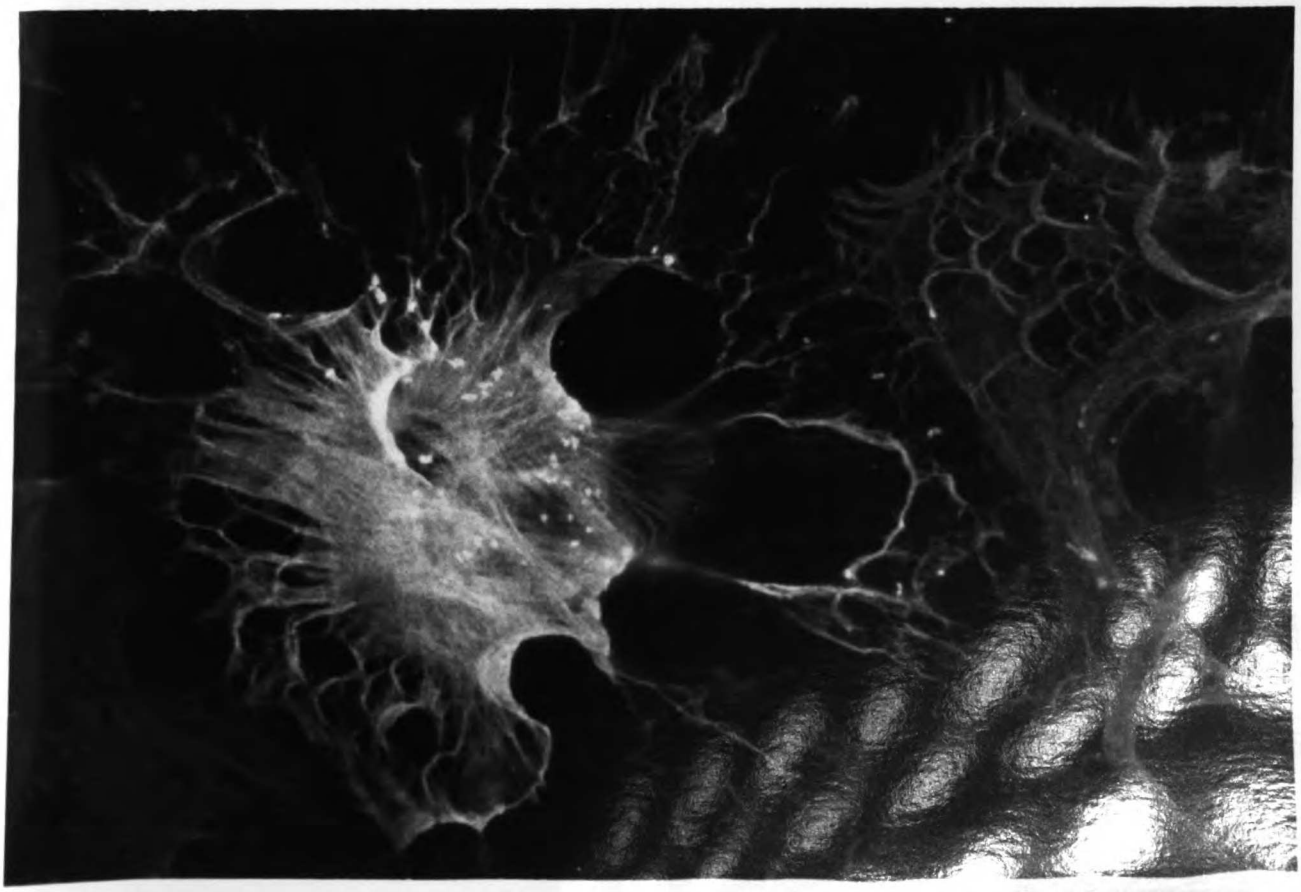
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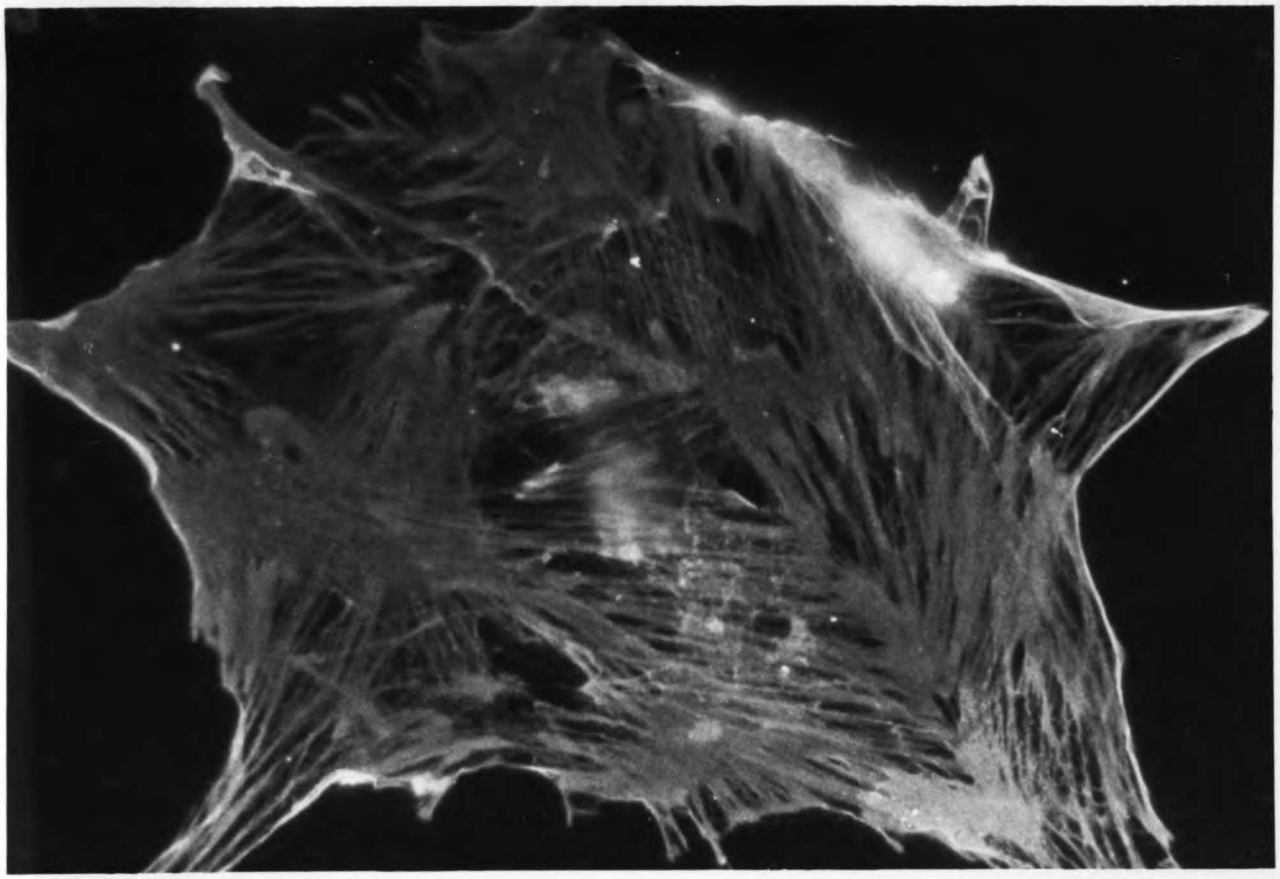
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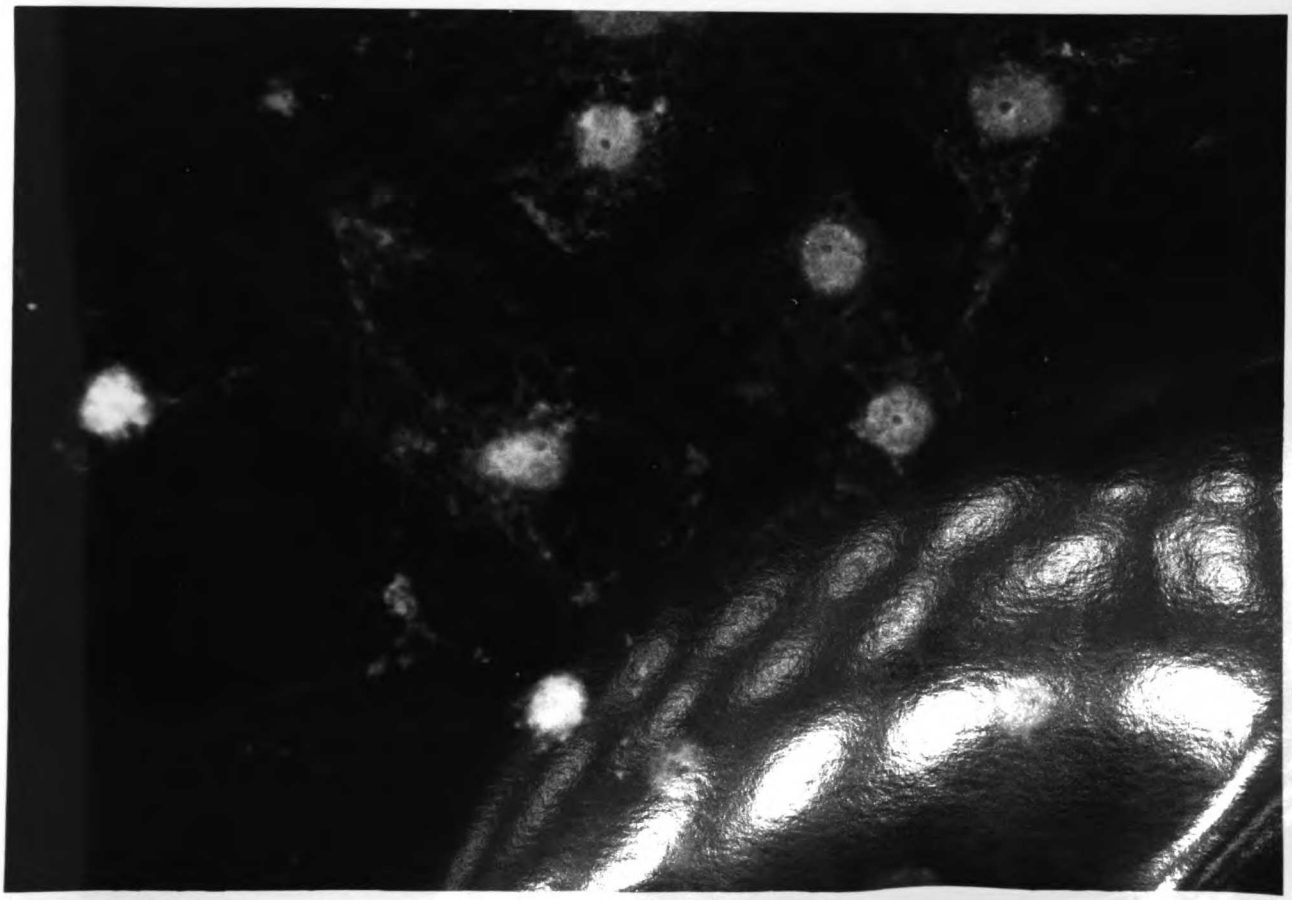
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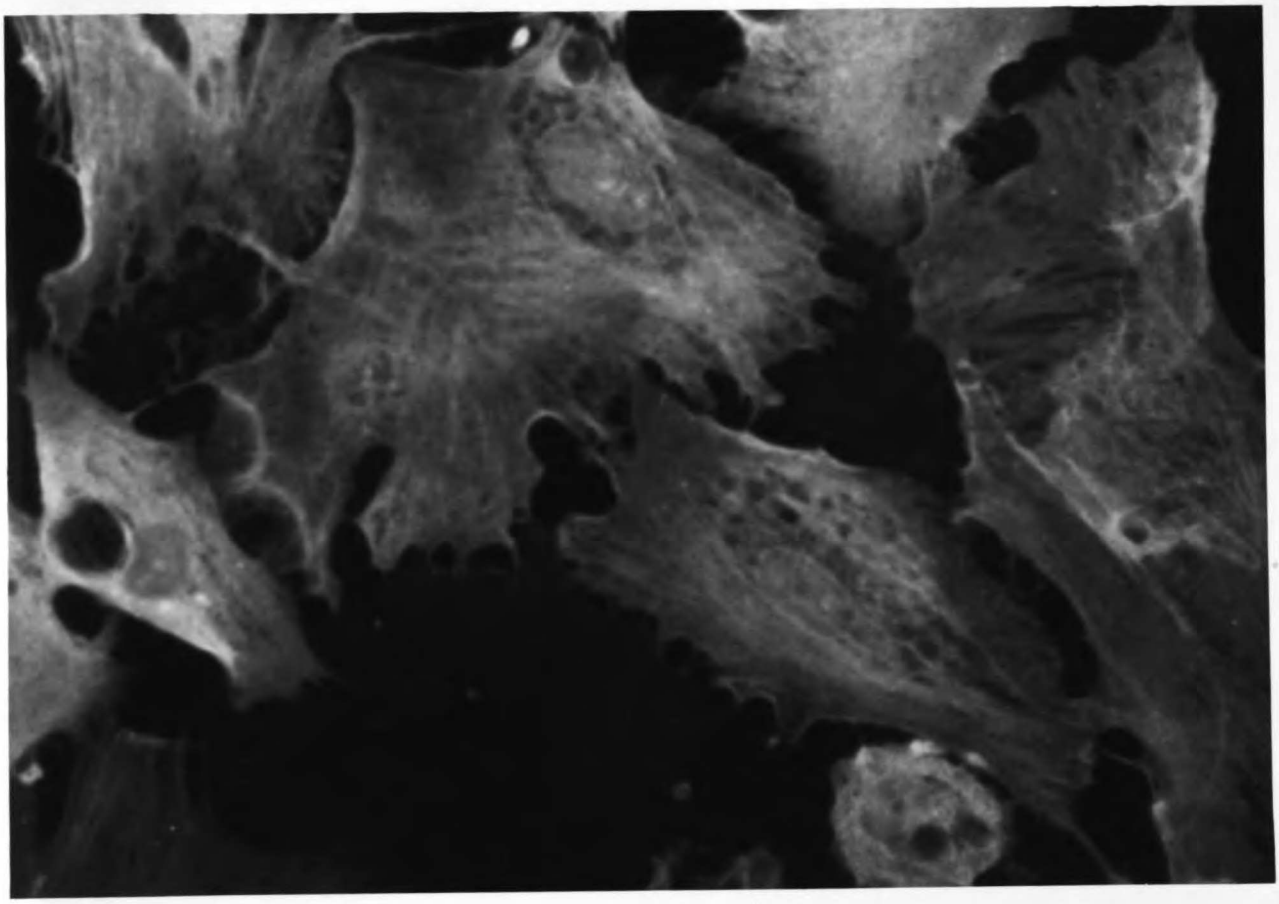
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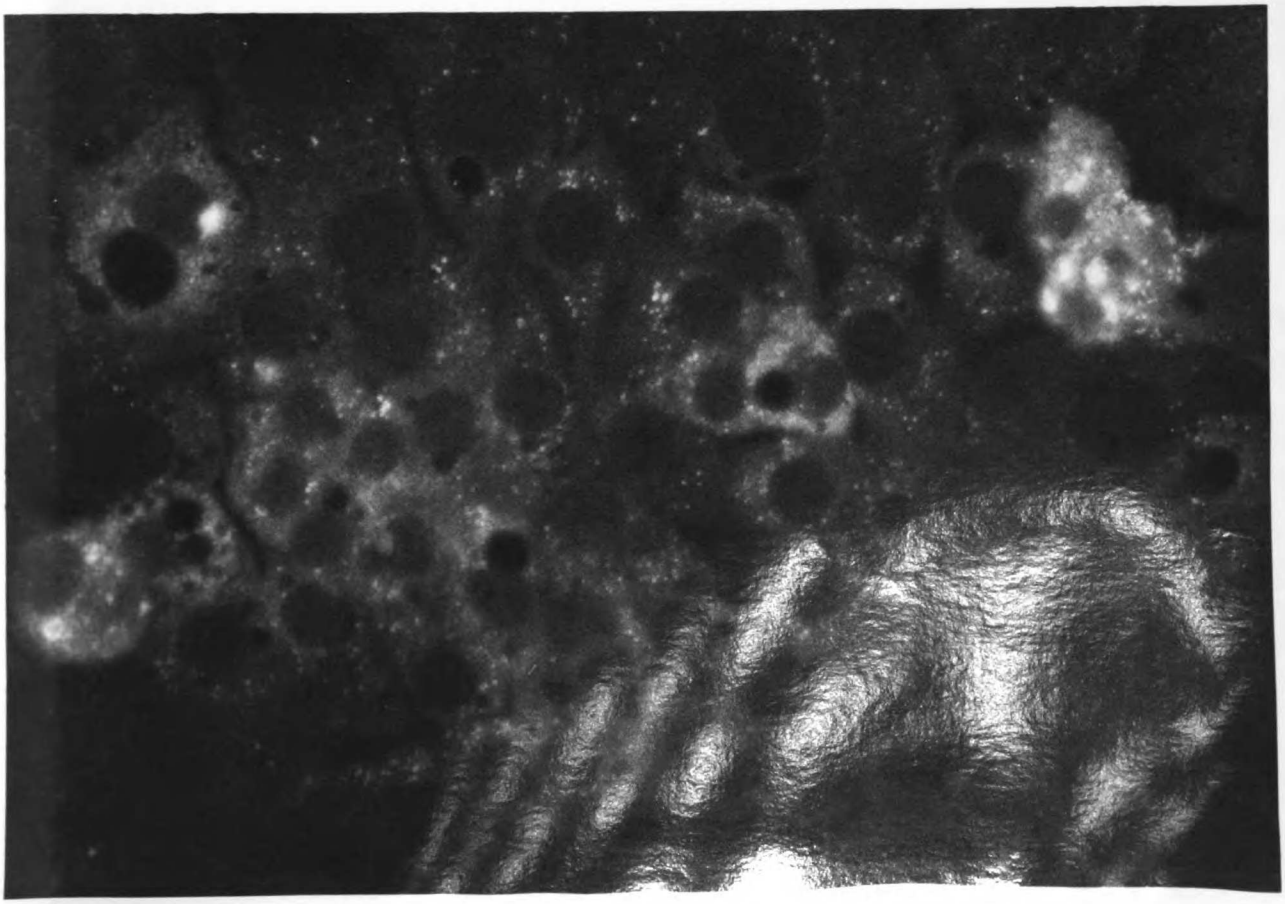
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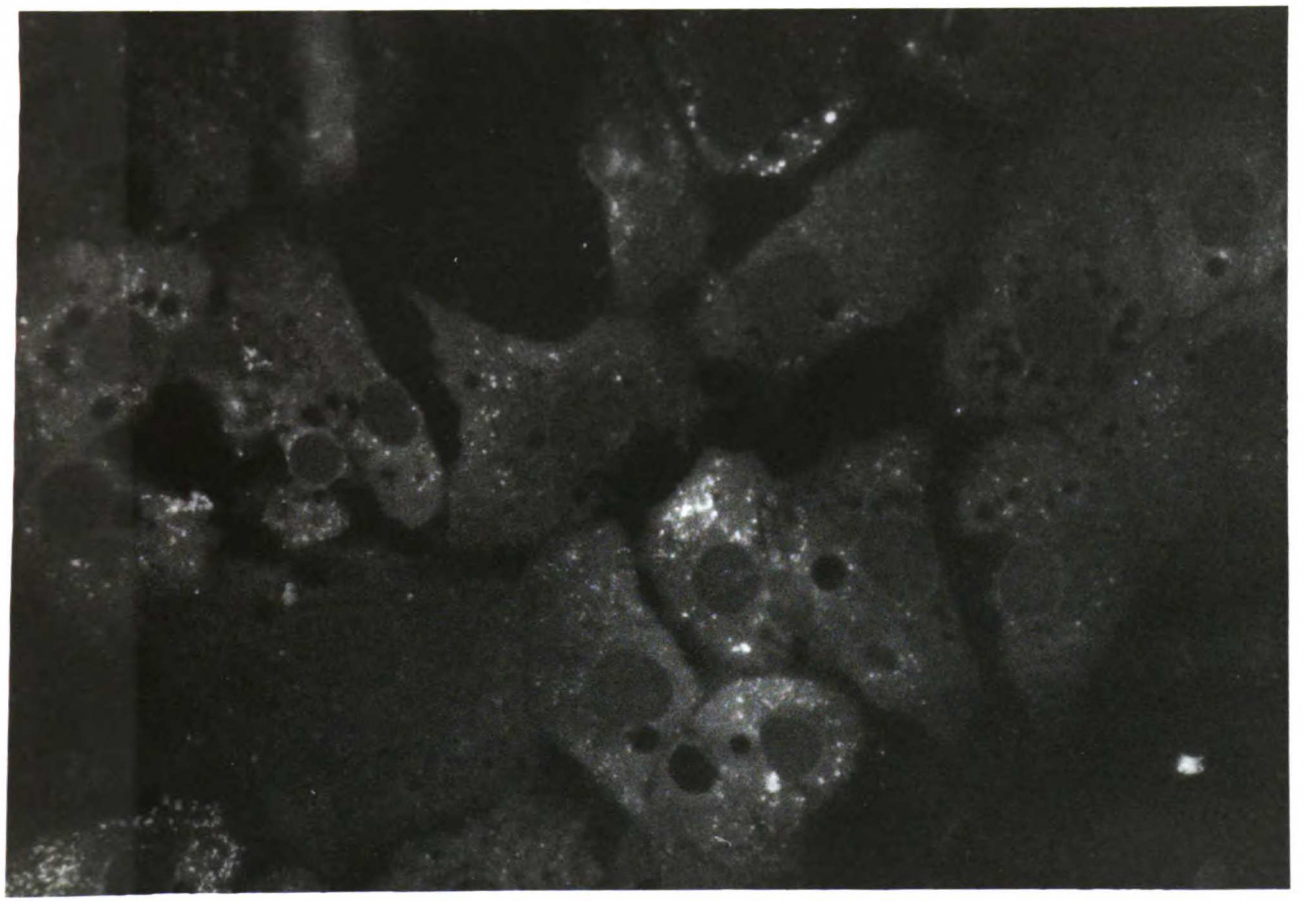


H.



I.

J.



# CHAPTER 4

# EVIDENCE FOR HORMONAL REGULATION OF RAT ADRENOCORTICAL ACTIN CONTENT

## The Initial Observation

The evidence reported thus far strongly indicates that ACTH regulates the organization of the cytoskeletal protein actin in cultured rat adrenocortical cells. That ACTH might influence adrenocortical actin content as well as organization was first indicated in early studies in which lysates of ACTH-treated adrenocortical cells were analyzed by SDS-PAGE. It was noted in these studies that when cultured rat adrenocortical cells were induced to retract by chronic incubation with ACTH, and total cellular proteins separated by SDS-PAGE, these cells contained less protein staining relative to untreated cells in the band on the gel co-migrating with purified muscle actin, i.e., the band at a molecular weight of approximately 45,000 daltons. Densitometric scans of gels from ACTH-treated and untreated cells confirmed that treated cells indeed contained less protein in this band. Furthermore, the loss of protein in this band seemed to be rather specific to this band, for the intensity of other bands in the gel remained unaltered with hormone treatment. That this band represents adrenocortical actin is indicated both by its co-migration with purified muscle actin and by the fact that this band is the same band which is specifically recognized by guinea pig anti-actin antisera generated against muscle actin (Figure 9).

Attempts to reproduce the effect of ACTH on adrenocortical actin, demonstrated in Figure 11, have confirmed this initial observation. Here, rat adrenocortical cells have been either treated (bottom panel) or not treated (top panel) with 2.2nM ACTH for 24 hrs., and equivalent numbers of cells lysed in SDS and analyzed by SDS-PAGE on a 10% polyacrylamide gel. Scans of dried gels confirm the visual loss of protein in the band at 43,000 daltons. Densitometric analysis of the peak at this position relative to other peaks indicates that this band amounts to 10-12% of the total cellular protein in untreated cells, and 7-9% of the total protein in treated cells, a decrease in content of this band of approximately 25% relative to control. Neglecting other minor changes in protein contents not quantifiable by this crude analytical technique, a drop of 25% in this one band suggests a decrease of approximately 2% of the total cellular protein in response to ACTH. Such a major change in protein is not trivial, and must have tremendous consequences for cell function. Moreover, the observation that this change seems to be under hormonal control suggests the exciting possibility that this change might be involved in the mechanism of action of ACTH in this cell, and in particular in the hormonal control of adrenocortical cell shape.

The change in actin content induced by ACTH follows a time course which parallels the effect of the hormone on cell shape. Figure 12 is a composite of densitometric scans of gels of adrenocortical cell lysates prepared from cells treated with ACTH for various times. Panel A shows unstimulated cells. Panels

B,C, and D show scans from cells treated with 2.2nM ACTH for 24, 96, and 144 hrs., respectively. It is apparent that the ACTH induces loss of protein from this band, and that the extent of this loss is a function of time of treatment. Furthermore, hormone-induced actin loss is dose-dependent, as shown in Figure 13. Here, adrenocortical cells were treated with various concentrations of ACTH for 24 hrs., and cell lysates analyzed by SDS-PAGE. The apparent relationship between ACTH dose and actin loss closely parallels the relationship between hormone concentration and cell rounding both in magnitude of change and in range of hormone concentration. Finally, as with ACTH-induced cell rounding, the effect of ACTH on actin content is hormone-specific (Figure 14). Insulin and beta-endorphin have no significant effect on the content of the actin band. Interestingly, the naturally occurring peptide corresponding to residues 7-38 of ACTH (panel C), also has no effect on actin content. This peptide lacks steroidogenic activity, and is a full antagonist of ACTH in terms of stimulation of cAMP production (462), yet has no effect on cell shape. This observation implies that whatever structural aspect enables ACTH to influence steroidogenic activity in this cell also confers on the molecule the potential to alter adrenocortical cell shape and actin content.

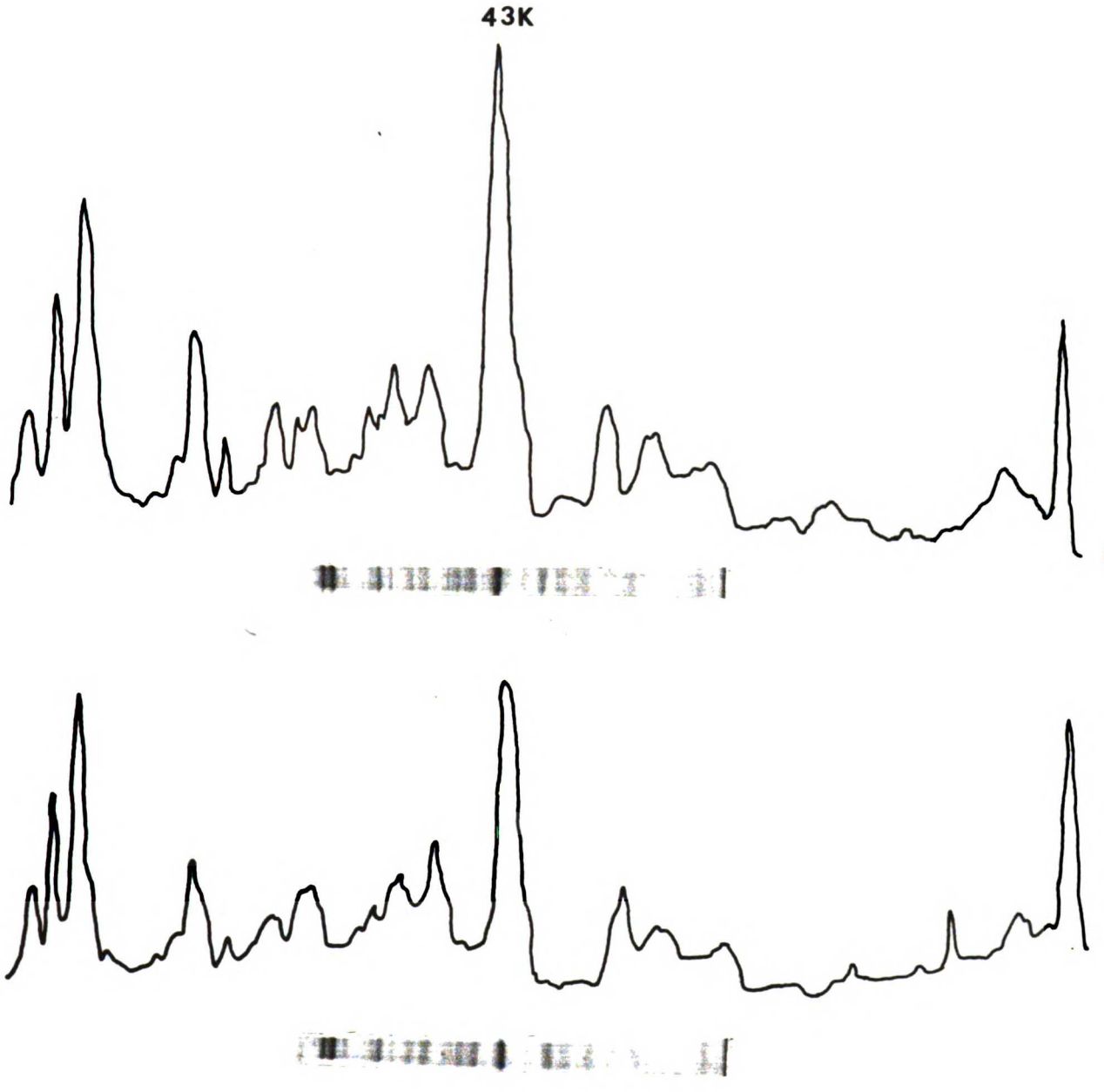



Figure 11. Densitometric Analysis of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Gels. Rat adrenocortical cells were incubated with (bottom) or without (top) 2.2nM ACTH for 24 hrs. and processed as described under "Methods" section, Chapter 3, for SDS-PAGE. Gels were scanned with a GCA/McPherson Model EU 205-11 scanning densitometer at $OD_{550nm}$ at a rate of 5cm/min at 1mM sensitivity.

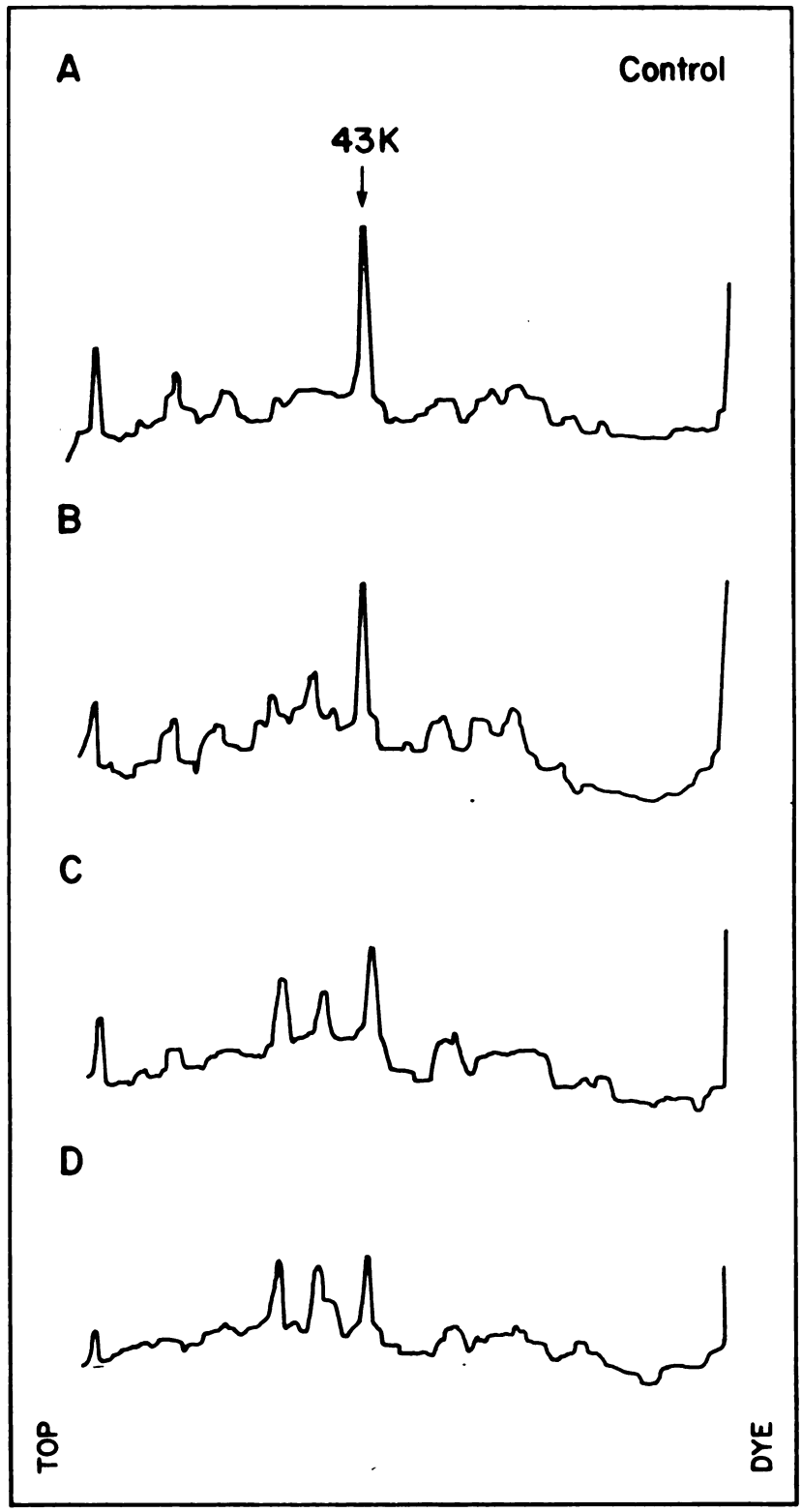



Figure 12. Time Course of ACTH-Induced Actin Loss Detected by SDS-PAGE. Rat adrenocortical cells were cultured for 7 days, then treated with 2.2nM ACTH for 0 hr. (A), 24 hrs. (B), 96 hrs. (C), or 144 hrs. (D), and processed as described in the legend to Figure 11. Scans were aligned at the stacking gel end (edge labelled "Top"). The position of G-actin (43K) is indicated by the arrow.

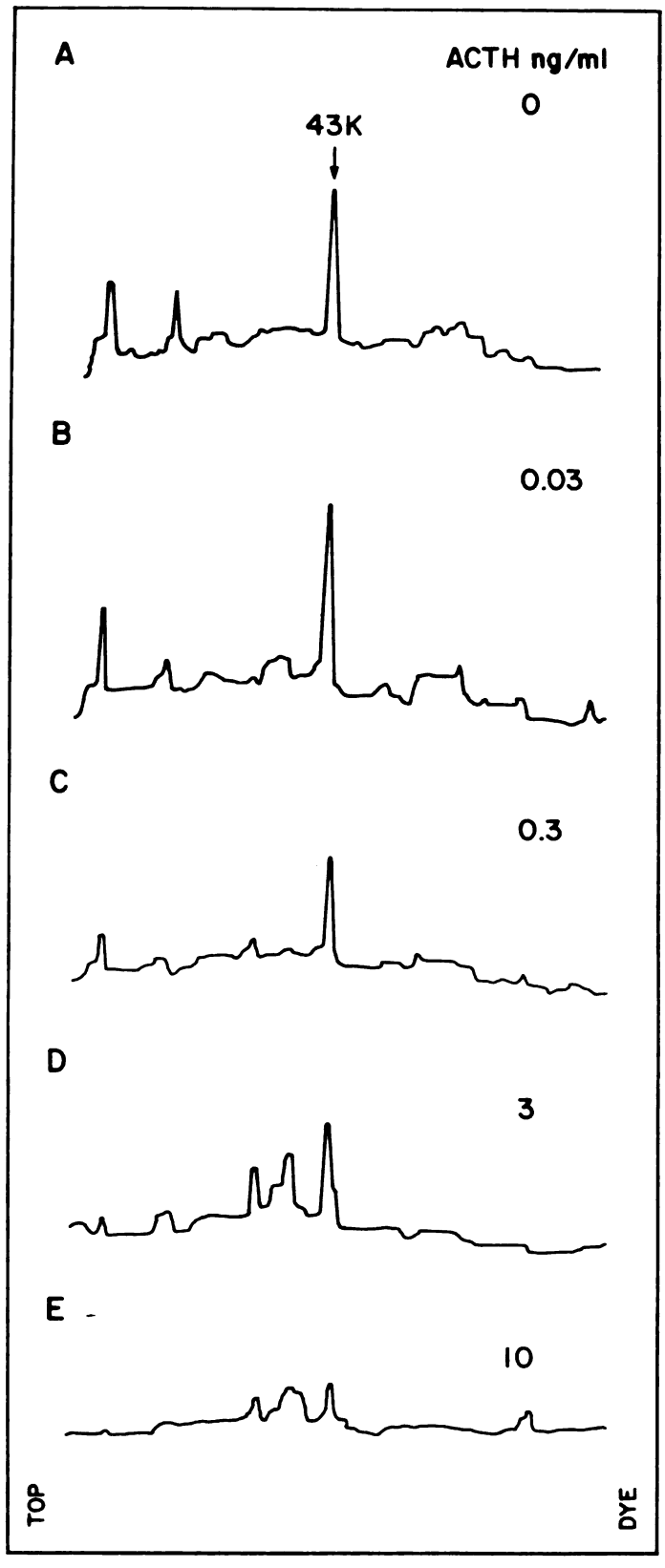



Figure 13. Dose-Response Relationship of ACTH-Induced Actin Loss Analyzed by SDS-PAGE. Rat adrenocortical cells were cultured for 7 days, then treated with the various concentrations of ACTH noted for 48 hrs. Cells were processed and gels scanned for protein as described in Figure 11. The arrow labelled "43K" indicates the position of G-actin.

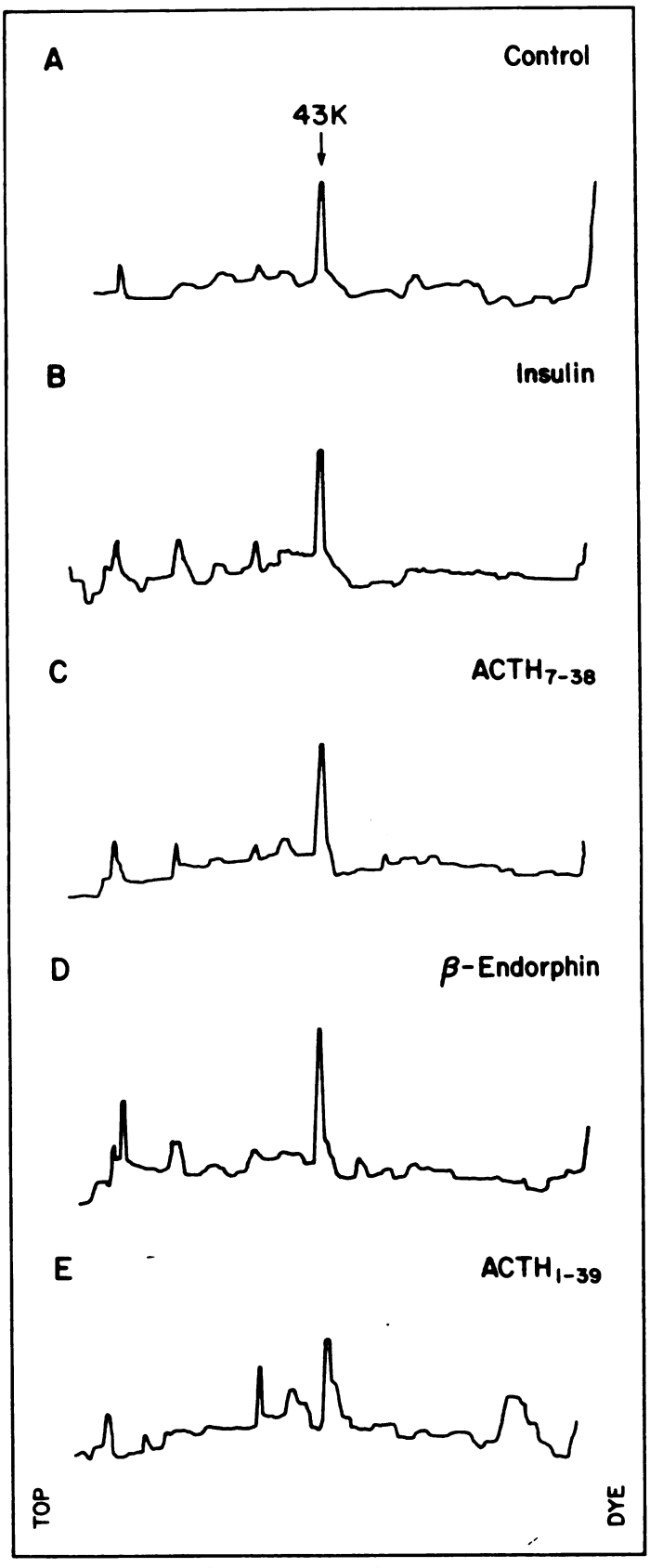



Figure 14. Specificity of ACTH Effect on Adrenocortical Actin. Densitometric scans of SDS gels of rat adrenocortical cells treated with various hormones. All peptides were used at 10ng/ml, and treatment was for 24 hrs. The arrow labelled "43K" corresponds to the position of G-actin.

Further Quantitation of ACTH-Induced Actin Loss: The DNase I Inhibition Assay

The results presented thus far strongly suggest that actin content and organization in the rat adrenocortical cell may be under hormonal control, and that changes in the integrity of the cytoskeleton in response to ACTH may be involved in the underlying molecular mechanism of action of ACTH-induced cell rounding. In order to further explore ACTH control of adrenocortical microfilament structure and organization, we felt that a more accurate means of quantitating actin content was required.

To this end, the DNase I inhibition assay for actin developed by Blikstad et al. (456) was utilized. This assay has been used previously to accurately measure actin contents in a diverse number of tissues (456,487,488,489). Moreover, this assay has the capability of estimating the size of cellular pools of monomeric and polymeric actin. This assay is based on the fact that monomeric actin (G-actin) binds tightly to the pancreatic enzyme deoxyribonuclease I (DNase I), and forms an essentially irreversible 1:1 complex with this protein such that it inhibits the DNase degrading activity of the enzyme (490). High affinity binding of actin to DNase I is limited to monomeric actin; therefore, under conditions which do not disturb the organizational state of actin in the cell upon solublization of proteins from the cell, this assay can be used to distinguish monomeric actin from polymeric actin. Moreover, previous studies have shown that muscle and nonmuscle actins effect inhibition of DNase enzymatic activity in an identical fashion (459).

Therefore, this assay does not discriminate between actins from different sources, and for this reason, offers a distinct advantage over immunological approaches to quantitating actin contents which are generally plagued with problems related to heterogeneity of antigenic determinants in different actins.

For the purpose of measuring total actin contents from adrenocortical cells, guanidine hydrochloride at a final concentration of 0.75M was included in the extraction buffer to ensure complete actin depolymerization. This concentration of guanidine-HCl is known to effect reversible depolymerization of actin filaments without denaturation of the protein (491).

To prepare actin from adrenocortical cells in culture, cells were lysed in a buffer containing Triton X-100 and immediately mixed 1:1 with a second buffer containing 1.5M guanidine-HCl. Insoluble material was removed by centrifugation, and aliquots of lysates diluted prior to assaying. The assay itself measures the degree of inhibition of a known amount of DNase I on calf thymus DNA, using the increase in absorbance at $OD_{260nm}$ as the means of monitoring DNA degradation (456). As G-actin forms a stable 1:1 complex with DNase I, the degree of inhibition of enzyme activity is a direct index of the amount of actin present. Standard curves are generated in this assay using purified rabbit muscle actin treated with extraction buffer as a standard, and cell lysates are always diluted such that inhibition occurred in the linear part of the dose-response curve.

Figure 15 shows that the DNase I inhibition assay yields a linear dose-response relationship between actin amount and inhi-

bition of DNase activity. Here, purified muscle actin at the amounts indicated is plotted as a function of inhibition of enzymatic activity. It is clear that in the range of 0.3 to 1.5ug of actin, this dose-response relationship is linear.

With this assay, the effect of ACTH on rat adrenocortical cell actin content observed by SDS-PAGE was reinvestigated. The results shown in Table I confirm that ACTH initiates a dose-dependent decrease in actin content. At 0.22nM ACTH, a dose at which no visible sign of cell rounding is evident in the light microscope, there is a small but significant decrease in total actin content relative to untreated cells; at 2.2nM ACTH, a dose at which these cells fully retract, there is a loss of more than 20% of the total actin. This loss can not be attributed to ACTH-induced changes in cell number, for there is no significant difference in DNA content between treated and untreated cells over the 12 hour incubation period. This is an important consideration in light of the fact that ACTH inhibits DNA synthesis in these cells in culture (438).

The time course of ACTH action on actin content, as measured by the DNase inhibition assay, is shown in Figure 16. It is apparent that ACTH induces a maximal loss of 21-25% of the total actin within 12 hours after hormone administration. Again, the time course of ACTH action on adrenocortical actin content parallels that of hormone-induced cell rounding and stress cable loss. The specificity of the change in actin content induced by ACTH is shown in Figure 17. Other basic polypeptide hormones such as beta-endorphin and insulin (not shown), which neither

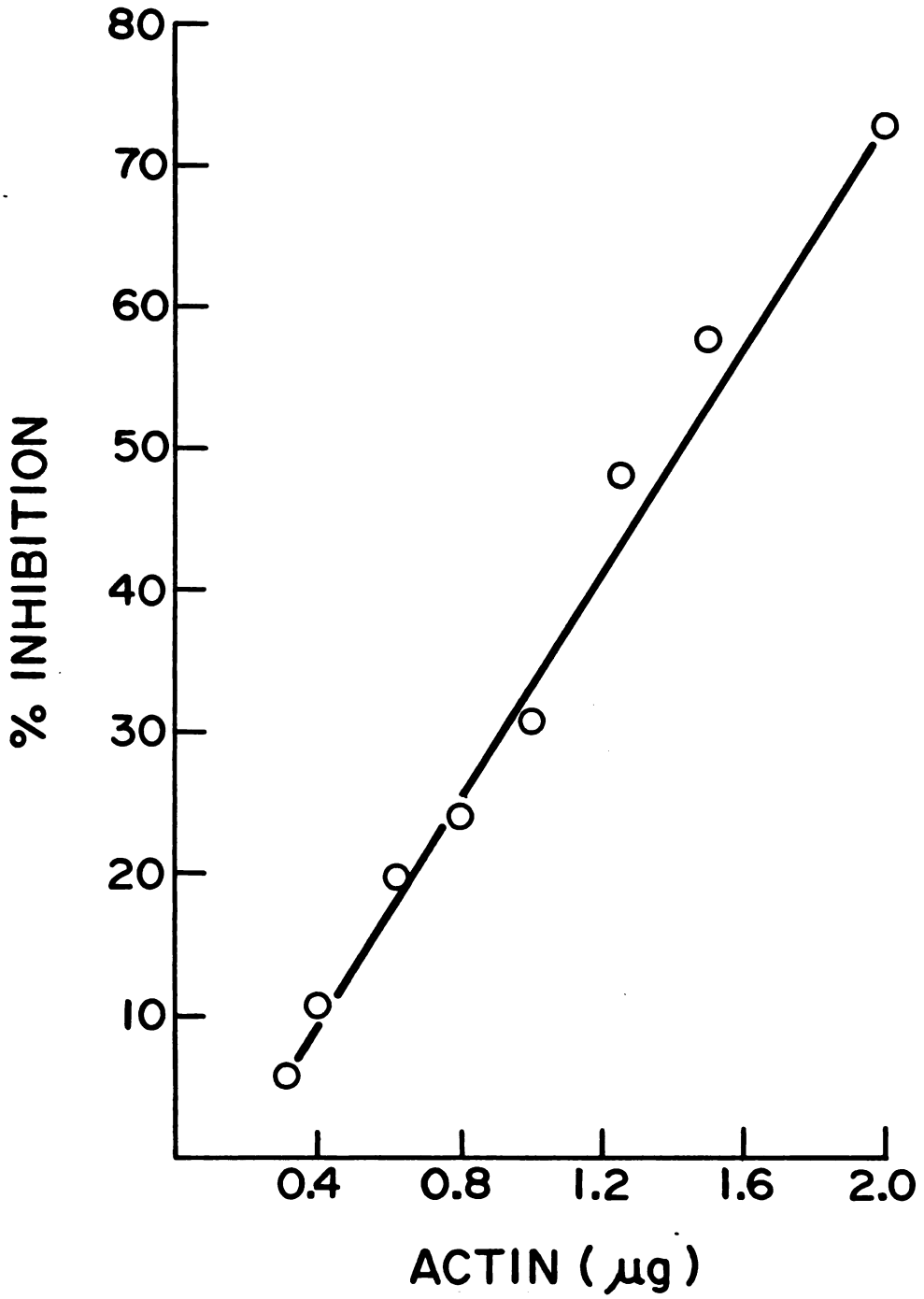



Figure 15. Standard Curve for Actin Measurement by the DNase I Inhibition Assay. Purified rabbit skeletal muscle actin was used to inhibit DNase I as described in the "Methods" section of Chapter 3. Each point represents the mean of three determinations. The correlation coefficient is 0.992.

TABLE I

Effect of ACTH on Rat Adrenocortical Cell Actin

Adrenocortical cells (5 x $10^5$/well) were incubated with or without 2.2nM ACTH for 12 hrs., 7 days after plating. The cells were processed, and actin content determined by the DNase inhibition assay as described in "Methods" section, Chapter 3. DNA content was assessed according to Short et al. (467). Values are mean $\pm$ S.E. of the number of separate determinations shown in parentheses.

| Hormone | Actin | DNA | Actin/DNA | Decrease |
|---|---|---|---|---|
| | ug | ug | | % |
| None | 18.84±0.64 (4) | 4.05±0.02 (3) | 4.65±0.16 | |
| ACTH (0.22nM) | 16.70±0.00 (8) | 4.08±0.07 (3) | 4.09±0.07 | 12 |
| ACTH (2.2nM) | 14.85±0.07 (6) | 3.98±0.04 (3) | 3.67±0.04 | 21 |

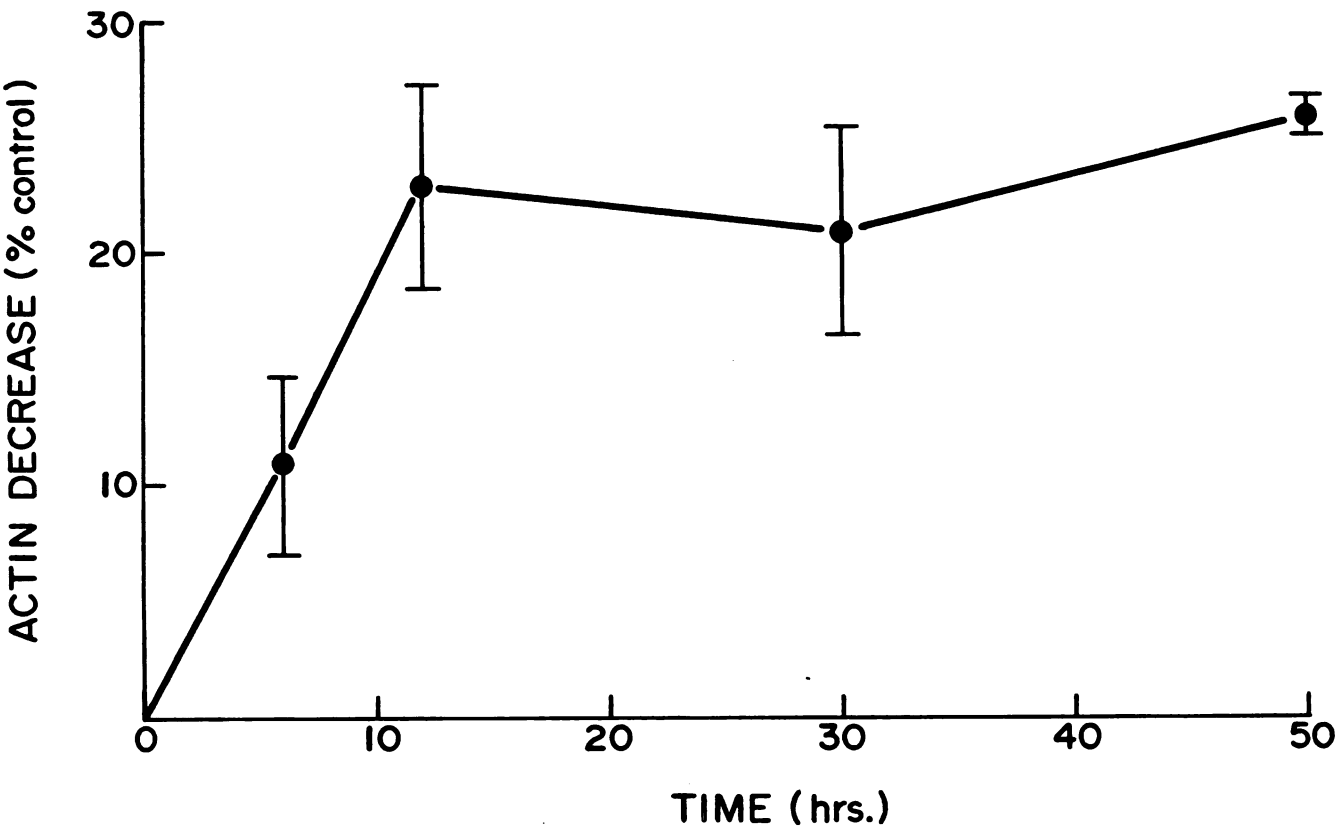



Figure 16. Time Course of the Decrease in Adrenocortical Actin Content Induced by ACTH. Cells were incubated with or without 2.2nM ACTH, and at the specified times, processed for measurement of actin by DNase inhibition. DNA was also measured at each time point. Results are from four separate experiments. Each point represents the mean $\pm$ S.D. of at least three experiments. In each experiment, cells were incubated in triplicate with or without ACTH. The decrease in actin, expressed as a percent of that level found in untreated cells, was calculated from the weight ratio micrograms of actin/ug DNA at each time point.

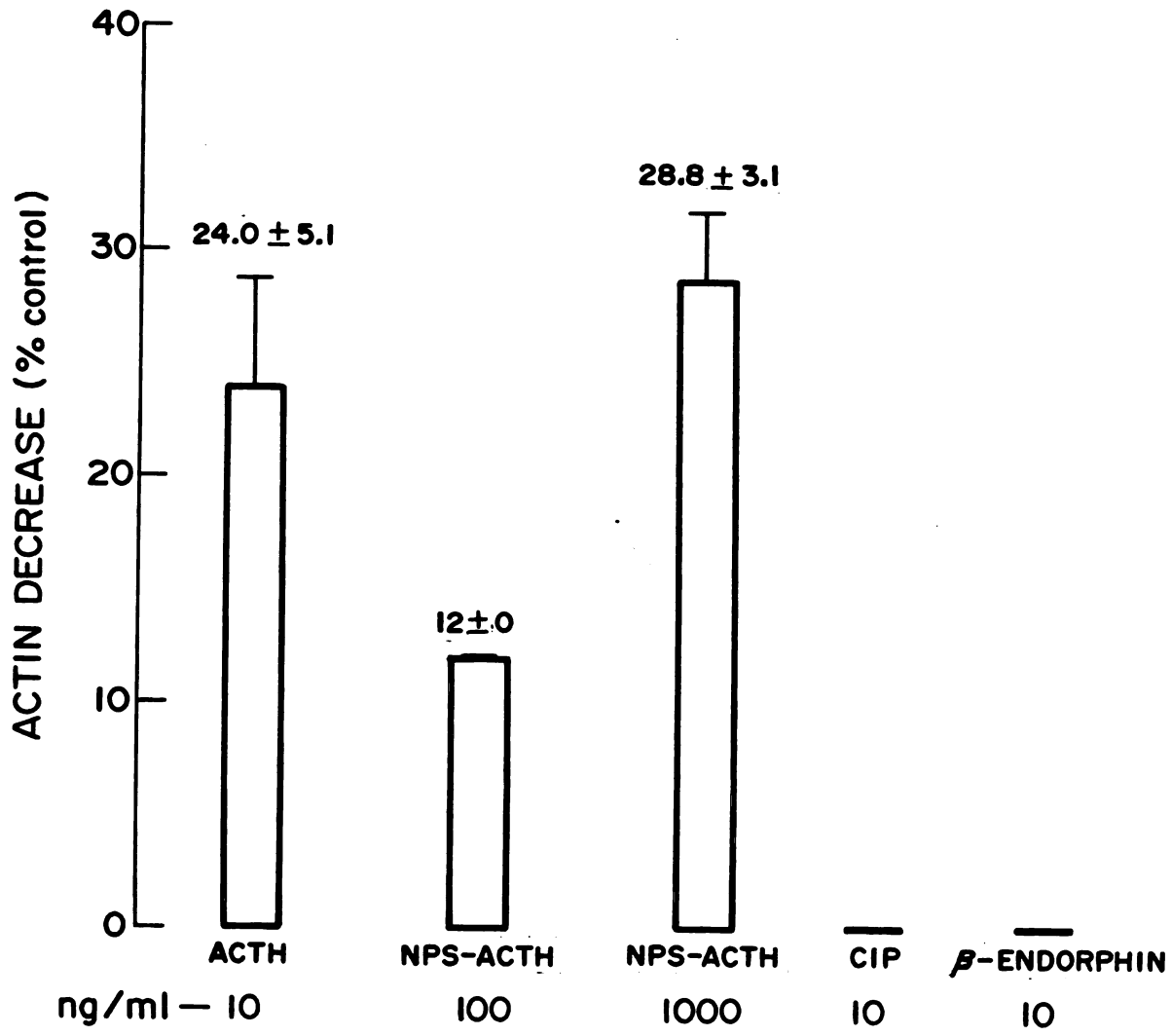



Figure 17. Specificity of ACTH-Induced Actin Loss. Cells were incubated with the various peptides for 24 hrs., then processed for actin determination by the DNase I inhibition assay. Values are the means ± S.D. of triplicate incubations. CIP, corticotropin-inhibiting peptide; NPS, o-nitrophenyl sulfenyl.

stimulate adrenocortical steroidogenesis nor promote changes in cell shape, have no effect on actin content. Corticotropin-inhibiting peptide (CIP), a naturally occurring peptide corresponding to $ACTH_{7-38}$, also has no effect on actin content. This peptide has no intrinsic steroidogenic activity, and is a full antagonist of the native hormone on stimulation of steroidogenesis (462). Interestingly, o-Nitrophenylsulfenyl-$Trp^9$-ACTH, which is a weak ACTH agonist (492), also produces a concentration-dependent decrease in actin but only at higher doses. Not shown is the further observation that 8-Br-cAMP can produce an identical drop in actin content in the absence of ACTH.

As with ACTH-induced cell rounding and stress cable loss, the effect of ACTH on adrenocortical actin content is reversible upon hormone removal. Cells incubated in the absence of ACTH for 20 days had 5.38 $\pm$ 1.23ug of actin/ug of DNA, compared to a value of 2.45 $\pm$ 0.43ug/ug DNA for cells treated for an equivalent time with 2.2nM ACTH. Cells treated with ACTH for 14 days then incubated for 6 more days in the absence of ACTH had 6.35 $\pm$ 1.12ug of actin/ug of DNA.

The results of the effect of ACTH on actin content obtained with the DNase I inhibition assay are strikingly similar to those obtained by densitometric scanning of SDS gels of cell lysates. Both assays reveal a hormone-induced loss of 20-25% of the total cellular actin at physiological doses of ACTH. Both assays reveal an effect of ACTH on actin content over a similar time course. Furthermore, both assays demonstrate that this effect on actin

content is dose-dependent and hormone-specific for ACTH, and that ACTH analogs which can stimulate steroidogenesis can also promote cell retraction and actin loss. The fact that these assays yield similar results testifies to their accuracy in recording an identical cellular event by two very different approaches.

Since the discovery that actin is the naturally occurring inhibitor of deoxyribonuclease I (482), several groups have used the binding of actin to this protein as the basis of assays to identify, isolate and quantitate actins from a variety of cell sources. The DNase I inhibition assay of Blikstad et al. (456) described above has been extensively used to identify actin in nonmuscle tissues, and to quantitate the contents of monomeric and polymeric actin in a host of cells. The ability to distinguish G- and F-actin pools by this assay is responsible for much of the evidence that hormones and other extracellular stimuli can influence the state of intracellular actin assembly (488,493). Binding of actin to DNase I immobilized to agaorse (394,491) or sepharose (494) has been used to purify actins from such diverse sources such as platelets (456), retinal ganglion cells (494), and from isolated epithelial microvilli (453). Moreover, DNase I affinity chromatography has been extensively used to identify actins in cellular extracts (496), and has become a common criterion for positive identification of actin.

Several immunological studies have attempted to characterize nonmuscle actins using antisera generated against actin. Morgan et al. (495) developed a radioimmunoassay to explore the antigenic properties of a series of purified actins from rabbit

skeletal muscle, chicken embryo brain, bovine brain and bovine cardiac muscle. All four unlabeled actins were capable of some degree of inhibition of binding of $^{125}I$-labeled chicken embryo brain actin to homologous antiserum, demonstrating shared or similar antigenic determinants. However, the $ED_{50}$ values varied over two orders of magnitude between chicken embryo brain actin and rabbit skeletal muscle actin in this assay. In the cardiac actin radioimmunoassay, unlabeled cardiac and skeletal muscle actins inhibited the binding of $^{125}I$-labeled cardiac actin to homologous antiserum, but neither brain actins inhibited the binding, suggesting that muscle actins possess at least one antigenic determinant not expressed in the brain actins, in addition to the share determinants. Furthermore, cardiac actin and skeletal actin generated different inhibition curves in the cardiac actin radioimmunoassay, demonstrating that, although antigenically related, they did not react identically in this assay. These results suggested that actins from different sources possess both shared and unique antigenic determinants. Correlations of immunoreactivity of these actins with existing amino acid sequence data (496) have strongly suggested that alterations in only a few residues of the actin molecule can significantly alter its antigenic properties. In other words, actins from different sources are not antigenically identical.

Several groups have generated anti-actin antibodies to establish other forms of immunoassays for the quantitation of actins from cell sources. Lessard et al. (497) experimentally produced anti-rabbit muscle actin antibodies in order to set up a

solid phase assay for actin using $^{125}$I-labeled protein A as the means of detecting cross-reactivity. Though they were successful in producing anti-actin antibodies, their assay was limited in usefulness, for they found that although rabbit skeletal muscle actin could displace 80-90% of labeled actin, extracts of neuroblstoma cells could only produce 60-70% displacement. Consequently, to use this assay to accurately quantitate neuroblastoma actin would necessitate purification of this protein to be used as the proper displacing ligand. Moreover, this assay was found to be much less sensitive than the actin radioimmunassay of Morgan et al. ($ED_{50}$ = 5-10ug/ml versus 0.2-1ug/ml). Charlemagne et al. (498) have developed an actin ELISA using rabbit anti-actin antibodies generated to rabbit muscle actin chemically coupled with dinitrophenyl to break immunological tolerance. Unfortunately, this assay was not tested for its ability to detect nonmuscle actins. As with the solid phase immunoassay, this assay was quite insensitive relative to the actin immunoassay. Kurki (499) has developed a similar actin ELISA using spontaneous human anti-actin and experimentally produced rabbit anti-actin antibodies to compare the specificity of this assay with that of indirect immunofluorescence staining techniques. He found that although human anti-actin antibodies reacted well with the "native" actin in tissue sections prepared for immunofluorescence, rabbit anti-bovine muscle actin antibodies preferentially reacted with "denatured" actin bound to microtitre wells, again suggesting different specificities of anti-actin antibodies.

These studies have clearly shown that high affinity anti-actin antibodies are not only difficult to generate, but also that their specificity is unpredictable. Therefore, such antibodies are difficult to use in heterologous immunoassays to identify and quantitate actins from diverse sources.

The observation that actins of widely diverse origin, from skeletal muscle to sea urchin eggs to human platelets, all bind to pancreatic DNase I with similar affinity (456) has been a key event in the development of assays to accurately quantitate nonmuscle actins. The use of DNase I in lieu of a primary anti-actin antibody to bind actin avoids the difficulty of generating high affinity anti-actin antibodies which do not distinguish between actins from different sources.

The DNase I immunoprecipitation assay of Snabes et al. (459) is an extremely sensitive immunoprecipitation assay based on the binding of DNase I to actin. Using this assay, they have reported that in a series of nonmuscle actins tested, all actins tested compete with radiolabelled rabbit muscle actin to give identical displacement curves, indicating that these actins interact with the enzyme in an identical manner. These results suggest a high degree of conservation of the DNase binding site for actin. Moreover, their estimates of actin contents in cellular extracts agree well with other estimates of actin contents using other means of detection, thus supporting earlier conclusions concerning the specificity of DNase binding to actin (456). This assay can detect actin in the nanomolar concentrations, and thus compares favorably in terms of sensitivity to that of the actin

radioimmunossay of Morgan et al. (495).

The binding of actin to DNase I has been well characterized. Bovine pancreatic deoxyribonulease I is one of two proteins (the other is profilin) known to interact specifically with monomeric actin (80). The binding of DNase I to muscle actin is of high affinity ($K_d = 10^8 M^{-1}$), and is extremely stable in a variety of solvents (500). DNase I can prevent actin polymerization in the presence of high salt (456). Furthermore, this protein can also bind to F-actin, and by formation of DNase-F-actin complexes, can promote actin depolymerization (501). Recently, the detailed structure of rabbit skeletal muscle actin complexed with bovine pancreatic DNase I has been determined by x-ray crystallographic methods at 6-Å resolution (500). From the dimensions of both molecules and their spatial relationship, it is clear that one molecule of DNase I interacts with one molecule of actin, and that this interaction involves specific regions of both molecules.

Due to the nature of the interaction of DNase I with actin, and in light of the broad cross-reactivity of the enzyme with actins of different origins, the DNase immunoprecipitation assay of Snabes et al. (459) was an attractive approach to confirming our earlier estimates of adrenocortical actin content using both SDS-PAGE and DNase inhibition assays. In this assay, actin in cell lysates competes with radioactively labelled actin for a limited amount of DNase I. Bound label is separated from free label by antibody immunoprecipitation using rabbit anti-DNase antibody to bind all DNase I, and sheep anti-rabbit IgG second

antibody to immunoprecipitate actin-DNase-antibody complexes. While this assay relies on the same principle of DNase binding as does the DNase inhibition assay, the immunoprecipitation assay is at least two orders of magnitude more sensitive, and thus potentially more sensitive at detecting subtle changes in actin levels.

The following sections describe the setup and characterization of this immunoassay for the detection and quantitation of adrenocortical cell actin. During the process of adapting this assay for this purpose, several major changes in the original protocol were made which we believe greatly improve the accuracy of the assay. These modifications, and the reasoning behind their use, are also presented. Finally, this assay is used to quantitate rat adrenocortical actin in order to confirm our earlier evidence that ACTH promotes loss of actin in these cells.

## Purification of Rat Adrenal Actin

At this time, we felt that rigorous use of the DNase I immunoprecipitation assay would eventually demand demonstration that rat adrenal actin reacts identically with muscle actin standard in this assay. For this reason, rat adrenal actin was purified to homogeneity.

The general protocol followed for the purification of rat adrenal actin was that developed by Uyemura et al. (502) for the isolation of Dictyostelium actin, and is outlined in Figure 18. Rat adrenals were collected and stored at -20°C until further processed. Adrenals were defatted on ice and homogenized in 8mls

of 10mM triethanolamine, pH 8.0, containing 40mM sodium pyrophosphate, 30% (w/v) sucrose, 0.01% LTI, 0.01% bacitracin, 0.01mM PMSF using an ultrasonic polytron (Bronwill Scientific) for three 2-second bursts at 30 setting. The homogenate was immediately centrifuged for 15 mins. at 18,000 x g, then the supernatant spun for 90 mins. at 100,000 x g. High speed supernatant (24 mls) was concentrated by ultrafilatration using an Amicon PM-10 filter (Diaflo) to 1.5mls, and applied to a G-150 column (1.5 x 105.5cm) equilibrated with depolymerization buffer (10mM Triethanolamine, pH 8.0, 20mM sodium pyrophosphate, 0.4mM DTT, 50uM $Na_2ATP$, 50uM $MgCl_2$, 0.005% $NaN_3$) containing 10% sucrose. Fractions (2.15mls) were collected at gravity flow rate.

Figure 19 shows the protein profile of fractions off the G-150 column, and SDS-PAGE analysis of selected fractions off the column. Those fractions containing the majority of actin (#52-61) were pooled, dialyzed against depolymerization buffer for 2 days at 4°C, and centrifuged for 2 hrs. at 150,000 x g to remove residual F-actin. The supernatant of this spin (21mls) was diluted 1:1 with 10mM triethanolamine, pH 8.0, 0.4mM DTT, 50uM $Na_2ATP$, 50uM $MgCl_2$ and applied to a DEAE-cellulose column (37.7mls bed volume) equilibrated with 10mM triethanolamine, pH 8.0, 5mM sodium pyrophosphate, 20mM KCl, 50uM $Na_2ATP$ and 50uM $MgCl_2$ (DEAE buffer). Proteins were eluted with an increasing KCl gradient of 0.02M to 1M in DEAE buffer.

Figure 20 shows the protein profile of fractions off this column, and the corresponding SDS-PAGE of selected fractions. Again, those fractions containing actin (#35-41) were pooled,

dialyzed against depolymerization buffer for 24 hours to depolymerize actin. This solution was then centrifuged for 2 hrs. at 150,000 x g, and the supernatant immediately gel filtrated on a precalibrated TSK3000SW column equilibrated with depolymerization buffer lacking ATP and DTT. Eluted fractions were monitored for protein at $OD_{280nm}$. The perfusion pressure was maintained at 350-500psi, and the flow rate was 0.5 or 1.0ml/min. The panels in Figure 21 show the protein profiles of purified rabbit skeletal muscle actin (A) and presumptive rat adrenal actin (B) run on this column. Those fractions of the adrenal actin DEAE prep co-eluting with those containing purified muscle actin, at a position expected for a globular protein of 43,000-45,000 daltons (arrow), contained actin at a purity of 93-99% as judged by SDS-PAGE. Purified rat adrenal actin was stored as G-actin in TSK column buffer containing 0.5mM $Na_2ATP$ in aliquots at $-20^{o}C$.

Figure 22 represents a composite of densitometric scans of various steps in the purification of rat adrenal actin. In these scans, the position of muscle G-actin standard is indicated by the arrow. Table II presents a summary of actin yields throughout the purification. It is evident that this purification protocol results in isolation to near homogeneity of a protein (actin) which is less than 6% of the total protein in the starting adrenal homogenate.

The final purification protocol presented in Figure 18 consistently yields adrenal actin which is greater than 93% pure as judged by SDS-PAGE. Though the final yield of adrenal actin

(0.14mg/3.7mg total protein, or 3.8% of S100,000 x g starting material) is significantly lower than that reported for Dictyostelium actin (31mg/97mg total protein, or 32% of starting material, see reference 502), the yields of actin at each step of the purifications after the initial homogenizations are comparable. The bulk of adrenal actin loss seems to have occurred in the initial low speed centrifugation, most probably due to incomplete solublization of the starting material.

One significant deviation in the protocol for adrenal actin purification from that for Dictyostelium actin is the inclusion of a final gel filtration step. The TSK3000 column is a fast, efficient means of separating highly purified adrenal actin from residual contaminants and actin degradation products. That these final contaminants copurify with actin through initial chromatography steps suggests that they are either associated in some macromolecular complex which does not disassemble upon gel filtration and which does not sediment at 100,000 x g, or that they are specifically bound to actin. In light of the number of actin-associated proteins now known to associate with nonmuscle actins, the probability that these contaminants represent actin-associated proteins is quite high. In fact, one of the contaminating proteins which can be seen co-eluting with actin through the DEAE-cellulose column (Figure 20), migrates to a position on the SDS gel (16,000 daltons) which corresponds to profilin, a well-known actin-binding protein which is known to bind specifically to actin and to influence its state of assembly (91,92,480). Why such contaminants separate from actin during the

# PROTOCOL FOR ADRENAL ACTIN PURIFICATION

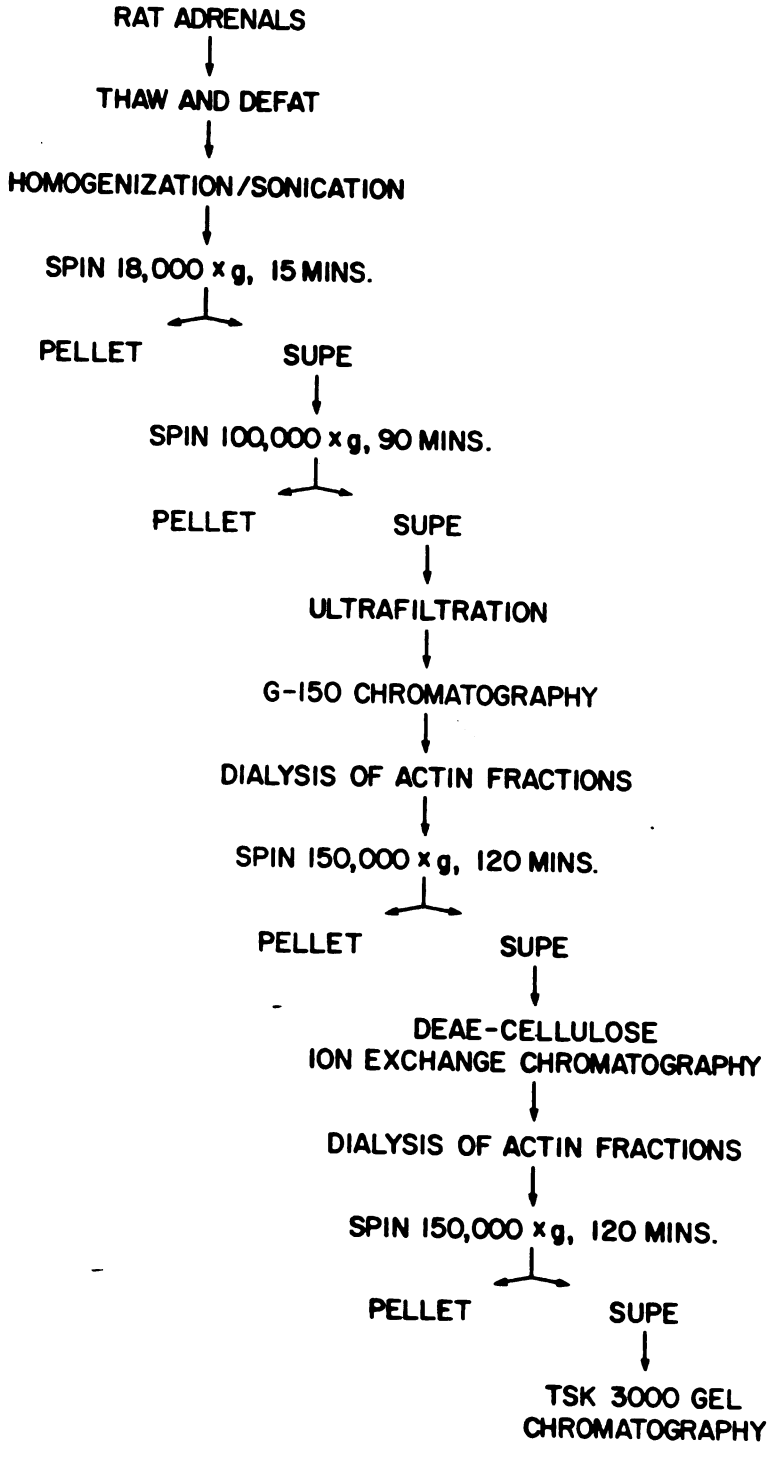



Figure 18. Purification Scheme for the Isolation of Rat Adrenal Actin. The details of this protocol are discussed in the text.

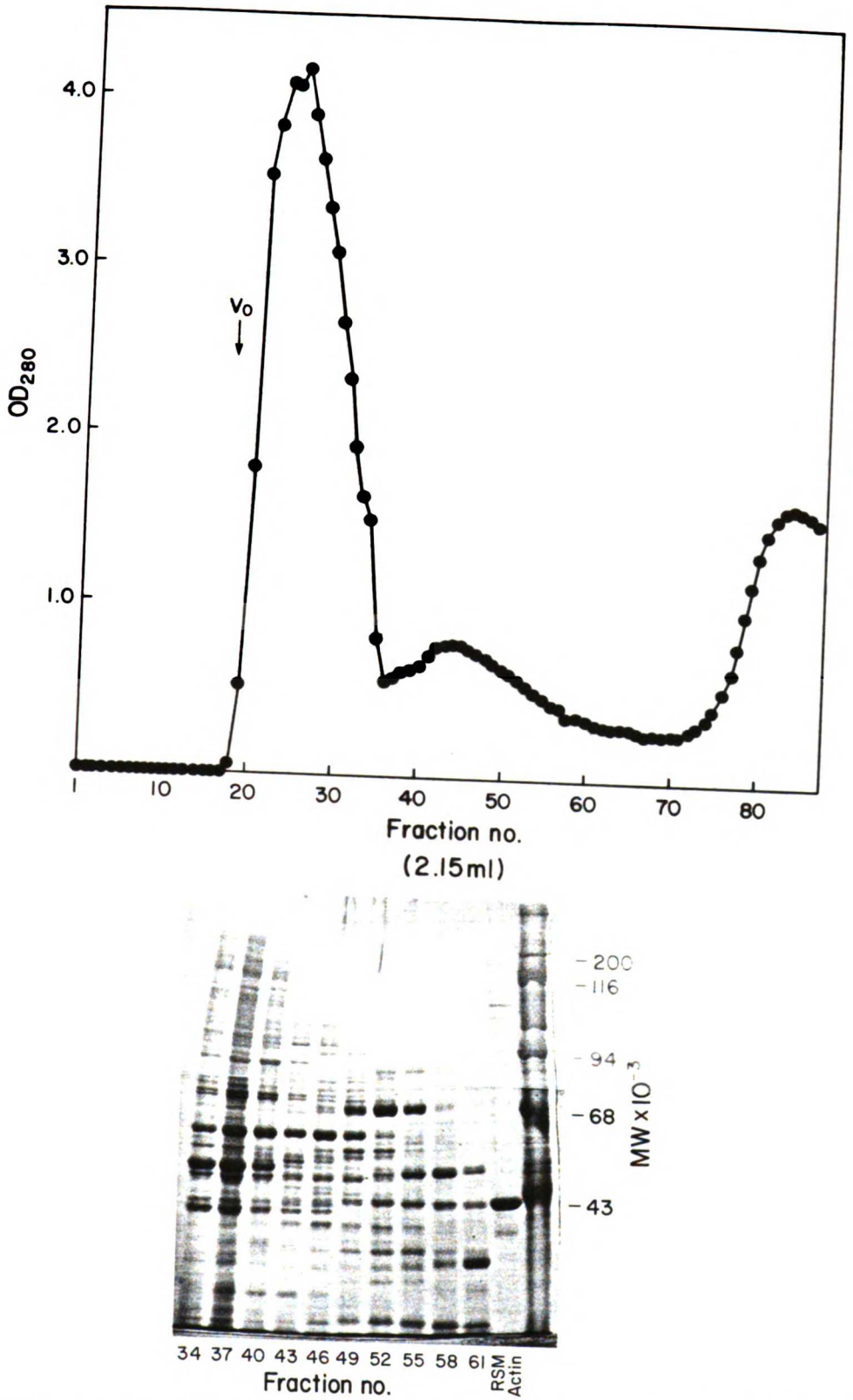



Figure 19. G-150 Gel Chromatography of Rat Adrenal Homogenate. A) Protein profile off the G-150 column (1.5 x 105.5cm) of the 100,000 x g supernatant. Fractions (2.15mls) were collected at a flow rate of 6.6 mls/hr.; B) 10% polyacrylamide SDS gel of selected fractions off the G-150 column. Ten ul each of fraction was run per gel lane. Fractions #52-61 were pooled for DEAE-cellulose chromatography. $V_o$ indicates the void volume of the column.

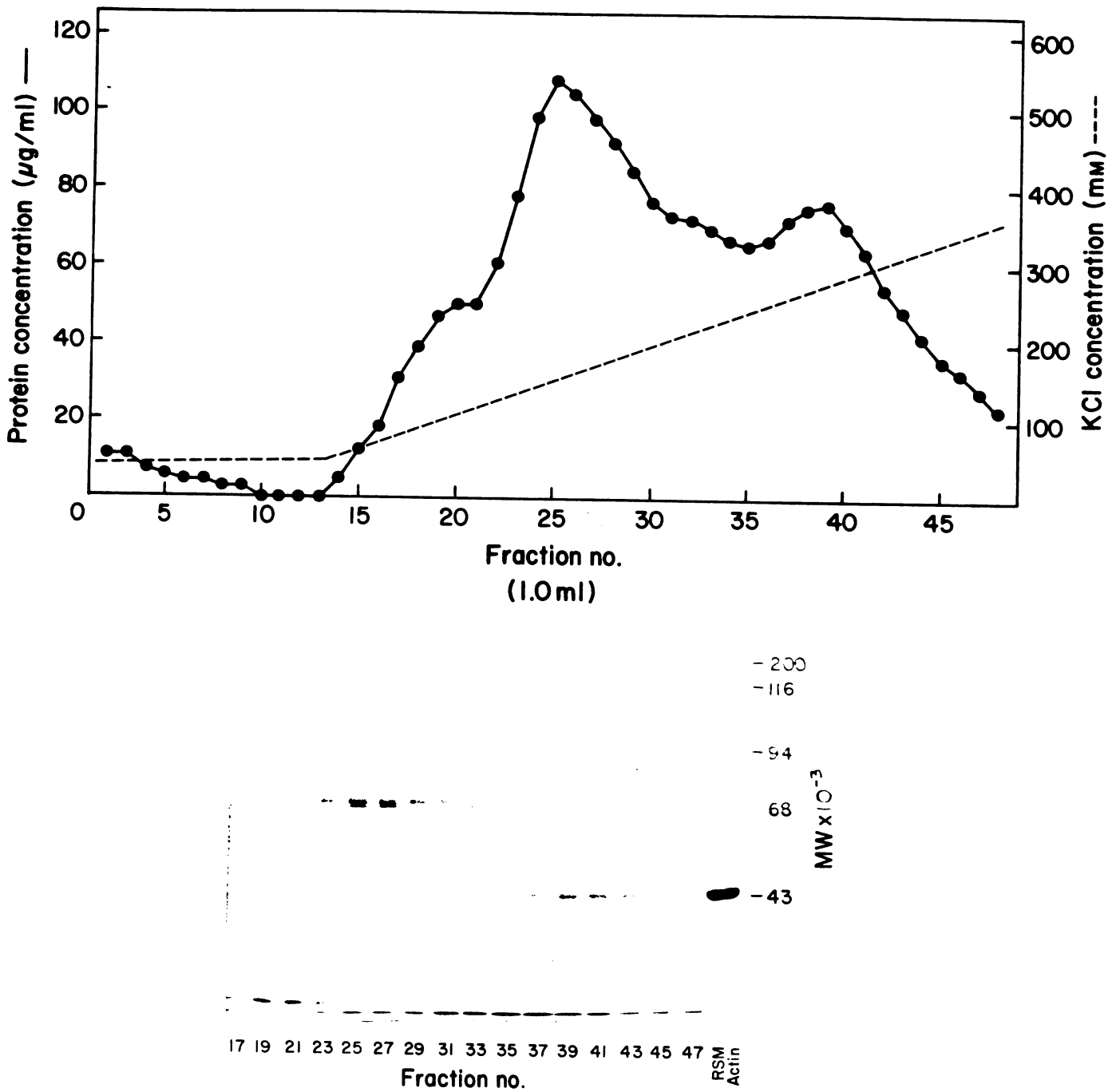



Figure 20. Ion Exchange Chromatography of Pooled G-150 Fractions. A) Profile of fractions off DEAE-cellulose column (1.2 x 10cm, 37.7mls bed volume) monitored for protein by Bradford (465) and for KCl concentrations; B) SDS-PAGE of selected DEAE fractions. Ten ul of each fraction was analyzed. Fractions #35-41 were pooled for further processing.

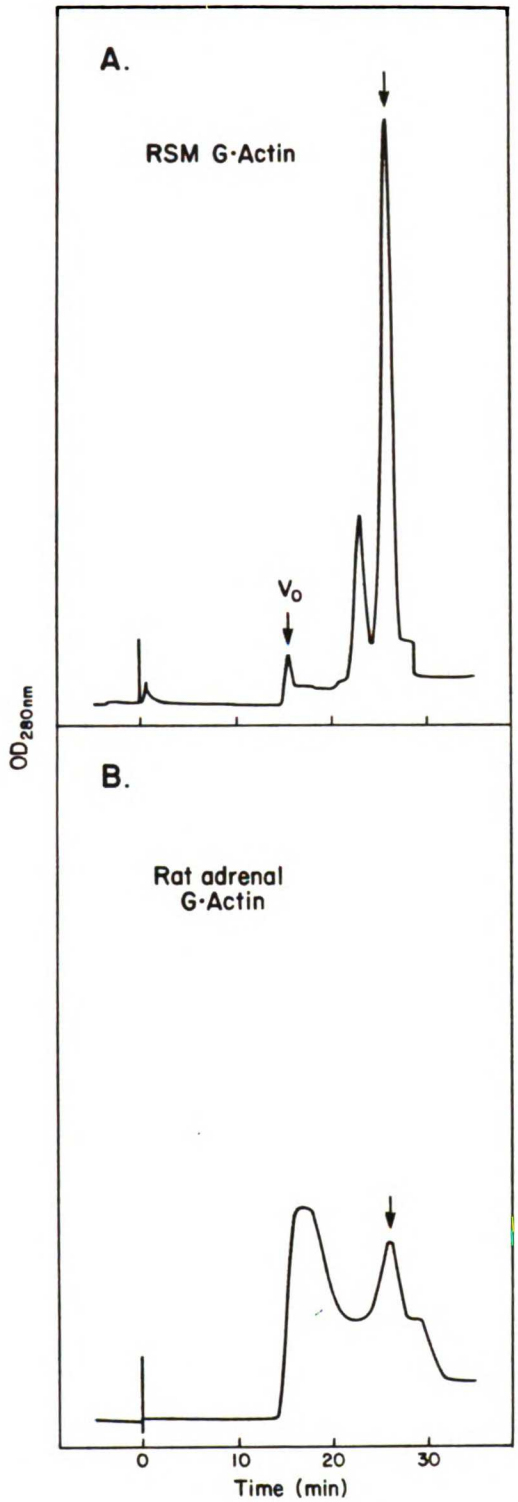



Figure 21. TSK3000SW Gel Filtration of Pooled DEAE Fractions. Column buffer was 10mM Triethanolamine, pH 8.0, containing 20mM sodium pyrophosphate, 0.4mM DTT, 50uM $MgCl_2$, and 0.005% $NaN_3$. The flow rate was set at 0.5ml/min. Column void volume ($V_o$) is approximately 7 mls. Protein was monitored at $OD_{280nm}$. A) protein profile of purified rabbit skeletal muscle G-actin; B) profile of pooled DEAE fractions (0.5mg protein) after dialysis and re-centrifugation. Unlabelled arrow indicates the elution position expected of a globular protein of 43,000-45,000 daltons, i.e., monomeric actin (G-actin).

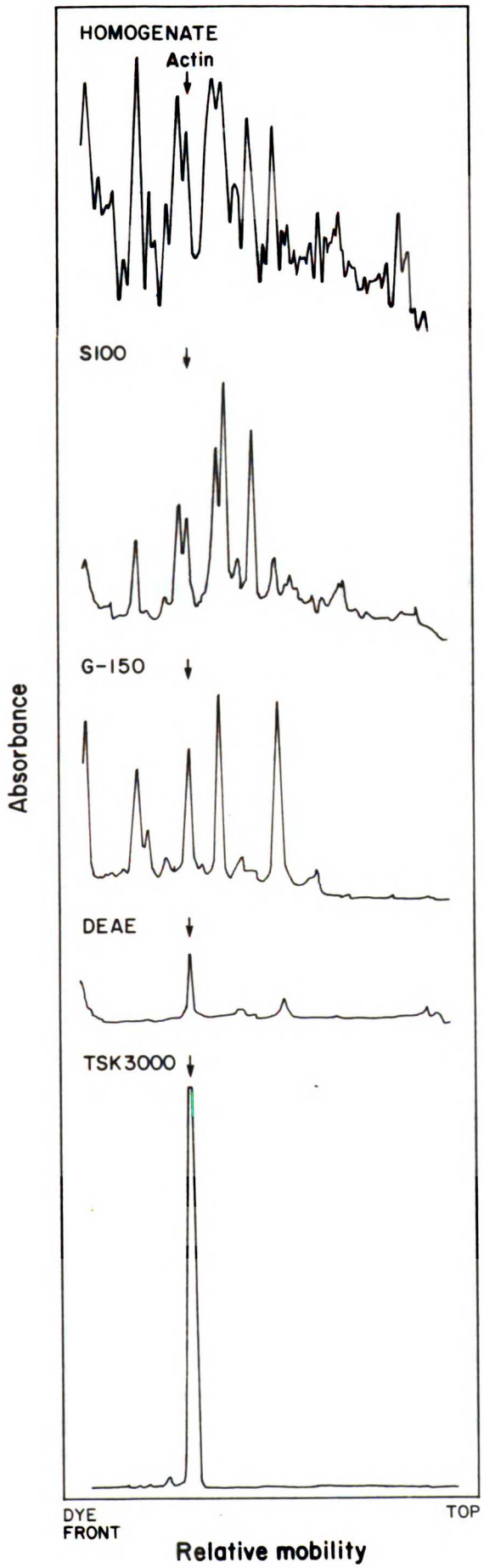



Figure 22. Summary of Rat Adrenal Actin Purification. Densitometric scans of SDS-PAGE gels of samples of selected steps in the purification of rat adrenal actin. As the protein loads of samples were different, note only the purity of the actin peak (arrow) relative to other proteins.

| Step | Protein (mg) | Actin/ Protein* (%) | Total Actin (mg) |
|---|---|---|---|
| I. Homogenate | 366.0 | 5.5 | 20.1 |
| II. S100,000 x g | 62.9 | 5.5 | 3.7 |
| III. G-150 Pool | 8.7 | 11.0 | 1.0 |
| IV. DEAE Pool | 0.6 | 23.0 | 0.14 |
| VI. TSK3000 | 0.15 | 93-99 | 0.14-0.15 |

* actin/protein ratios express actin content as the percent of total protein.

Table II. Summary of Yields at Various Steps in the Purification of Rat Adrenal Actin. Protein was determined by Lowry et al. (464). All actin contents were determined by planimetry of densitometric scans of actin bands in SDS gels.

final gel filtration step is not known, but may be due to loss at some earlier step of other factors, such as ions, which might regulate their interaction.

Positive Identification of Rat Adrenal Actin

Though we have not here further biochemically characterized adrenal actin, this actin does share some physicochemical properties with other nonmuscle actins: similar molecular size as judged by co-migration with muscle actin on SDS-PAGE (Figure 22), acidic nature by virtue of its ability to bind strongly to DEAE (Figure 20), and ability to polymerize as evidenced by precipitation upon centrifugation after addition of high KCl. These observations all support the conclusion that the protein purified is indeed actin.

In addition to the observation that presumptive adrenal actin co-migrates with purified muscle actin on SDS gels, three other approaches were taken to prove that the protein purified is indeed adrenal actin. First, as it is known that G-actin binds to DNase with such high affinity that it can only be dissociated from the enzyme with strong denaturing agents such as guanidine-HCl (503), affinity chromatography on DNase I-sepharose was employed to selectively identify actin from an adrenocortical cell lysate. Adrenocortical cells, solublized and sonicated in a buffer containing Triton X-100, were passed through a DNase-sepharose column. The column was then washed with a series of buffers containing increasing amounts of guanidine-HCl, and all eluates analyzed by SDS-PAGE for the presence of a protein co-

migrating with actin at 45,000 daltons. Figure 23 shows the coomassie-stained gel results of such an experiment. Here, the protein profile of the starting adrenocortical cell homogenate is compared with the protein patterns of the various washes off the DNase affinity column. It is clear that the major band retained on the column and specifically removed with 4M guanidine-HCl co-migrates with the purified presumptive adrenal actin at a position expected for a 45,000-dalton protein. This is exactly the position at which purified muscle actin migrates. As both the major protein eluted with high guanidine-HCl and presumptive adrenal actin co-migrate with muscle actin on SDS gels, these results strongly support the conclusion that this protein is adrenal actin.

The second means of positively identifying the purified protein as adrenal actin used is actually a variation on the theme of DNase affinity chromatography. In this case, rat adrenocortical cells in culture were labelled for 24 hrs. with $^{35}S$-methionine under conditions which promoted heavy incorporation of label into cellular protein. Cells were then lysed and subjected to DNase-sepharose affinity chromatography as described in the experiment in Figure 23. Figure 24 shows the radioactivity profile of fractions off the column during the different washing conditions. Though the majority of the radioactivity passed unretarded through the column (bars labelled "lysate" and "effluent"), a significant amount of labelled protein was retained on the column and eluted in 4M guanidine-HCl. When pooled fractions from fraction #1 of this high guanidine-HCl wash

were analyzed by SDS-PAGE (insert), the major band of radioactivity was seen to co-migrate with muscle actin actin 43,000 daltons, again suggesting that the majority of labelled protein specifically retained on the DNase column was actin.

Cross-reactivity of presumptive adrenal actin with specific anti-actin antibodies was a third criterion used to identify this protein as actin. Giunea pig anti-actin antibodies, generated against rabbit muscle actin, were used in a solid phase radioimmunoassay to test for their ability to recognize presumptive adrenal actin. Figure 25 shows the results of this immunoassay. Here, purified muscle and adrenal actins were separately attached to microtitre wells, then reacted with anti-actin antiserum. Binding of anti-actin antibody to both antigens was assayed and quantitated by reacting bound antibodies with iodinated protein A. It is evident that the antibody used recognizes both muscle and adrenal actins, although with widely different reactivities: although antibody cross-reacts with adrenal actin, it does so at one-tenth the reactivity with muscle actin. Increasing the amount of actin plated results in increasing radioactivity; these curves reach a similar plateau when the amounts of actin plated are increased further, indicating that the anti-actin antiserum used recognizes an antigenic determinant(s) common to both adrenal and muscle actins.

Purified adrenal actin was also tested in an ELISA for its ability to cross-react with anti-actin antibodies. The procedure for this immunoassay involves plating presumptive adrenal actin onto microtitre wells, then reacting this protein first with

dilutions of guinea pig anti-actin antisera then with a rabbit anti-guinea pig IgG-horse radish peroxidase conjugate. Enzyme activity is then quantitated by color development, monitored at $OD_{414nm}$, which is produced by oxidation of the substrate. Figure 26 shows the results of three anti-actin antisera tested with this ELISA. It is clear that all three of the antisera tested strongly cross-react with adrenal actin.

Taken together, the affinity chromatography and immunoreactivity experiments presented here leave little doubt as to the identity of the purified protein as adrenal actin. Two different properties of actins in general, i.e., their affinity for DNase I and their recognition by anti-actin antibodies, have been used to prove that this protein is adrenal actin. Moreover, the observation that the guinea pig anti-muscle actin antisera used cross-react strongly with adrenal actin is consistent with other reports that anti-actin actin antibodies often recognize actins of diverse origin (495), and further supports the conclusion that these antibodies specifically stain actin-containing microfilaments in adrenocortical cells.

## The DNase Immunoprecipitation Assay

Purified rabbit skeletal muscle actin was used throughout these studies as an actin standard and as labelled ligand in the immunoprecipitation assay. Actin standards were stored in aliquots at -70°C in TSK column buffer containing 0.5mM $Na_2ATP$ for up to five months without any affect on their ability to bind to DNase. In fact, we have found that use of one stock of actin

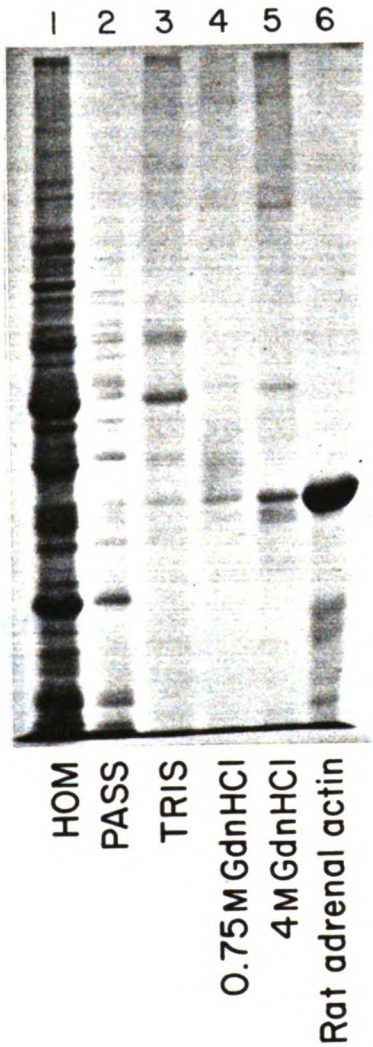



Figure 23. Identification of Rat Adrenocortical Actin by DNase-Sepharose Affinity Chromatography. Adrenocortical cell lysate (72ug protein) was applied to a 0.7ml DNase-sepahrose column. Lane 1) starting homogenate; lane 2) proteins passing unretarded through column; lane 3) 10mM Tris-HCl wash; lane 4) 0.75M guanidine-HCl wash; lane 5) 4M guanidine-HCl wash; lane 6) purified muscle actin.

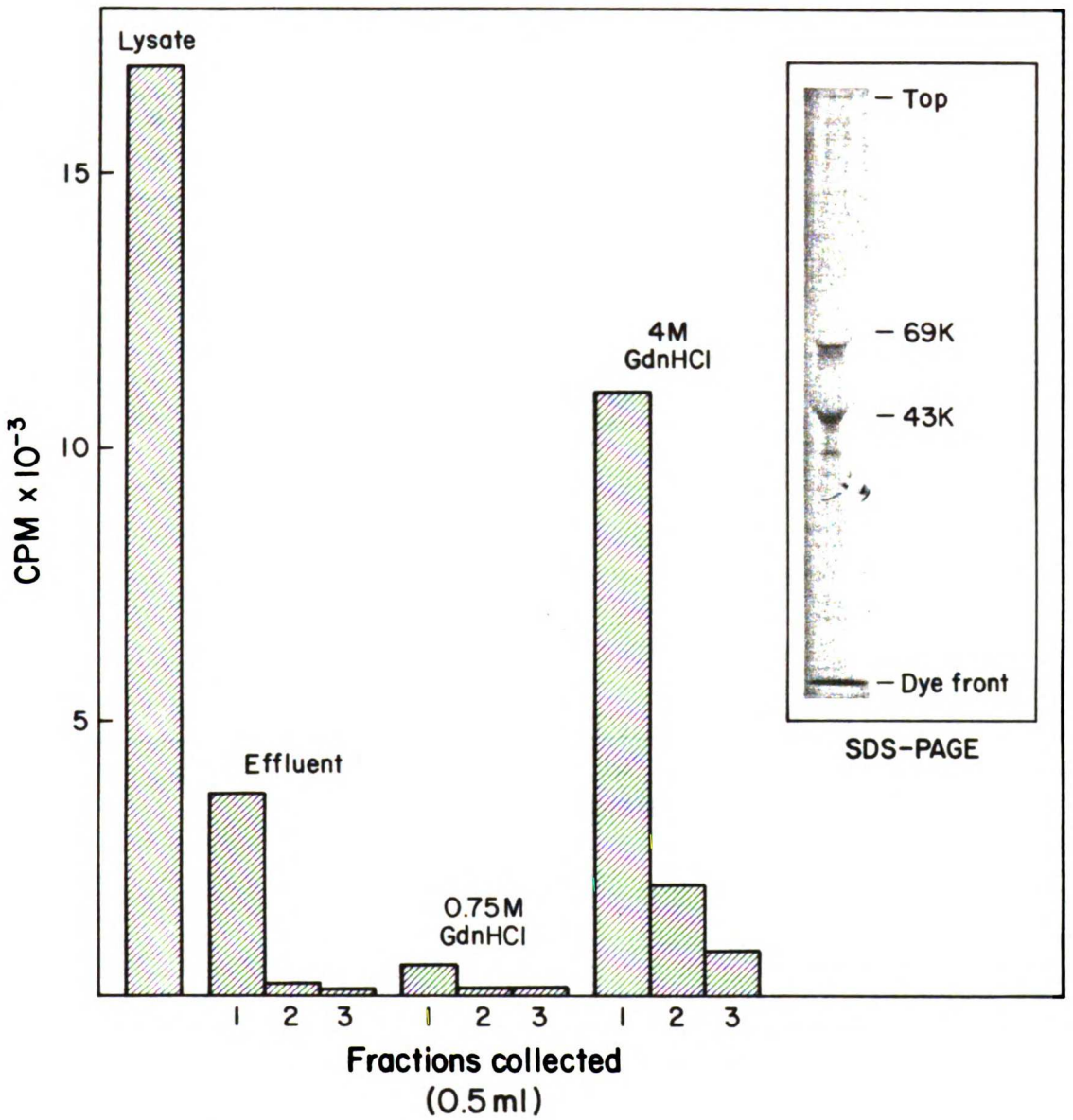



Figure 24. Retention of Rat Adrenocortical Actin on DNase-Sepharose. Rat adrenocortical cells were labelled with $^{35}S$-methionine for 24 hrs., then solublized and applied to a DNase-sepharose column exactly as described in Figure 23. Fractions (0.5ml) of each wash were counted for radioactivity (hatched bars), and fraction #1 of the guanidine-HCl wash was analyzed by SDS-PAGE (insert). Note the prominent radioactive bands in this fraction located at 43K and 72K daltons.

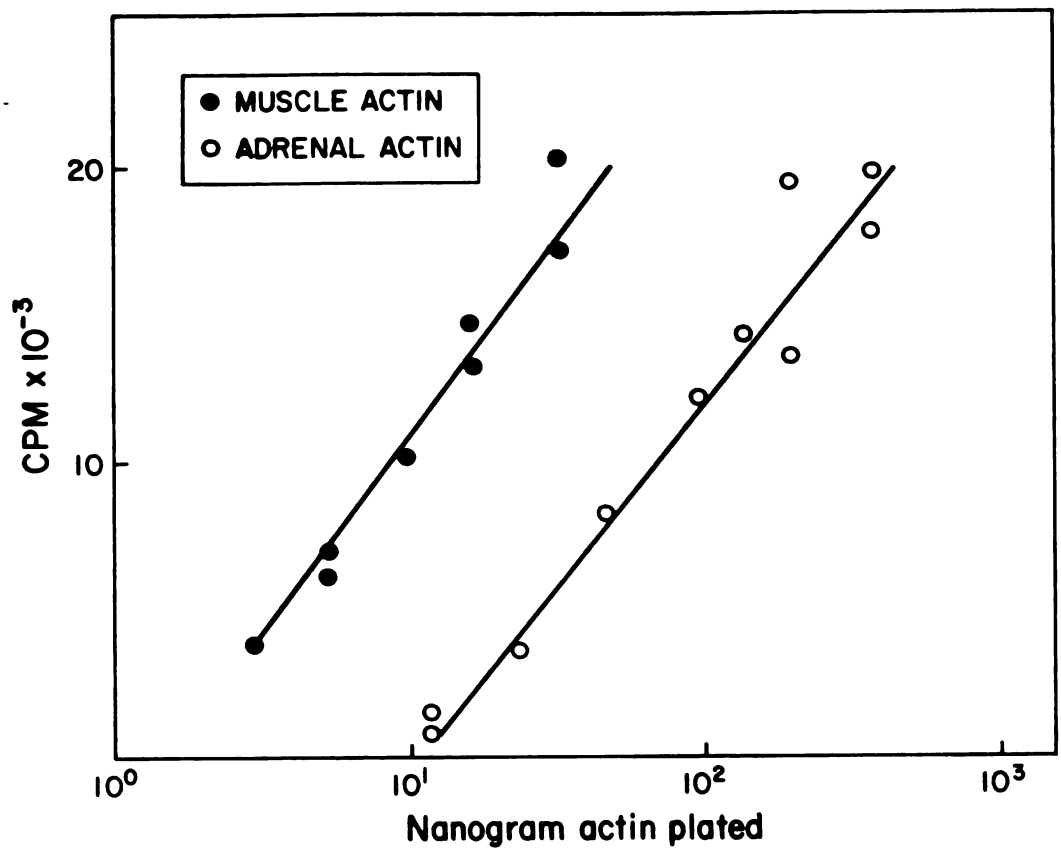



Figure 25. Solid Phase Radioimmunoassay of Muscle and Adrenal Actins. Purified rabbit skeletal muscle (closed circles) and rat adrenal (open circles) actins were used as antigens. Increasing amounts of actins were plated in microtitre wells as described in "Methods" section, Chapter 3, then treated with guinea pig anti-actin antiserum at 1:50 dilution. After washing, remaining antibody was detected by reaction with $^{125}$I-protein A (10,000cpm). Background counts were determined by incubation with guinea pig preimmune serum in lieu of anti-actin antiserum, and all values reported have been corrected for background levels of radioactivity.

significantly reduces interassay variation.

Rabbit skeletal muscle actin (5ug) was iodinated by the cloramine T method as described in Chapter 2 for the iodination of protein A. 2-Mercaptoethanol was removed from actin prior to iodination by exchange over G-25 into the same storage buffer lacking reducing agent. Immediately after iodination, one milligram of purified BSA was added, and the reaction mixture gel filtrated on a sephacryl S-200 column in 2mM Tris-HCl, pH 7.5, containing 0.2mM $CaCl_2$, 0.2mM ATP, 0.5mM 2-MeOH, 0.58uM BSA and 0.005% $NaN_3$. Panel A in Figure 27 shows the radioactivity profile off this column. A single peak of radioactivity appears at the position where unlabeled monomer actin elutes (arrow labelled "G-actin"); this peak is well retarded on the column and represents $^{125}I$-labeled G-actin. This peak is cleanly separated from free radioactivity ($^{125}INa$).

The lower two panels in Figure 27 demonstrate that iodination of actin using chloramine T does not chemically damage the protein. This is an important consideration in light of the harsh iodination conditions employed. Figure 27B shows the profile of polymerized muscle actin (F-actin) which has been depolymerized with 0.75M GdnHCl prior to iodination, then gel filtrated under conditions identical to those in panel A of Figure 27. The majority of radioactivity elutes at a position expected for monomer actin, demonstrating that GdnHCl treatment promotes actin depolymerization. However, when this iodinated G-actin is collected of the column, immediately mixed with 1 mg of unlabelled actin, and reapplied to the same S-200 column now

equilibrated with S-200 buffer containing 150mM KCl, 2mM $MgCl_2$ and 1mM $Na_2ATP$, the radioactivity profile shown in Figure 27C is obtained. Under these conditions which favor actin polymerization, the major peak of radioactivity appears at the void volume of the column ($V_o$), a position expected for polymerized actin. Note also that in both Figures 27B and 27C, G- and F-actin are cleanly separated from one another. Since actin used for iodinations retains its ability to polymerize and depolymerize under appropriate ionic conditions, we can safely conclude that this protein remains chemically intact after iodination.

Purification of Anti-DNase Antibodies by Protein A-Sepharose Affinity Chromatography

To reduce nonspecific background binding in this assay, anti-DNase antiserum was further purified by affinity chromatography using protein A-sepharose (Sigma). Lyophilized antiserum was reconstituted to its original concentration with $H_2O$, and passed twice through 3.5 mls of protein A-sepharose equilibrated with 50mM Tris-HCl, pH 8.6, 150mM NaCl, and 0.02% $NaN_3$. Column fractions were eluted sequentially with buffers of increasing acidity, and monitored for protein by absorbance at $OD_{280nm}$. Figure 28 shows the protein elution profile from the protein A-sepharose column. Although the majority of protein passed through the column unretarded, some protein did interact with column material, but could be removed by either pH 4.3 or pH 2.3 buffers. According to the published procedure, immunoglobulins of the IgG class elute from this column in pH 4.3

buffer (504). That this is the case is shown in Figure 29. Here, each of the protein peaks off the protein A-sepahrose column is tested at different dilutions in an ELISA using rabbit IgG as primary antigen and sheep anti-rabbit IgG-horse radish peroxidase as second antibody-enzyme conjugate. It is evident that the protein which elutes in pH 2.3 buffer (peak 2) is the the only material retained on the column which reacts positively in this assay, other than the starting material (rabbit anti-DNase antiserum). As protein A specifically binds rabbit immunoglobulins of the IgG class (505), the observation that this affinity chromatography selectively removes DNase-positive reactivity from whole anti-actin antiserum suggests that the anti-DNase antibodies in this antiserum are of the IgG immunoglobulin class.

As further proof that protein A-sepharose affinity chromatography purifies anti-DNase antibodies from whole antiserum, fractions off this column were used in the DNase immunoprecipitation assay to compare their reactivity with that of whole anti-DNase antiserum. As shown in Figure 30, fraction #2 off the affinity column, but not the same fraction from rabbit preimmune serum, is able to immunoprecipitate $^{125}$I-actin. The ability of fraction #2 to immunoprecipitate labelled actin compares favorably to unfractionated whole rabbit anti-DNase antiserum. We have found that background radioactivity levels in this assay are significantly reduced when affinity purified anti-DNase antibodies is used in lieu of whole antiserum; therefore, protein A-sepharose fraction #2 was routinely used for all

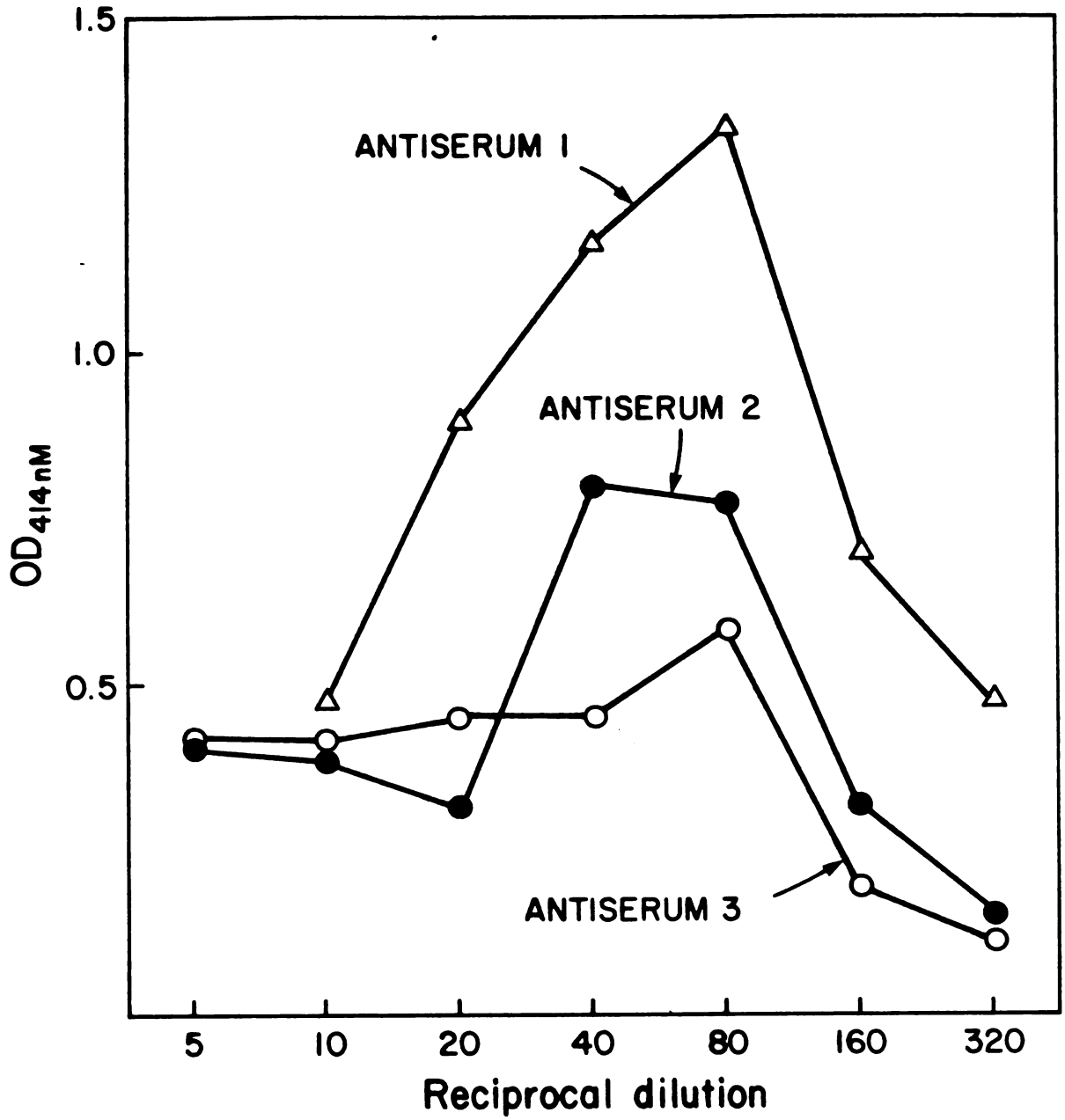



Figure 26. ELISA Cross-Reactivity of Adrenal Actin with Anti-Actin Antisera. Conditions for ELISA are described in "Methods" section, Chapter 3. Purified rat adrenal actin (200ng) was plated as antigen and reacted with several guinea pig anti-actin antisera at the various dilutions indicated. Antisera numbers refer to individual animals immunized.

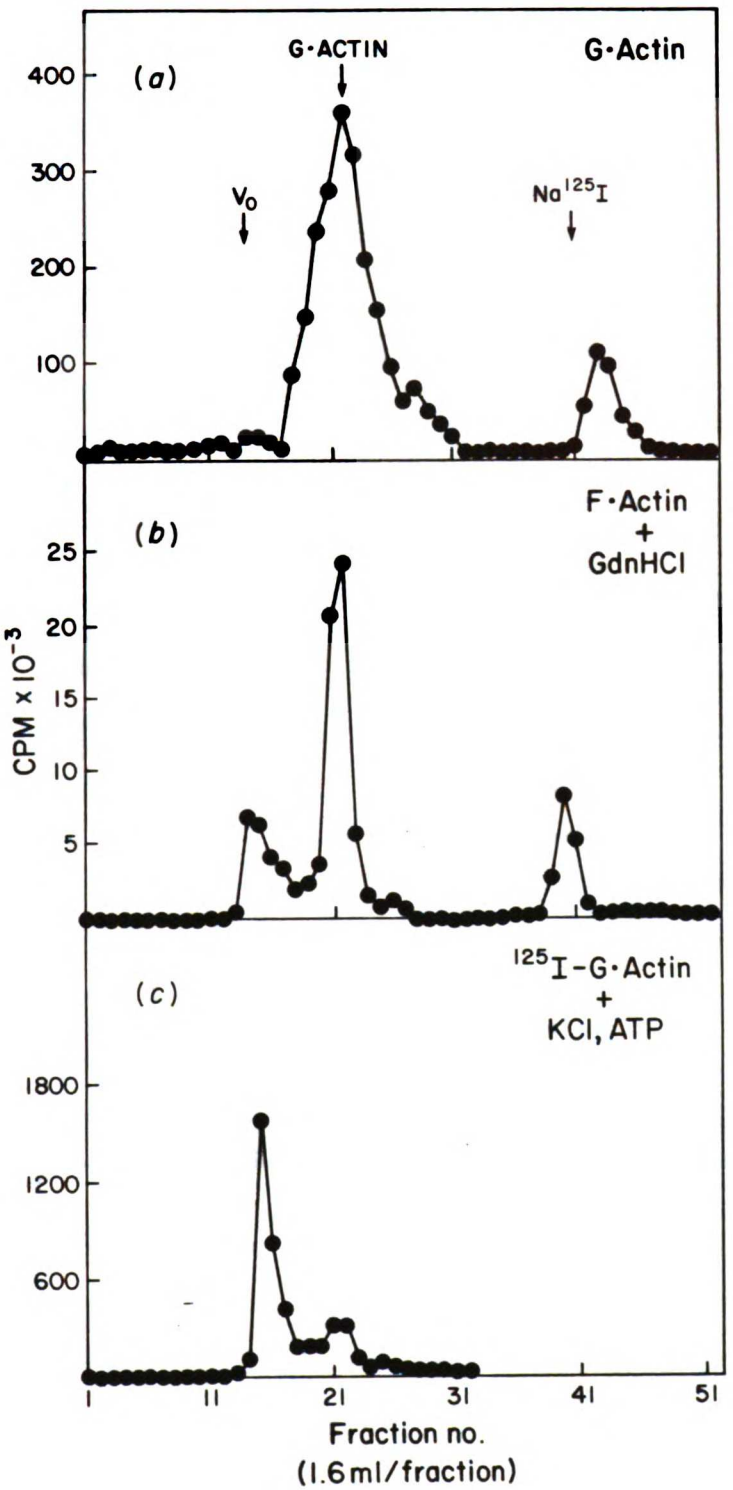



Figure 27. Profiles of Iodinated Muscle Actin Run on Sephacryl S-200 under Various Ionic Conditions. Column buffer was 2mM Tris-Cl, pH 7.4, containing 0.2mM $CaCl_2$, 0.2mM $Na_2ATP$, 58uM BSA, 0.5mM B-MeOH, and 0.02% $NaN_3$. A) Iodinated G-actin; B) F-actin treated with guanidine-HCl as described by Blikstad et al. (456) prior to filtration; C) iodinated G-actin off column re-chromatographed in above column buffer containing 1mM ATP, 2mM $MgCl_2$, and 150mM KCl. $V_o$ indicates column void volume. Sixteen ul of each fraction were counted for radioactivity.

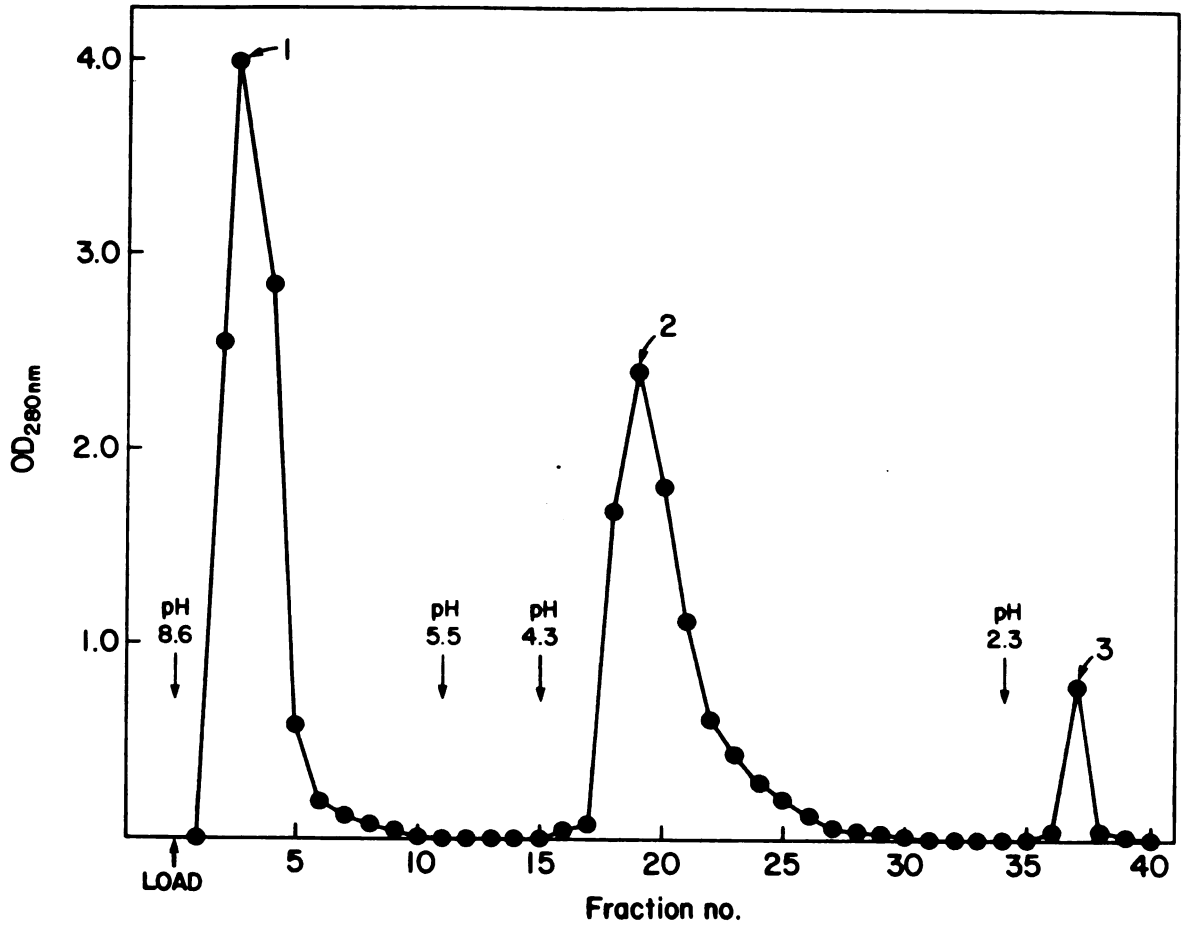



Figure 28. Purification of Anti-DNase Antiserum by Protein A-Sepharose Affinity Chromatography. Rabbit anti-DNase antiserum (58mg of lyophilized antiserum) was applied to a Protein A-sepharose column (1 x 9cm, 7.1mls bed volume) equilibrated with 50mM sodium phosphate, pH 8.6, containing 150mM NaCl, 0.02% $NaN_3$. Column was then washed with buffers of varying pH, and fractions (1.0ml) monitored for protein by absorbance at $OD_{280nm}$. Peak #1) protein passing unretarded through column; Peak #2) protein eluting in 50mM sodium acetate, pH 4.3, containing 150mM NaCl and 0.02% $NaN_3$; Peak #3) protein eluting in 50mM glycine-HCl, pH 2.3, containing 150mM NaCl and 0.02% $NaN_3$.

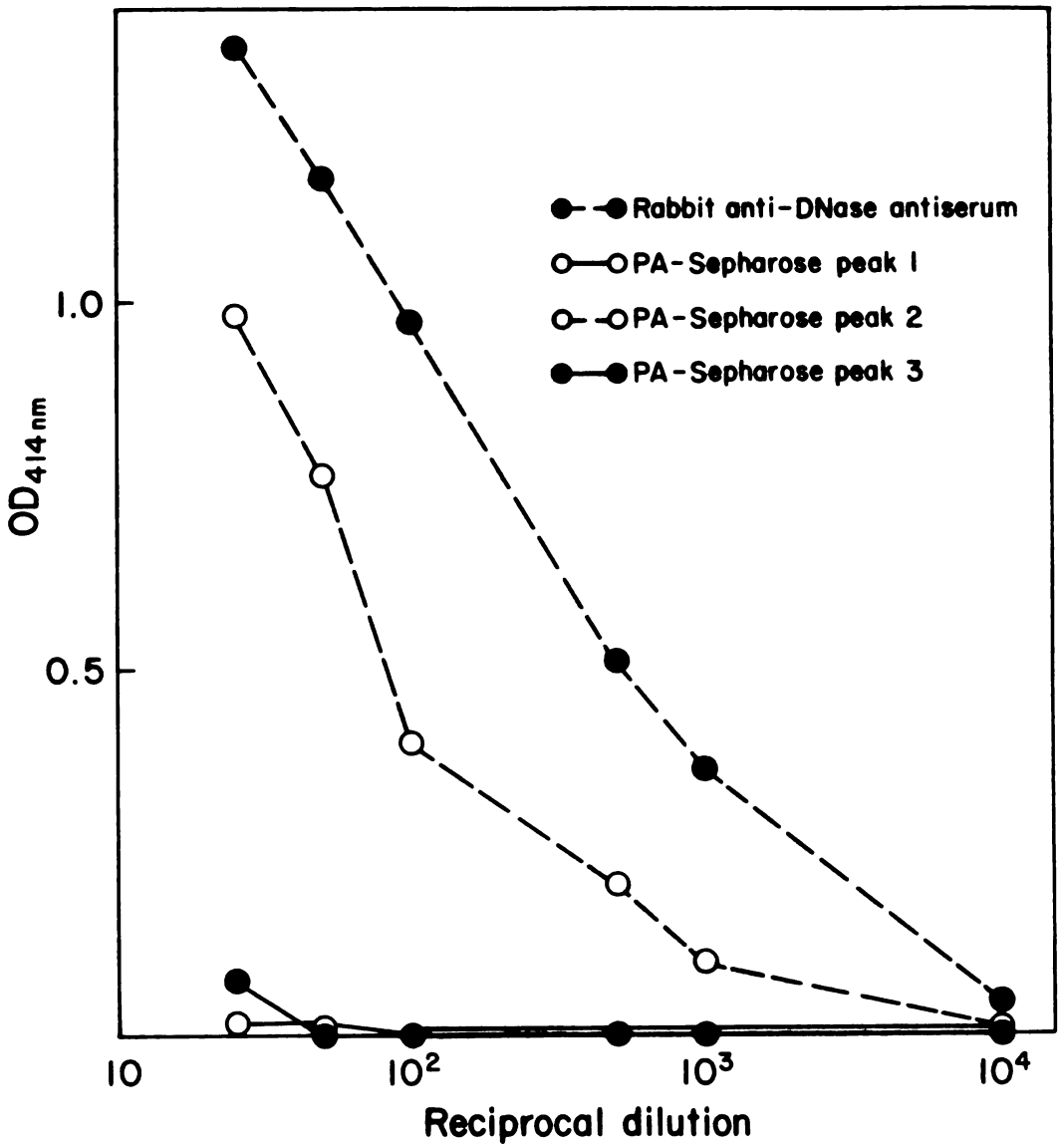



Figure 29. ELISA of Peaks off Protein A-Sepharose Column for Detection of Anti-DNase Immunoreactivity. Dilutions of individual peaks off the protein A-sepharose column were tested for cross-reactivity with DNase I (200ng) against unfractionated rabbit anti-actin antiserum. The protocol for this ELISA is described in detail in "Methods" section of Chapter 3.

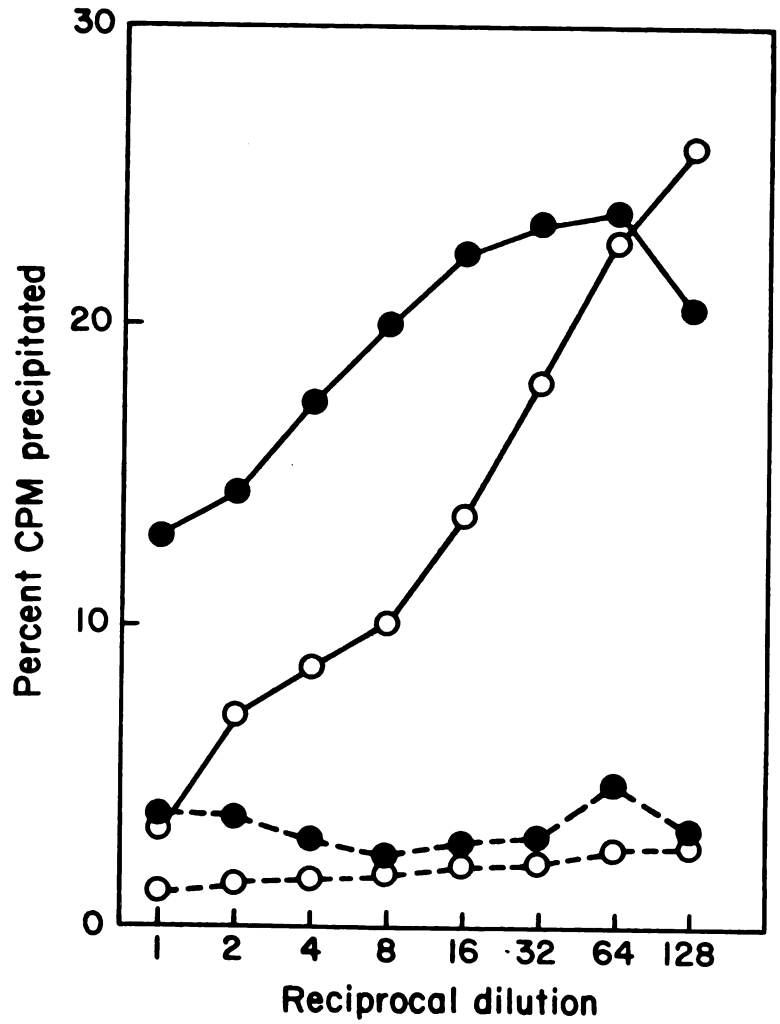



Figure 30. Setup of the DNase Immunoprecipitation Assay. Titration of whole anti-DNase antiserum, guinea pig preimmune serum, and protein A-sepharose affinity purified fraction #2 from anti-DNase and rabbit preimmune preparations. ●——●, whole antiserum, unfractionated; ●---●, whole preimmune serum, unfractionated; o——o, affinity purified whole antiserum fraction #2; o---o, affinity purified preimmune serum fraction #2.

subsequent immunoprecipitation experiments.

DNase/Anti-DNase Immunofluorescent Staining of Adrenocortical Stress Cables

Wang and Goldberg (506) have developed a staining procedure utilizing anti-DNase antibodies in conjunction with purified DNase I which can be used in immunofluorescent staining procedures to visualize actin-containing microfilaments in fixed, demembranated cells. In this protocol, DNase I replaces primary anti-actin antibody, and anti-DNase antibody, in conjunction with FITC-labelled second antibody, is used to visualize DNase I bound to microfilaments. The filaments stained with this DNase/anti-DNase technique were identical to those stained with anti-actin antibodies, supporting the conclusion that this technique specifically stains microfilaments.

To further confirm that peak #2 off the protein A-sepharose column contains anti-DNase antibodies, and as indirect corroboration that indirect immunofluorescence using anti-actin antibodies does in fact specifically stain actin-containing structures in the cell, we have used fraction #2 to visualize adrenocortical stress cables. For this experiment, rat adrenocortical cells were solublized with Triton X-100 and fixed with methanol exactly as described above in the protocol for indirect immunofluorescence. Cytoskeletons were first incubated with DNase I, washed several times with PBS, then incubated with fraction 2 at a 1:50 dilution in PBS. Cytoskeletons were washed again with PBS, incubated for one hour at 22$^{o}$C with FITC-labelled goat anti-

rabbit IgG, and finally visualized by epifluorescence.

Figure 31 shows two photographs of one such cell stained by this procedure. The upper photograph reveals abundant diffuse fluorescence as well as heavy staining of filaments which extends longitudinally along the ventral surface of the cell. These structures resemble the actin-containing stress cables visualized by indirect immunofluorescent staining with anti-actin antiserum. Careful focusing of the microscope on the surface of the cell away from the substratum (the dorsal side, lower photograph in Figure 31) reveals that the highlighted stress cables are specifically located on the ventral surface of the cell adjacent to the substratum. This asymmetric distribution of stress cables is commonly observed in electron micrographs of cultured cells (113), and further supports the conclusion that these structures are actin stress cables. Staining in which either DNase I or anti-actin antiserum are omitted from the protocol reveals the diffuse fluorescence evident in these photographs without the distinct staining of stress cables (not shown).

## Characterization of the DNase Immunoprecipitation Assay

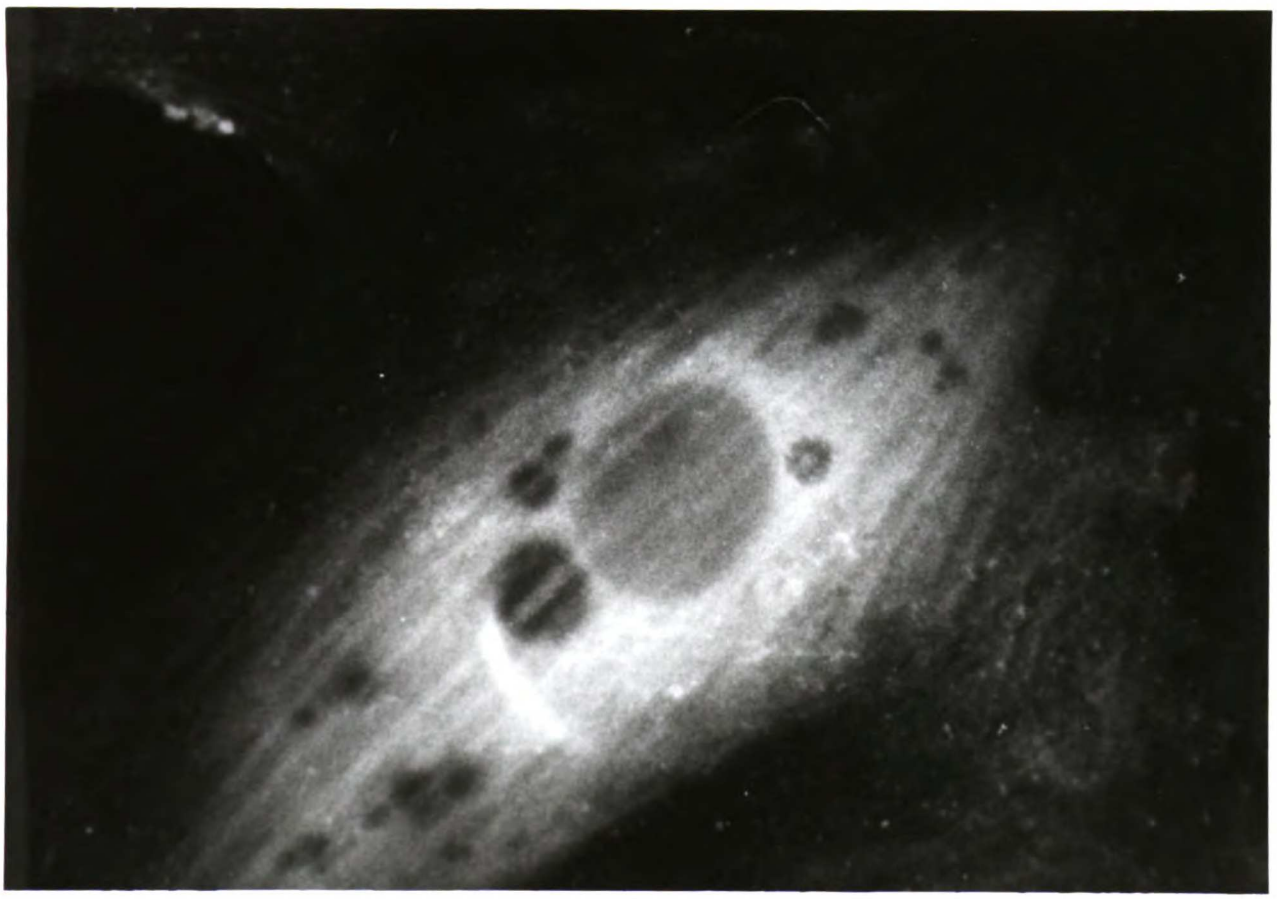
Figure 32 shows the final, composite results of several complete displacement curves using both rabbit skeletal muscle and purified rat adrenal actins as displacing ligands. Affinity purified rabbit anti-DNase antibody was used at 1:350 final dilution; sheep anti-rabbit IgG precipitating antibody was used at a 1:7 final dilution. These displacement curves are remarkably reproducible, and background cpm always measures less

A.



Figure 31. DNase Immunofluorescence of Rat Adrenocortical Cells. Cells were grown on glass coverslips, and solublized and fixed as described in "Methods" section, Chapter 3, for indirect immunofluorescence. Cytoskeletons were then incubated sequentially with 100ng DNase I, affinity purifiied protein A-sepharose fraction #2 rabbit anti-DNase I at 1:50 dilution, and FITC-labelled sheep anti-rabbit IgG at 1:25 dilution. A) objective focused on that side of the cytoskeleton facing away from the substratum; B) focused on that side of adjacent to the substratum.

FIGURE 31B.



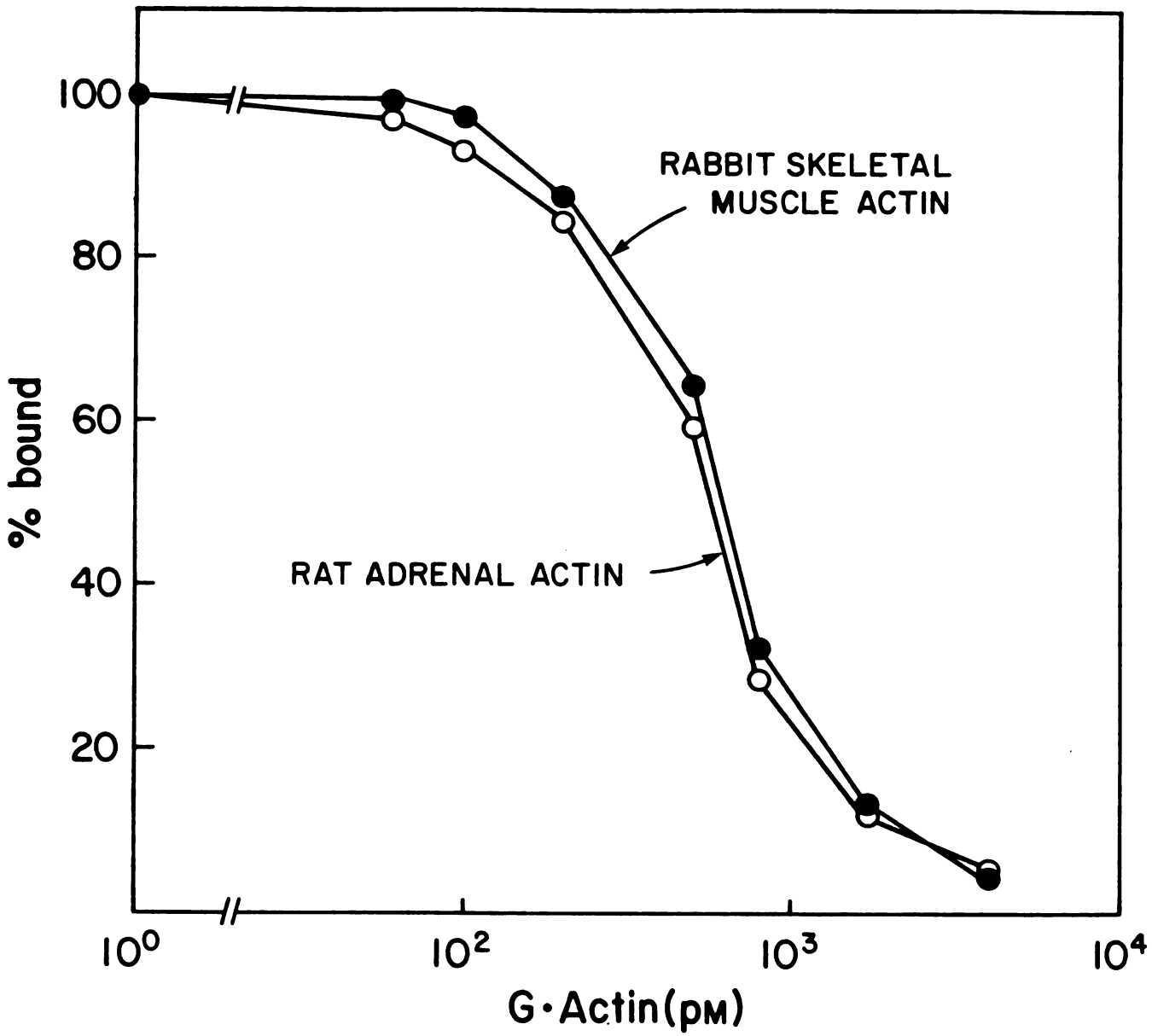



Figure 32. Complete DNase Immunoprecipitation Competition Curves. Curves were generated using rabbit skeletal muscle (closed circles) and purified rat adrenal (open circles) actins as displacing ligands. In all experiments, label was $^{125}$I-muscle actin, rabbit anti-DNase antibody used at 1:350 final dilution, and sheep anti-rabbit IgG used at 1:7 final dilution. All values used to calculate percentages reported have been corrected for nonspecific binding, which is defined as that value obtained in the absence of DNase in the incubation tube. Muscle actin curve = 7 experiments; adrenal actin curve = 3 experiments. All raw data points are in duplicate. $ED_{50}$ muscle actin = 16.517 $\pm$ 1.12; $ED_{50}$ adrenal actin = 16.151 $\pm$1.88.

than 7% of the maximal counts precipitable. By increasing the amounts of DNase I and anti-DNase antiserum used ten-fold, more than 85% of the total radioactivity can be precipitated, again indicating that labelled actin remains intact during the conditions of storage and use in the assay. The chemical integrity of the label is further confirmed by the appearance of one radioactive band, centered at 43,000 daltons on SDS gels, when labelled actin is analyzed by SDS-PAGE. Storage of label for several weeks did not significantly alter the percent of total counts precipitable or alter the appearance of the label on SDS gels.

The concentration of displacing ligand required for half-maximal displacement ($ED_{50}$) is 524 $\pm$ 36 pM for rabbit muscle actin, and 510 $\pm$ 60pM for rat adrenal actin. This assay is able to reproducibly detect less than 2.5ng/ml (55.6pM) actin. The sensitivity of this assay for muscle actin matches that obtained by Snabes et al. (459), and is comparable to the results of the actin radioimmunoassay of Morgan et al. (495). The fact that the displacement curves using rabbit muscle actin and rat adrenal actin exactly overlap one another is ultimate proof that this assay does not distinguish between these actins. This is in striking contrast to the results using guinea pig anti-actin antibodies, presented in Figure 25, which reveal a great disparity between muscle and nonmuscle cross-reactivities. That muscle and adrenal actins react identically in the DNase immuno-precipitation assay supports the belief that this assay does not distinguish between actins from different sources, and is our

rationale for using this assay in conjunctin with muscle actin standards to quantitate adrenocortical actin.

The original procedure for the preparation of cell and tissue samples for use in the DNase immunoprecipitation assay involves extraction of samples with a buffer containing 0.1% Triton X-100, followed immediately by addition of guanidine hydrochloride to a final concentration of 0.75M. The addition of guanidine at this concentration, it has been presumed, will ensure complete depolymerization of F-actin present in the lysate without irreversibly denaturing the actin. Though there is ample evidence that experimentally produced actin filaments, and microfilaments in cell lysates, do disassemble in response to low concentrations of guanidine-HCl, there is no evidence that this treatment results in <u>complete</u> depolymerization of actins in Triton extracts. Moreover, though it is claimed that the binding of DNase I to F-actin will itself promote depolymerization, there is again no direct evidence that complete depolymerization will be achieved in the case of detergent-solublized actin preparations. In fact, evidence presented in Figure 27B argues that guanidine-HCl treatment of purified muscle F-actin does not result in complete depolymerization: note that there is a significant peak of radioactivity which elutes at the void volume of the column. This is the elution position expected for filamentous actin. Thus, treatment with guanidine-HCl, at least by the original protocol used, does not ensure complete disassembly of filamentous actin. Since the DNase immunoprecipitation assay (as well as the DNase inhibition assay)

relies on the tight binding of monomer actin to DNase (456), accurate quantitation of actin contents in cell lysates demands that all actin be in monomer form in the assay. As residual F-actin (or aggregated, denatured G-actin) is not measured in this assay, the presence of filamentous actin in cell lysates put into this assay would result in underestimation of actin contents. Such an artifact has in fact been proposed as an explanation for discrepancies in past estimations of cellular actin contents between assays based on immunological recognition and those based on DNase-actin binding (497).

In an attempt to circumvent this potential complication, we have searched for a more efficient method of sample preparation which will ensure complete depolymerization of solublized cellular actin without disturbing the interaction of actin with DNase. As sodium dodecyl sulfate is a very efficient ionic detergent for the solublization of proteins (483), and has been used extensively in other immunoprecipitation protocols to selectively extract proteins from cell lysates without disrupting antigen-antibody interactions (507), this agent was tested to determine whether treatment of actin with SDS had any effect on the performance of actin in the DNase immunoprecipitation assay.

To this end, known amounts of rabbit muscle actin were treated with a series of buffers consisting of 1% NP40, 0.1% DOC, and several concentrations of SDS, then diluted at least ten-fold with DNase incubation buffer (PBS containing 0.1% gelatin, 0.2mM $CaCl_2$, 0.2mM ATP, 0.02% $NaN_3$) prior to use in the DNase immunoassay. Figure 33 shows displacement curves generated with

rabbit muscle actin treated with several buffers. SDS at a concentration of 0.1% had no effect on ability of actin to compete with label for DNase. However, higher concentrations of SDS did disrupt the displacing potency of actin: treatment of actin with buffer containing 0.5% SDS weakened the ability of actin to compete with labelled actin for DNase, and treatment of actin with 1% SDS completely destroyed the ability of actin to interact specifically with the enzyme. As expected, treatment of actin with the original lysis buffer used by Snabes et al. (0.1% Triton X-100 and 0.75M GdnHCl) had no effect on actin performance.

As actin standards treated with SDS were greatly diluted prior to use in the immunoassay, the effects of 0.5 and 1% SDS on the displacement curves cannot be attributed to effects of the detergent on the enzyme; rather, these effects are most likely due to varying degrees of irreversible actin denaturation upon treatmnent of the protein with higher concentrations of SDS. These results demonstrate that SDS at a concentration of 0.1% can be used to solublize actin without deleterious effects on actin performance in the DNase immunoprecipitation assay.

Figure 33 also illustrates several other interesting characteristics of the DNase immunoassay not reported in the original paper of Snabes et al. (459). Heating actin to 90°C prior to use in this assay destroys the ability of actin to displace labelled actin from DNase I, again most likely due to irreversible denaturation (or degradation) of the molecule. This observation, as well as the results with higher concentrations of

SDS, suggests that the binding of actin to DNase I is dependent of the tertiary conformation of the protein. Furthermore, as the lysis buffers tested all contain 2% 2-MeOH, it appears that reduction of disulfide bridges in actin and in DNase I does not affect their specific interaction. This is particularly interesting in light of the fact that reduction of one of the two disulfide bridges in the DNase molecule destroys its ability to cleave DNA (500).

Figure 33 further shows that F-actin, introduced into the assay in a high concentration relative to label, is able to completely displace label from DNase. This indicates that the conditions of the assay promote actin depolymerization. However, iodination of F-actin somehow alters its ability to interact specifically with DNase, as indicated in Figure 33 by the fact that excess of unlabelled G-actin cannot displace $^{125}$I-F-actin bound to DNase. This suggests that either iodinated F-actin is irreversibly bound to DNase, or that iodination itself destroys the ability of F-actin to depolymerize in the presence of DNase I.

Finally, Table III defines the specificity of this immunoassay for actin. Other intracellular cytoskeletal proteins (myosin, laminin, and beta-tubulin) as well as major extracellular matrix proteins (collagen and fibronectin) can not significantly displace actin from DNase I, suggesting that this assay is specific for actin. Furthermore, the observation that RNase A, in the presence of excess unlabelled G-actin and in the absence of DNase I, does not result in loss of counts

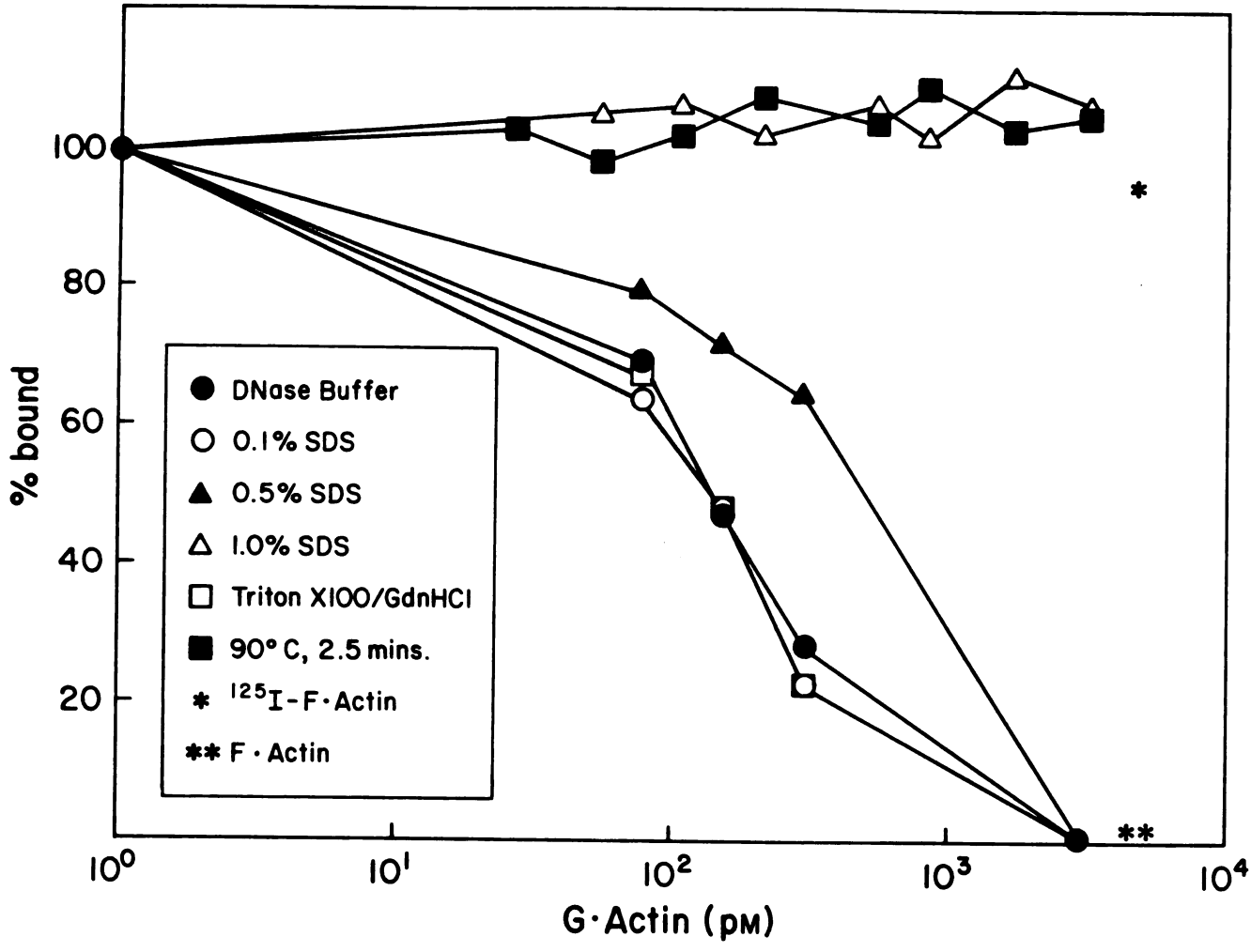



Figure 33. Effect of Various Treatments on Actin Performance in the DNase Immunoassay. In each experiment, G-actin was treated as described prior to use as displacing agent in the assay.
* F-actin iodinated and used in lieu of $^{125}$I-G-actin; ** F-actin used as displacing agent with $^{125}$I-G-actin.

| Displacing Ligand | Final Conc. (nM) | Percent of Control |
|---|---|---|
| None | — | 100 |
| G•Actin | 3.2 | 38 |
| RNase A | 10.4 | 112 |
| | 104.3 | 113 |
| α, β-Tubulin | 1.4 | 116 |
| | 14.3 | 103 |
| Myosin | 0.3 | 108 |
| | 3.2 | 121 |
| Laminin | 0.2 | 103 |
| | 2.2 | 118 |
| Fibronectin | 0.3 | 105 |
| | 3.2 | 87 |
| Collagen, Type I | 1.4 | 106 |
| | 14.3 | 103 |

Table III. Specificity of the DNase Immunoprecipitation Assay for Actin. All proteins were introduced into the assay in DNase incubation buffer. "Control" is defined as the amount of radioactivity precipitated in the absence of displacing actin; all values are reported as percent of "control" values. In all three experiments performed, values have been corrected for nonspecific background radioactivity.

precipitated demonstrates that RNase cannot replace DNase I in this assay. This further confirms the dependence of this assay on the specific interaction of actin with DNase I.

Confirmation of the DNase Immunoprecipitation Assay: Quantitation of Adrenocortical Actin

To test the accuracy of this modified DNase immunoprecipitation assay in quantitating actin contents in cellular extracts, and to confirm the estimates of adrenocortical actin levels obtained by SDS-PAGE and DNase inhibition analyses, this assay was used to determine actin levels in cell lysates of adrenocortical cells. Furthermore, lysates were prepared from adrenocortical cells treated with or without ACTH in order to confirm the effect of this hormone of adrenocortical actin content. Cultured rat adrenocortical cells were incubated with or without 2.2nM ACTH for 24 hrs., then solublized in the NP40/DOC lysis buffer containing 0.1% SDS. Lysates were immediately centrifuged at 1500 x g for 15 mins. to remove insoluble material, and assayed for actin by this immunoassay.

Figure 34 shows the effect of ACTH on adrenocortical cell actin content as assayed by the DNase immunoprecipitation assay. ACTH induces a loss of 35-43% of the total actin in these cells without a significant change in total cellular protein. Actin content drops from a value of 7ug/95ug of total protein, or 7.4% of the total cellular protein, in untreated cells to 4ug/86ug, or 4.7% of the total protein, in treated cells. This corresponds to a loss of 36.5% of the total cellular actin. Note that the DNase

immunoassay estimate of the total actin content in unstimulated cells is slightly lower than that value obtained by densitometry of scans of cell lysates run on SDS gels (approximately 10% of the total protein, see Figure 11). Other estimates of total actin content by this assay (see Figure 35) more closely approximate the SDS-PAGE value.

Both the magnitude of the absolute amount of actin and of the extent of actin loss with ACTH treatment, measured here by the DNase immunoassay, are significnatly higher than those values obtained using the DNase inhibition assay (5.5% and 21-25%, respectively, see Table I, Chapter 3). The reason for this discrepancy is not known; however, we believe it involves the difference in lysis conditions between the two assays. Cell lysates for the DNase inhibition assay are prepared by lysis of cells in Triton X-100 followed by depolymerization of F-actin with guanidine-HCl. If this treatment does not effect complete depolymerization of filamentous actin, then we predict that the values obtained by this assay would be underestimates of the actual amounts of actin, and would be lower than the values obtained in the DNase immunoassay, where the use of 0.1% SDS in the preparation of cell lysates almost guarantees complete disruption of actin filaments. In light of the earlier experimental evidence suggesting that guanidine-HCl treatment does not result in complete actin depolymerization, we suggest that our lower values for actin contents obtained by the DNase inhibition assay support this hypothesis of inaccurate quantitation.

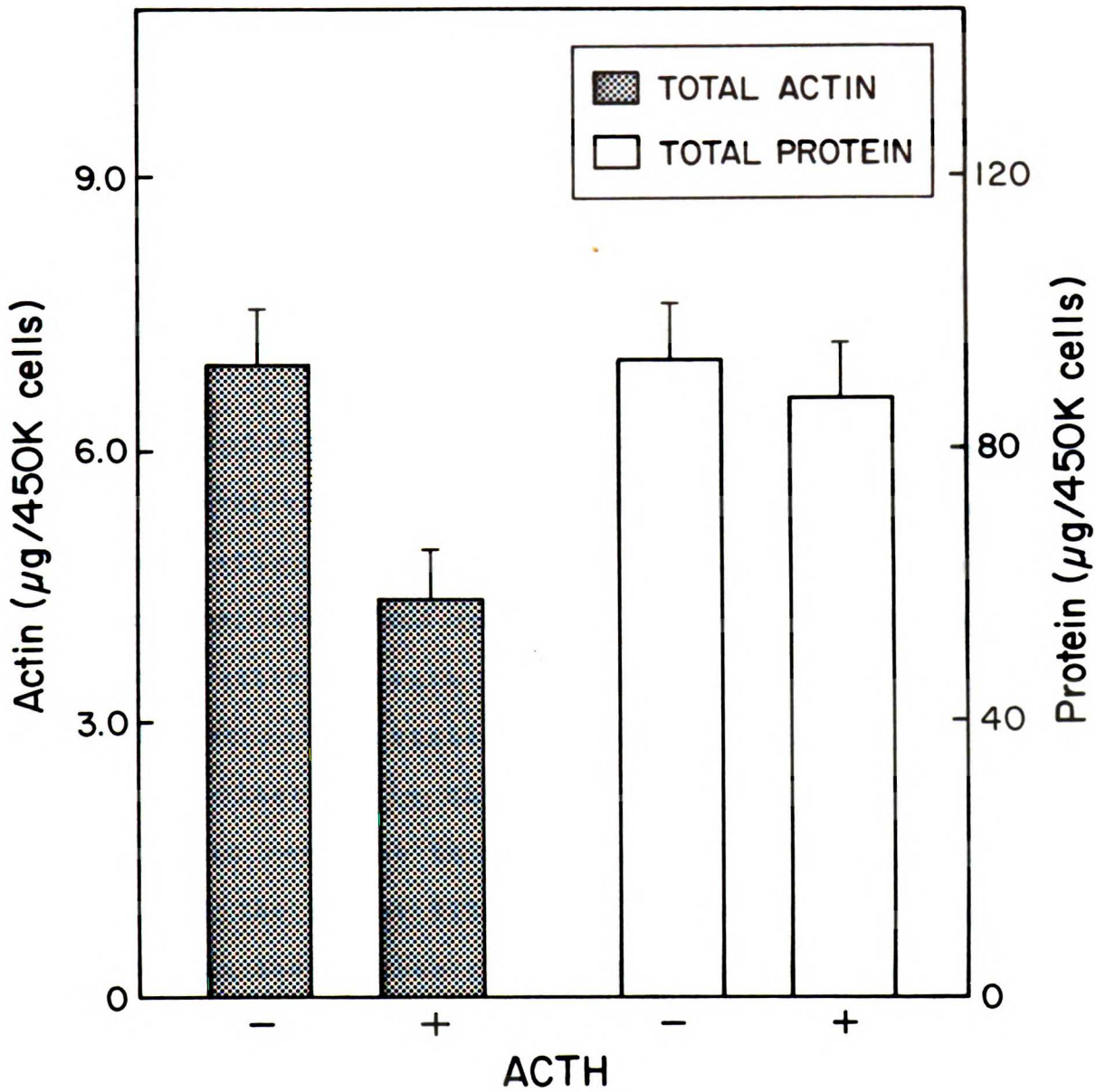



Figure 34. Effect of ACTH on Actin Content in Cultured Rat Adrenocortical Cells. Isolated adrenocortical cells from adult (400-450gm) male rats were cultured for 5 days in 1cm$^2$ culture dishes, then incubated with or without 2.2nM ACTH for 48 hrs. after which cells were washed 2X with PBS, lysed and assayed for actin content by DNase immunoprecipitation as described in "Methods" section, Chapter 3. Protein was determined by Lowry et al. (464) on 10% TCA-precipitates of cell lysates. Six separate actin determinations in triplicate for each treatment; four determinations four protein. Actin values reported are the percent of total protein which is actin.

The results presented here using the DNase immunoprecipitation assay fully confirm the earlier observations, made by SDS-PAGE and DNase inhibition analyses, that ACTH induces a loss of adrenocortical actin. Moreover, characterization of this immunoassay supports the conclusion that this assay accurately quantitates adrenal actin, and has given us confidence in using this assay to further explore and extend our investigations of the hormonal regulation of the cytoskeleton.

Measurement of Actin in the 3T3-L1 Preadipocyte/Adipocyte System

As further validation of the DNase immunoprecipitation assay in quantitating nonmuscle actins, and as an interesting collaboration, the DNase immunoprecipitation assay was employed to estimate actin contents in another well-known cell system. The 3T3 cell line is an established cell line of undifferentiated mouse fibroblast cells which convert to fully mature adipose cells when in response to steroids (508). 3T3-L1 is a daughter cell line of the parent 3T3 fibroblasts which undergoes diverse morphological and biochemical transformations in the process of hormonally-induced differentiation. During the conversion of preadipocytes to adiposites, cells lose their fibroblastic appearance and become spherical. At the same time, they gain increased activity of enzymes involved in triglerceride synthesis as well as sensitivity to various hormones known to affect mature fat cells. In addition, adipocyte maturation involves changes in the synthetic rates of several cellular proteins. Actin synthesis, for example, decreases dramatically during

differentiation, and results in a loss of 75% of the total cellular actin (509,510). This decrease has been correlated with both the change in cell shape and with a loss of actin stress cables in these cells. In preadipocytes, actin constitutes 12.1% of the total cellular protein; in mature fat cells, this protein constitutes only 3.2% of the total protein, as measured by densitometric scanning of cells run on SDS gels.

The 3T3 cell has much in common with rat adrenocortical cell. Both respond to specific hormones by diverse changes in cellular biochemistry and morphology. Hormonal stimulation effects a change in shape in both cells, from a highly asymmetrical to a spherical appearance. Coincident with cell rounding in both cells is a change in actin content, i.e., loss of total actin. These similarities make the adipocyte an ideal "positive control" cell in which to corroborate earlier estimates of actin content and loss in response to hormonal stimulation.

For this purpose, three populations of 3T3-L1 cells were analyzed for actin content: undifferentiated preadipocytes, adipocytes, and dexamethasone-treated adipocytes. Cells were harvested from tissue culture flasks, washed several times with PBS, resuspended to equal concentrations in PBS, and solublized in the same SDS lysis buffer used for adrenocortical cells. Lysates were further homogenized by ultrasonication, then spun for one hour at 100,000 x g to remove insoluble material. Supernatants were immediately diluted at least ten-fold in DNase incubation buffer, and assayed for actin by DNase immunoprecipitation and for total protein by Lowry. In three separate

experiments, actin contents in fibroblasts were found to be 10.4% of the total cellular protein; in mature adipocytes and dexamethasone-treated adipocytes, actin constituted 3.4% and 2.4%, respectively, of the total cellular protein. This corresponds to a loss of 67% of the total actin content in mature adipocytes, and a loss of 77% of total actin in dexamethasone-treated cells. These values, as well as the absolute amounts of actin in all three populations of cell, are remarkably similar to those values reported previously using scans of SDS gels as a means of quantitation (509). These results strengthen the contention that this assay accurately quantitates nonmuscle actin.

# CHAPTER 5
# FURTHER CHARACTERIZATION OF ACTH-INDUCED ADRENOCORTICAL ACTIN LOSS

## The Effect of ACTH on Adrenocortical Actin

Actin is a major protein in a variety of cell types. For example, actin comprises 15% of the total cellular protein in fibroblasts (512). Actin in macrophages constitutes 12% of the total cellular protein (599). In Acanthamoeba castellanii, actin is 7-8% of the total protein (513). In sea urchin eggs, actin makes up some 15 to 30% of the total cellular protein, depending of the developmental stage of the cell (514). In the rat adrenocortical cell, actin comprises approximately 10% of the total cellular protein as measured here by SDS-PAGE analysis, and by the DNase I inhibition and immunoprecipitation assays. These three techniques all demonstrate that ACTH initiates a loss of 20-60% of total adrenocortical cell actin. Therefore, 2-6% of the total cellular protein is lost in these cells in response to ACTH. Such a major change in protein must have tremendous consequences for cell function and cell shape.

Having established that ACTH promotes the loss of adrenocortical actin, we have employed the DNase immunoprecipitation assay in an attempt to determine the underlying molecular mechanism of hormone action on cell shape and cytoskeletal organization. One initial concern was that ACTH promotion of actin loss might not be a specific effect of this hormone on this one protein, but rather might reflect a nonspecific effect on total protein synthesis. Alternatively, ACTH-induced loss of actin might simply be the consequence of

cell loss upon hormone treatment, due to detachment of cells from culture dishes during cell rounding. Finally, loss of actin in ACTH-treated cells might result from decreased numbers of cells with time in culture, relative to untreated cells, due to ACTH-mediated inhibition of cell growth. Though the adrenocortical cells used in these studies were obtained from adult rats, and their doubling time in culture is quite slow ($t_{1/2}$ = 48 hours, see references 515,516), it is still possible that changes in cell growth might result in differences in cell number between treated and untreated populations, and thereby account for at least part of the apparent loss in adrenocortical actin.

To establish that the effect of ACTH on adrenocortical actin content was a specific effect, and not a consequence of differences in cell number due to cell loss or inhibition of cell growth, cells were treated for different times with or without 2.2nM ACTH, then analyzed for protein and for DNA contents. Figure 35 shows that over a time period of 48 hours of hormone treatment, ACTH has no significant effect on either total cellular protein or on DNA content. Assuming that DNA content directly reflects cell number, these results strongly suggest that changes in actin content are not a consequence of parallel changes in total cellular protein or in cell number, and therefore reflect a hormone-specific effect. Figure 35 also shows that the effect of 8-Br-cAMP on actin content cannot be the result of a decrease in total protein or loss of cells, for this agent again has no effect on either protein or DNA content over the 48-hour period in which it was administered.

Figure 36 shows the change in adrenocortical actin content, measured by the DNase immunoassay, as a function of ACTH concentration. Actin loss is apparent at physiological concentrations of the hormone. Note that at the lowest dose tested (22pM), a concentration at which no cell retraction is visible, actin content has dropped to a level achievable at higher doses of ACTH which do initiate cell rounding. Moreover, actin loss does not exceed more than 60% of the total cellular actin, even at near-pharmacological doses of ACTH.

As with both changes in cell shape and microfilament organization, ACTH-induced loss of actin is hormone-specific (insulin and beta-endrophin have no effect on actin content), and can be produced by exogenous 8-Br-cAMP in the absence of hormone (Figure 36). Furthermore, the effect of ACTH on actin content may specifically involve microfilament disassembly, for cytochalasin B, but not colchicine, can induce actin loss in the absence of the hormone (Figure 36). Though the effect of the cAMP analog on actin content again can not be attributed to differential cell loss, the drop in total cellular protein and DNA observed with cytochalasin B treatment (Figure 35) does suggest some loss of cells, and might in part explain the dramatic loss of actin in response to cytochalasin B. Nevertheless, a loss of even 35% of the cells upon exposure to cytochalasin B cannot fully account for a loss of over 60% of the total actin in these cells. Moreover, actin contents in Figure 36 are expressed as percent of total protein, and thus already take into account any companion changes in total cellular protein.

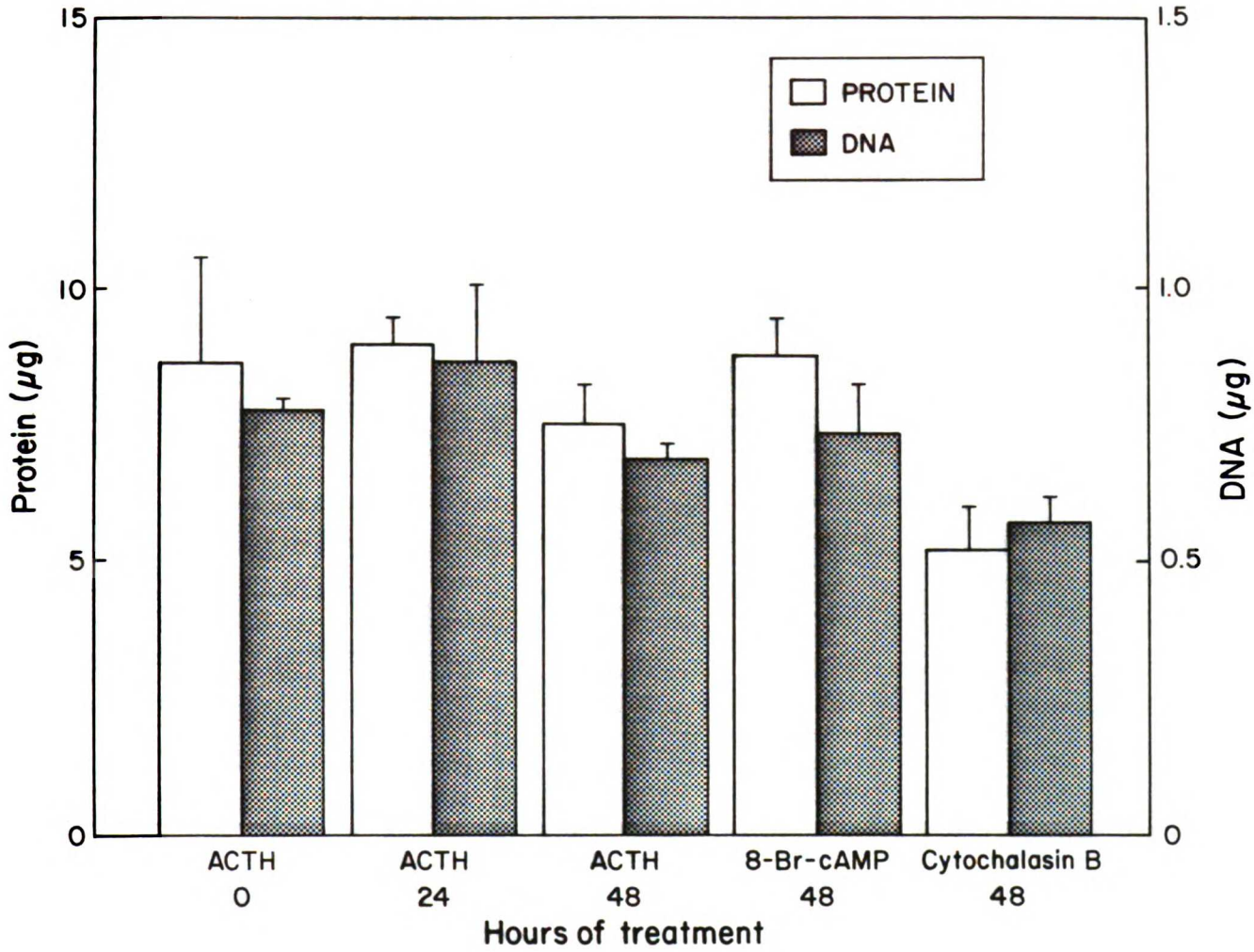



Figure 35. Effect of ACTH, 8-Br-cAMP, and Cytochalasin B on Rat Adrenocortical Protein and DNA Contents. Cells from young rats (200-250gm) were cultured for 6 days, then incubated with various agents for the times indicated. Cells were then washed with PBS and lysed in 500ul of 1N NaOH for 6 hrs. at 37°C. Protein was determined by Lowry et al. (464); DNA by Ceriotti (466). ACTH was used at 2.2nM; 8-Br-cAMP at 2mM; Cytochalasin B at 10uM.

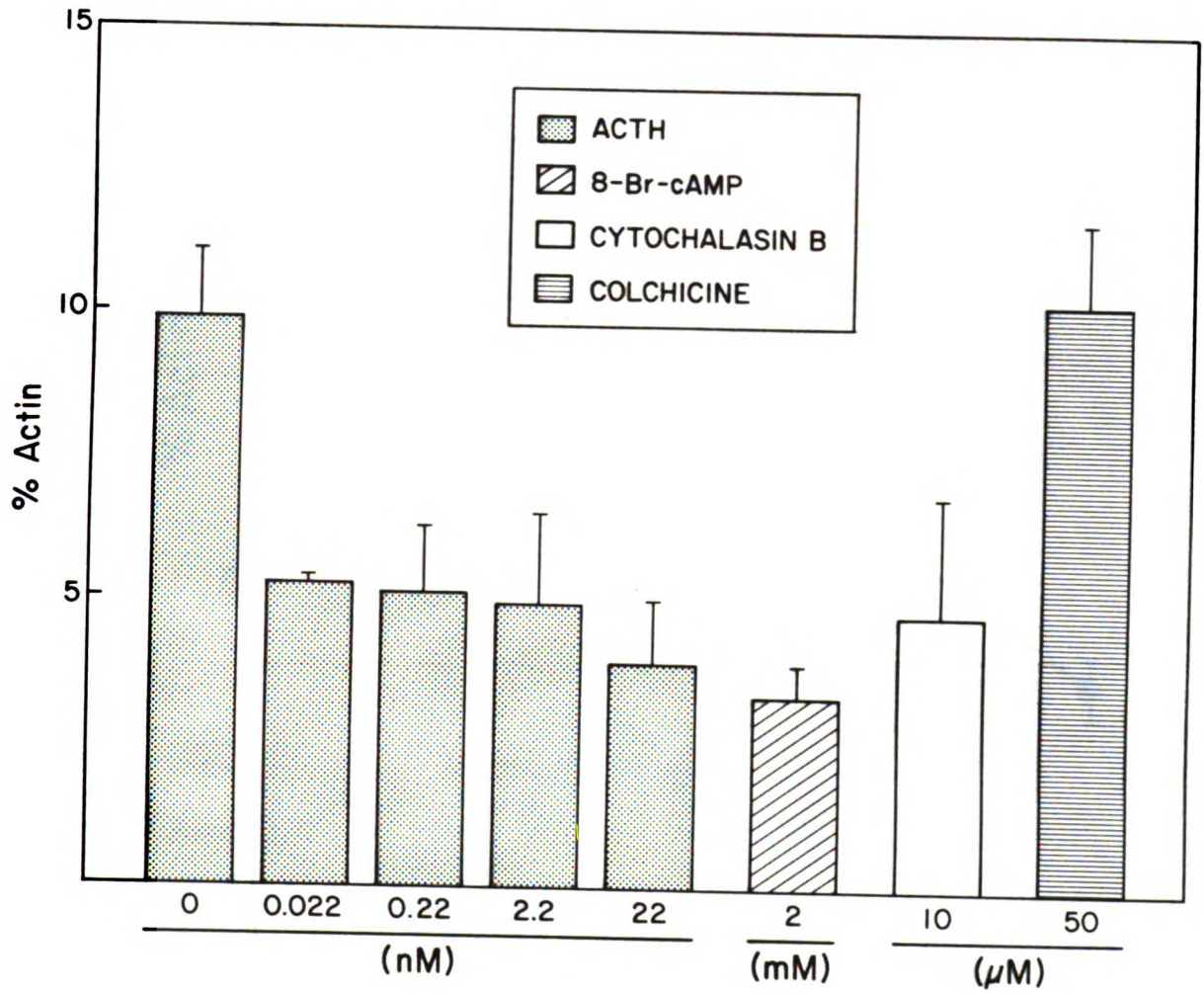



Figure 36. Dose Response of ACTH Effect on Adrenocortical Actin Content; The Effect of 8-Br-cAMP, Cytochalasin B, and Colchicine on Actin Content. Cells (450K) were plated for 7 days prior, then treated for 50 hrs. prior to lysis and measurement of actin content by DNase immunoprecipitation. Protein determined by Lowry et al. (464). Actin measurements represent three mean ± S.D. of three separate determinations each in duplicate, and all actin values are expressed as the percent of total protein. Protein determinations were done in triplicate.

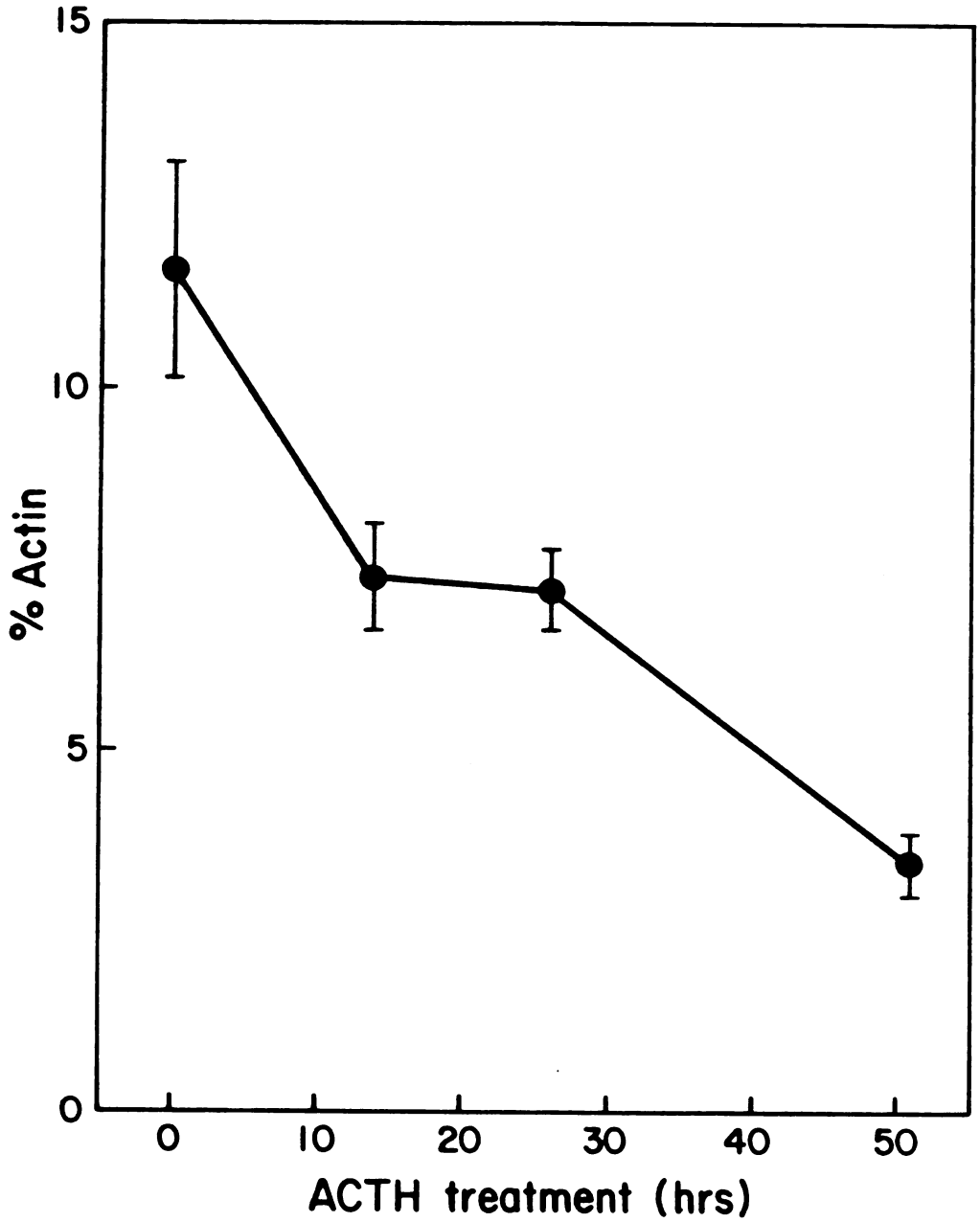



Figure 37. Time Course of ACTH-Induced Loss of Adrenocortical Actin. Cells (450K/well) were cultured for 5 days, then stimulated with 2.2nM ACTH for the time indicated prior to measurement of actin content by DNase immunoprecipitation. Actin is expressed as as percent of total protein. Actin values are the mean $\pm$ S.D. of three separate determinations, each in duplicate; protein for each point was determined on six separate aliquots of the lysate.

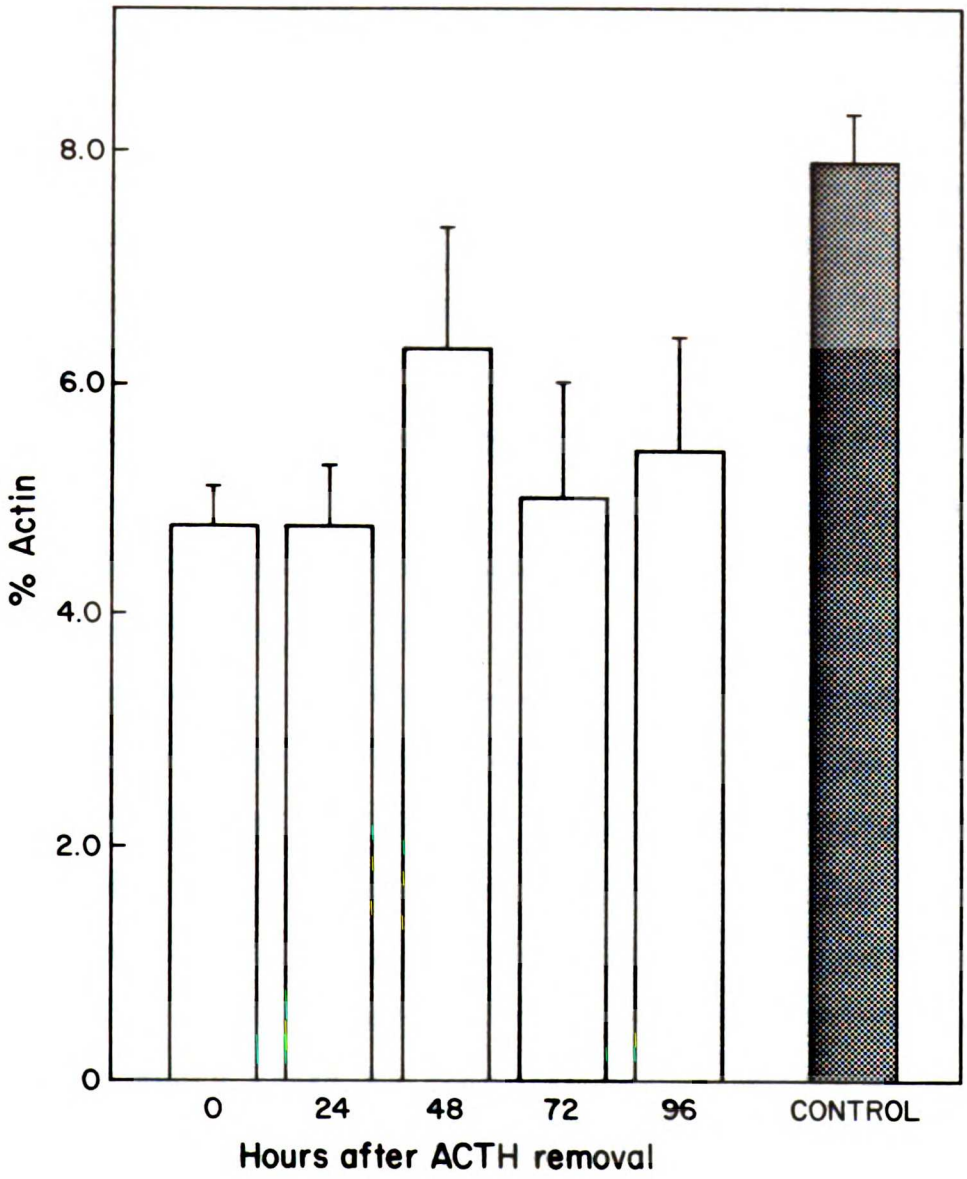



Figure 38. Effect of ACTH Removal on Actin Content of ACTH-Stimulated Cells. Cells (150K/well) were cultured for 4 days at which time they were incubated with or without 2.2nM ACTH for 48 hrs. Cells were then washed with fresh medium lacking hormone and incubated for the times indicated before determining actin content by DNase immunoprecipitation and protein by Lowry et al. (464). Bar graphs represent the mean ± S.E. of three separate experiments, each comprising quadruplicate actin determinations and triplicate protein determinations. "Control" refers to cells lysed at 96 hrs. which have never seen ACTH.

Figure 37 shows the time course of ACTH action of adrenocortical cell actin content. By twelve hours after hormone administration, a time at which cell rounding is first evident, this is a significant decrease in actin content which continues to increase in magnitude over the next 35 hours of treatment. Interestingly, the ACTH-promoted loss of actin, as a function of time, roughly parallels that of ACTH-induced cell retraction, and is on the same time scale as that of ACTH-mediated microfilament disassembly. The time correspondence of these events suggests some underlying co-ordination of events, and raises the intriguing possibility that these activities exist in some cause-and-effect relationship to one another.

One striking difference between ACTH-induced loss of adrenocortical actin and ACTH-induced alterations of cell shape and stress cable assembly is shown in Figure 38. Here, cells were stimulated with 2.2nM ACTH for 24 hours, at which time they were well retracted, then placed in fresh medium without hormone for various times as indicated. The bar labelled "Control" represents actin levels in cells never treated with ACTH. It is clear that even after 96 hours after hormone removal, a full two days after cells have returned to a flattened, asymmetric shape, and stress cables have reappeared, there is still an incomplete return of actin content to levels found in unstimulated cells. This is in marked contrast to both ACTH-induced cell retraction and stress cable loss.

## The Effect of ACTH on Adrenocortical Actin Content in Vivo

In an attempt to determine whether the effect of ACTH on actin content in cultured rat adrenocortical cells has any relevance to the action of the hormone in vivo, actin contents were quantitated in adrenals from hypophysectomized rats chronically treated with or without ACTH to determine whether a similar loss of adrenal actin could be detected. For this study, ten immature, male Sprague-Dawley rats (12 weeks old) were hypophysectomized, and one week later given either ACTH at 2.59ug/ml in 10% gelatin or gelatin vehicle intraperitoneally for four consecutive days as described by Ramachandran et al. (416). Twenty four hours after the last injection, rats were killed, adrenals extracted and defatted, and adrenals from each group pooled and weighed. Pooled adrenals were then solublized in 2.0 mls each of DNase immunoassay lysis buffer, and homongenized on ice with a polytron at high speed. One milliliter of each homogenate was immediately diluted into 2.5mls of lysis buffer and centrifuged for 1 hour at 100,000 x g. Supernatants were collected and promptly assayed for actin content by DNase immunoassay. Original homogenates were assayed for protein and for DNA.

The results can be summarized as follows: the pooled adrenal weight of the ACTH-treated adrenals (127.4mg/adrenal) was almost 50% greater than that of the untreated adrenals (89.1mg/adrenal), suggesting that chronic ACTH treatment in vivo promotes adrenal growth. This result is consistent with previous studies of the effect of ACTH on adrenal growth (436). Total adrenal protein from ACTH-treated rats also increased 50% relative to untreated

adrenals, from 705 + 35ug/adrenal to 1054 + 45ug/adrenal, whereas total adrenal DNA did not change significantly between ACTH-treated and vehicle-treated animals (19.68±2.55ug/adrenal with ACTH versus 20.01±3.01 without ACTH). This ACTH-induced increase in adrenal protein without a concomittant increase in DNA most likely reflects hypertrophy of adrenal cells without hyperplasia of the adrenal, a notion already put forth in a study of ACTH regulation of adrenal growth in vivo (441).

In contrast to total adrenal protein, adrenal actin content did not change significantly with ACTH treatment. ACTH-treated adrenals contained 11.546±4.365ug actin/adrenal and control adrenals contained 11.503±1.471ug actin/adrenal. Whereas total adrenal actin content does not appear to change in response to ACTH treatment if actin content is expressed in terms of DNA, it does drop significantly when expressed as the ratio of actin to total cellular protein. In fact, if actin is expressed in terms of total protein, ACTH appears to promote a loss of 29.5% of the total adrenal actin. As cell number (a reflection of DNA content) did not change significantly with ACTH treatment, the drop in actin content observed, relative to total protein, is entirely consistent with the loss of actin reported above in cultured adrenocortical cells.

The slight disparity between the extent of actin loss observed in vivo (29.5%) and that observed in cultured cells (35-55%), when measured by the same technique, may reflect the difference in adrenal cell populations in these experiments: whereas actin from whole adrenals represents actin from a host of

adrenal cell types, actin from cultured adrenocortical cells represents a particular population of relatively pure adrenal cells.

The fact that total protein increased in ACTH-treated adrenals relative to control adrenals, without a change in cell number, suggests that loss of adrenal actin relative to total protein reflects specific inhibition of actin synthesis in the face of general stimulation of adrenal protein synthesis, rather than loss of existing actin, a situation unlikely in cultured cells in light of the fact that ACTH-induced actin loss can be produced in the absence of protein synthesis. Alternatively, ACTH-induced actin loss may result from changes in the rate of actin degradation. Which of these scenarios is actually responsible for a change in adrenal actin relative to total protein awaits further study; nonetheless, the direction and magnitude of actin loss in response to ACTH, observed here relative to total adrenal protein, are similar to those seen in cultured adrenocortical cells, suggesting that hormonally-mediated changes in cytoskeletal content occur in vivo.

Investigation of the Mechanism of ACTH-Induced Actin Loss

In light of much direct and indirect experimental evidence supporting the notion that actin-containing stress cables are important cytoskeletal structures in defining cell shape, one possible mechanism underlying ACTH-induced adrenocortical cell rounding is that ACTH promotes detachment from the plasma membrane of stress cables involved in cell attachment and

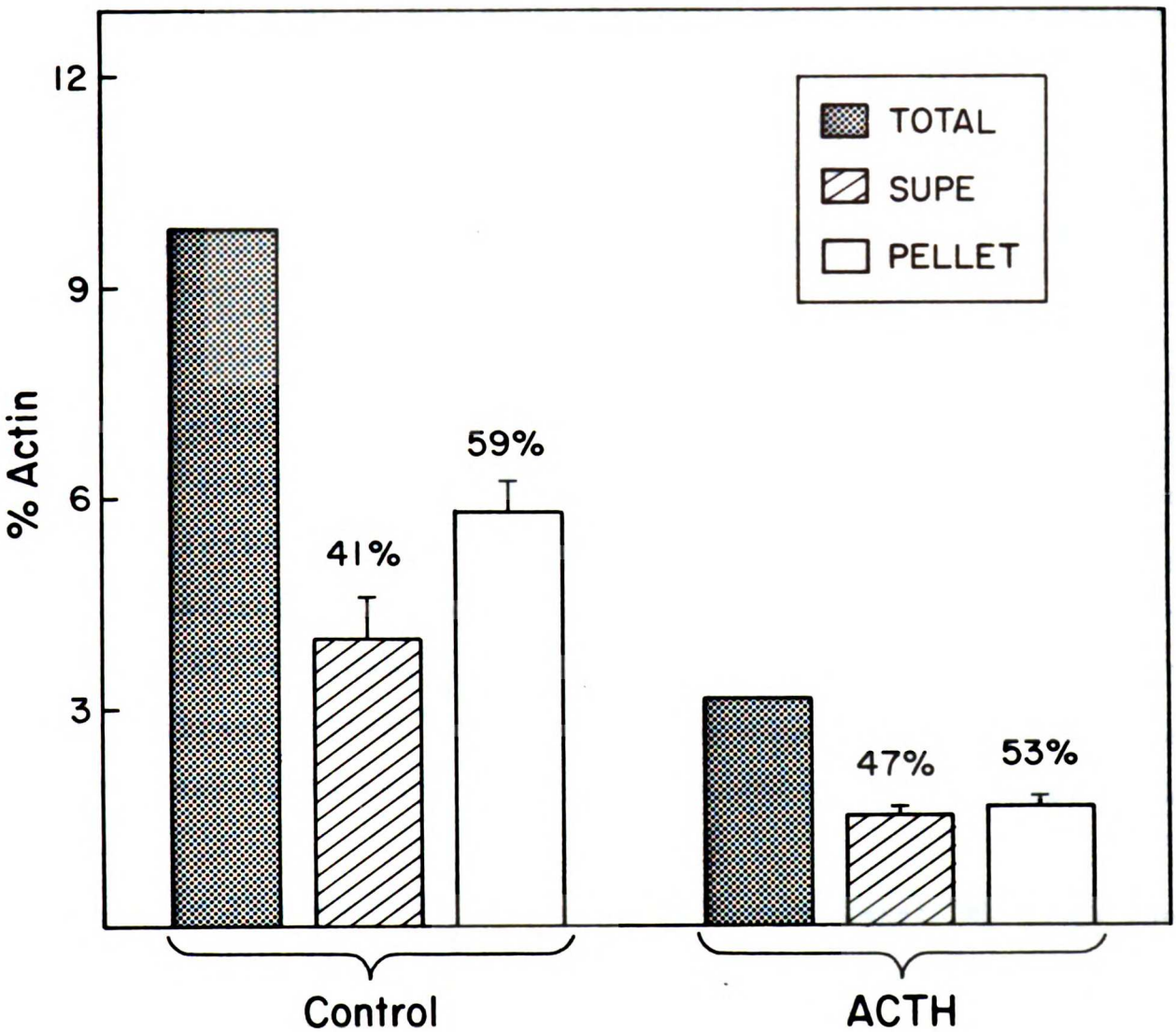



Figure 39. Effect of ACTH on Membrane-Associated Actin. Cells ($10^6$/ 35mm plate) were treated with or without 2.2nM ACTH for 72 hrs., then washed 2X with PBS and mechanically scraped into 200ul of ice-cold PBS containing 0.01% bacitracin, 0.01% LTI, 0.01mM PMSF. Cell membranes were pelleted at 1500 x g, then solublized in 200 ul of DNase lysis buffer and diluted 1:1 with DNase incubation buffer. One hundred ul of supernatant were diluted 1:1 with DNase lysis buffer. Each sample was assayed at three dilutions, each dilution in duplicate. All actin values are expressed as the percent of total protein. The percent of total actin found in each sample analyzed is reported above each bar. "Total" bars refer to the total actin in ACTH-treated and untreated populations.

flattening. Such a model for ACTH action is additionally attractive in that it explains the coincidence of hormone-induced microfilament disassembly and cell retraction by proposing a cause-and-effect relationship between these events.

To explore that ACTH promotes loss of membrane-associated microfilaments in the adrenocortical cell, cell membrane fractions were prepared from ACTH-treated cells, and from untreated cells, and both membrane-bound and soluble actin contents were determined. Cultured adrenocortical cells were incubated with 2.2nM ACTH for two days, then lysed in PBS buffer lacking detergents to prevent disruption of pre-existing membrane-microfilament associations. Membranes were separated from cytosolic proteins by centrifugation at 8700 x g for 15 mins. Membrane pellets and supernatants were then lysed in DNase immunoassay lysis buffer, and actin contents in both fractions measured.

Figure 39 shows the results of this experiment. Although the characteristic drop in total actin content is evident in ACTH-treated cells, there is no significant change in the distribution of remaining actin between membrane and soluble fractions. In unteated cells, 59% of the total actin is found in the membrane fraction; in ACTH-treated cells, 53% of the total actin is found in this fraction. This observation indicates that ACTH does not promote selective loss of membrane-associated actin, and argues against a simple detachment model as the modus operandi for ACTH-induced cell rounding.

Surface Labelling of Rat Adrenocortical Cells

Past observations of the effects of viral transformation on cytoskeletal organization and intracellular distribution suggest another mechanism for ACTH action on adrenocortical cell shape and actin organization. Transformation of normal cells in culture generally involves a host of characteristic changes in cellular function and morphology. Studies of normal cells and their virally-transformed counterparts have shown that transformation often induces flattened, asymmetrical cells to round up and retract away from the substratum to which they are attached (384). Coincident with cell rounding is a dramatic alteration in cytoskeletal organization (398). As a result of transformation, due in part to cell retraction, cells attach les firmly to surfaces, and lose sensitivity to many of the environmental controls which govern the growth of normal cells in culture. In some cases, viral transformation results in re-distribution of cytoskeletal proteins, e.g., appearance of actin and tubulin on the extracellular plasma membrane surface of the transformed cell (382). In other cases, loss of cytoskeletal proteins in virally-transformed cells results from secretion of these proteins into the medium (517). In light of these observations, we have tested the possibility that ACTH promotes actin loss from the rat adrenocortical cell by initiating the transfer of actin to the extracellular surface of the plasma membrane en route to secretion from the cell.

The experimental approach taken was to analyze surface proteins for the appearance of actin on the extracellular surface of the adrenocortical cell. This was done by labelling plasma

membrane-bound proteins on the extracellular face of the membrane by lactoperoxidase-catalyzed iodination of exposed tyrosine residues (384,523). If ACTH promotes actin secretion, then actin might appear on this face of the plasma membrane upon hormonal stimulation.

In all experiments, adrenocortical cells were cultured at low density in plastic dishes, and iodinated in the dishes as follows: cells were washed twice with PBS, then incubated with 330uCi of $^{125}$INa in 500ul of PBS. Iodination was initiated by addition of 50ul of lactoperoxidase (Sigma) at 2mg/ml and 20ul of $H_2O_2$ at 0.03% in $H_2O$. After 4 mins. at room temperature, 20ul of $H_2O_2$ were added and the reaction allowed to proceed for another 10 mins. Cells were then washed 3X with PBS containing 5mM KI, 2mM PMSF, 0.01% LTI, and 0.1%2-MeOH to quench the reaction and to inhibit proteolytic activity. Finally, labelled cells were solublized in SDS lysis buffer, and analyzed by SDS-PAGE.

Figure 40 shows a series of autoradiograms of SDS gels of adrenocortical cells surface labelled under different conditions of iodination. Lane 1 represents cells iodinated as described above. It is evident that may cellular proteins are labelled. This labelling is dependent on lactoperoxidase-catalyzed iodination, i.e., is not enzyme-independent, as shown by the lack of labelling when either lactoperoxidase (lane 2) or $H_2O_2$ and lactoperoxidase (lane 3) are omitted from the protocol. Moreover, that labelling conditions only result in iodination of surface proteins is shown by the lack of labelling when cytosolic proteins, extracted from surface-labelled cells, are analyzed by

SDS-PAGE (lane 4).

The controls for such labelling experiments are very important because conditions of iodination can easily damage cells and thereby expose intracellular proteins to labelling. To further demonstrate that the iodination protocol used here does not disturb the integrity of adrenocortical cells during labelling, mild trypsinization of surface-labelled cells was performed to demonstrate that only proteins on the extracellular surface of the plasma membrane are labelled upon iodination. If removal of plasma membrane-bound proteins exposed to the medium also results in loss of radioactivity from the cells, then the cells must have been intact during thelavelling procedure.

Rat adrenocortical cells were iodinated, and then trypsinized with 20ug/ml chymotrypsin-free trypsin (TPCK-trypsin, Worthington) for 10 mins. at 22°C or 37°C. Figure 41 shows the results of this experiment. Lanes A and B represent cells surface labelled as described by the standard protocol. Both lysates contain an abundance of labelled proteins. Lane E represents cells treated with trypsin after iodination. Much less radioactivity appears in this lysate, indicating that most of the radioactivity was associated with surface proteins which were remove by enzymatic digestion. Figure 41, lane F, shows that trypsinization itself is responsible for removal of radioactivity. Here, labelled cells were trypsinized in the presence of a molar excess of LTI. It is clear that loss of radioactivity is prevented when trypsinization is inhibited by LTI.

Lanes C and D of Figure 41 show the results of a slightly different experiment. Here, cells were first trypsinized with trypsin in the presence (lane D) or absence (lane C) of excess LTI, then iodinated by the standard procedure. Removal of surface proteins by trypsinization prior to iodination again removes most of the radioactivity from the cells, whereas inhibition of trypsinization by LTI prevents the loss of radioactivity. That removal of surface proteins removes labelled proteins again supports the belief that surface labelling specifically tags membrane proteins exposed to the extracellular medium.

Trypsinization itself does not introduce artifacts into the labelling procedure by damaging cells, for cells trypsinized at 22°C are still fully functional in terms of ACTH stimulation of steroidogenesis, as seen in Figure 42. Cells treated with trypsin for 10 mins. at 22°C are able to increase corticosterone production when treated with 2.2nM ACTH. The level of corticosterone production reached in trypsinized cells exactly matches that achieved in untrypsinized cells (not shown), and trypsinization itself has no effect on the basal rate of steroid production. Moreover, trypsinization at room temperature has no effect on ACTH stimulation of steroidogenesis, as indicated by the observation that cells treated with trypsin are as responsive to ACTH as cells trypsinized in the presence of LTI. However, cells trypsinized at 37°C for the same 10 mins. show reduced ACTH sensitivity, probably due to limited degradation of ACTH receptors. Taken together, these results strongly support the conclusion that the procedure used to iodinate surface proteins

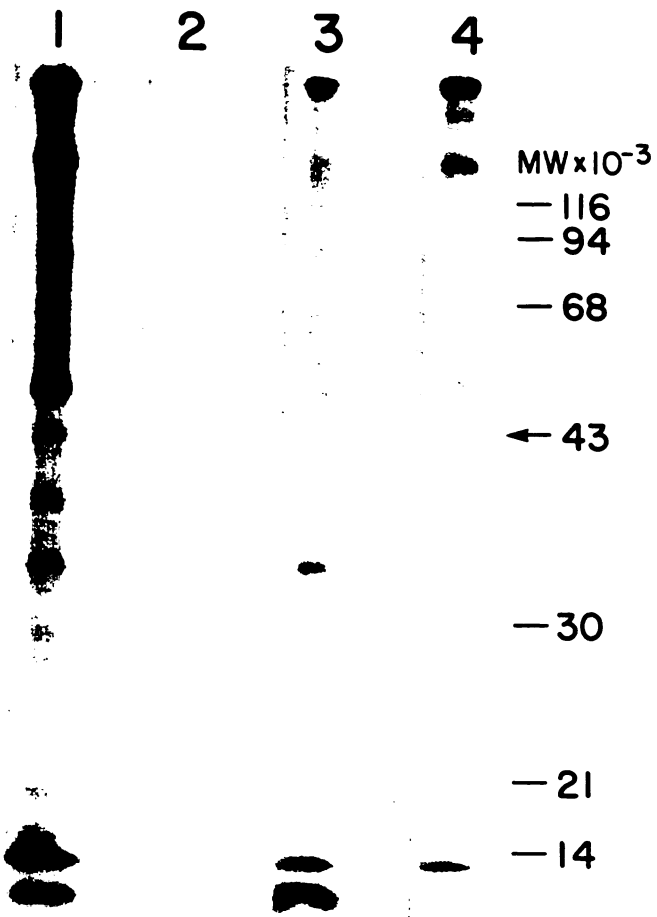



Figure 40. Surface Labelling of Rat Adrenocortical Cells. Cells were cultured in microtitre wells at 50K cells/well for 5-8 days, then surface labelled by lactoperoxidase-catalyzed iodination as described in the "Methods" section of Chapter 3. Lane 1) cells treated as described; 2) cells iodinated in the absence of lactoperoxidase; 3) cells iodinated in the absence of lactoperoxidase and $H_2O_2$; 4) soluble proteins in the supernatant from cells iodinated as in lane 1 which have been lysed in PBS by mechanical scraping and centrifuged at 1500 x g for 15 mins.

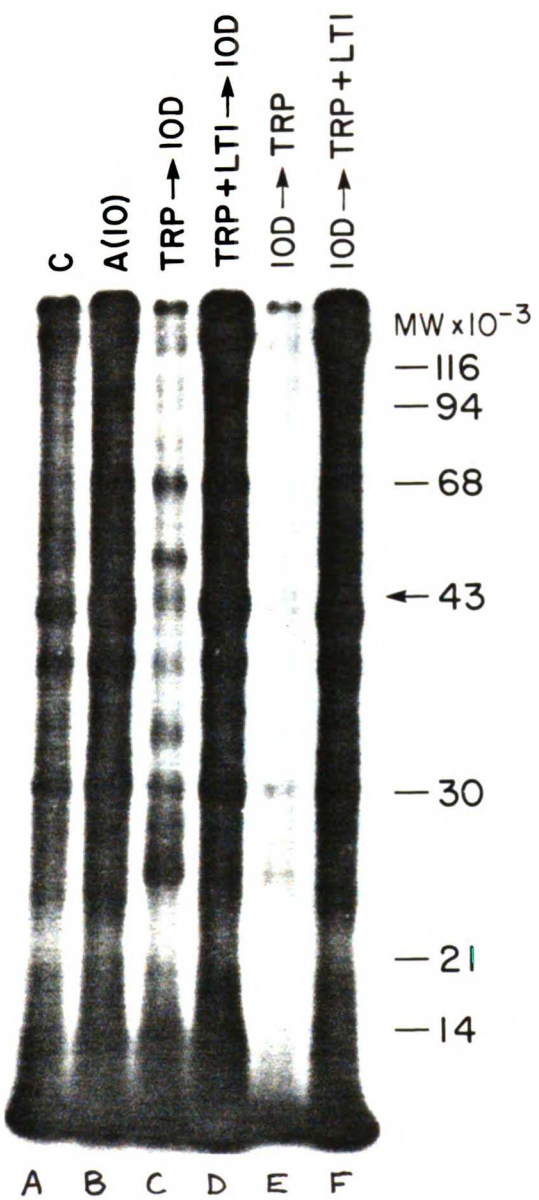



Figure 41. Trypsinization as a Means of Demonstrating the Specificity of Surface Labelling; The Effect of ACTH on the Pattern of Surface-Labelled Proteins. Cells were plated as described in Figure 40, then treated with (lane B) or without (lane A) 2.2nM ACTH for 48 hrs. before surface labelling. Unstimulated cells were first trypsinized with 10ug/ml TPCK-trypsin for 2 x 10mins. in the absence (lane C) or presence (lane D) of 10ug/ml LTI prior to iodination; other unstimulated cells were iodinated first, then trypsinized in the absence (lane E) or presence (lane F) of LTI.

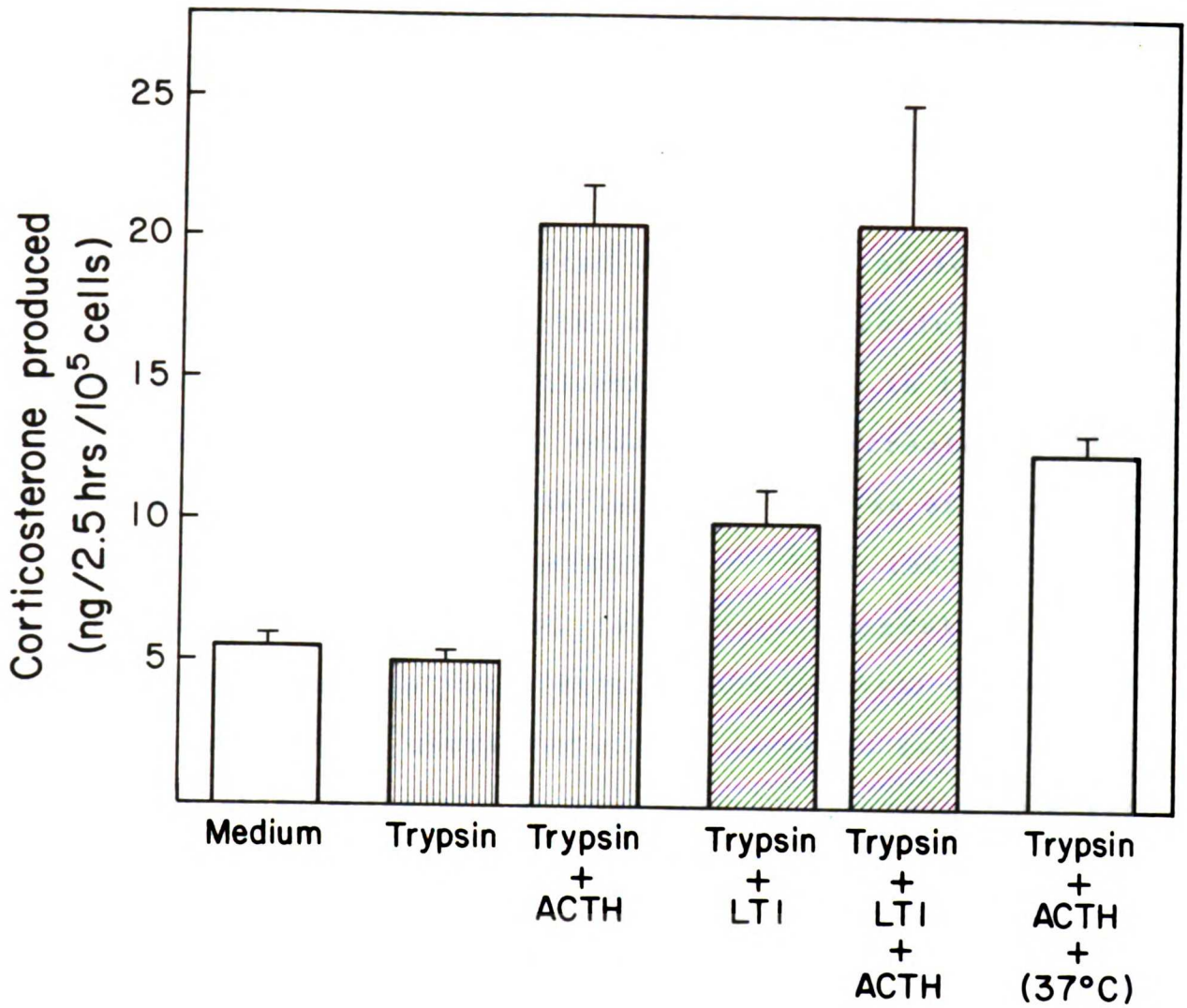



Figure 42. Effect of Trypsinization on Rat Adrenocortical Function. Cells ($10^5$) were cultured for 5 days at which time they were washed in serum-free medium, then typsinized with 20ug/ml TPCK-trypsin for 10 mins. at 22 °C in the presence or absence of 20ug/ml LTI. Cells were washed 2X with medium containing 10% fetal bovine serum, and incubated in 250ul of medium containing serum and 20ug/ml LTI in the presence or absence of 2.2nM ACTH for 2 hrs. at 37°C. Corticosterone secretion into the medium was assayed by specific RIA. The bar labelled "Trypsin + ACTH, 37 °C" refers to cells trypsinized at 37°C rather than at 22°C. "Medium" refers to cells which had not been trypsinized. LTI = Lima bean trypsin inhibitor.

specifically labels plasma membrane-bound proteins exposed to the extracellular medium without damaging the integrity of the cells.

Figure 41 shows the effect of ACTH on the pattern of surface-labelled proteins in rat adrenocortical cells. The lane labelled A respresents lysates of control cells; lane B that of cells which have been treated with 2.2nM ACTH for 48 hours. ACTH-treated cells were well-retracted at the time of iodination. Though these gels are overexposed, careful evaluation of these and other gels reveals no significant differences in the pattern of labelled proteins between treated and untreated cells. Furthermore, examination of these lanes, as well as of lane 1 of Figure 40, fails to reveal a distinct band of radioactivity centered at 43,000 daltons, strongly suggesting that adrenocortical actin does not reside on the external surface of either ACTH-treated or untreated cells. If actin exists on the extracellular side of the plasma membrane, it is unavailable for iodination.

In concert with these labelling studies, the DNase I immunoprecipitation assay was used to explore the possibilty that ACTH promotes secretion of adrenocortical actin. No actin could be detected in the medium of cells either treated or not treated with ACTH at any dose. Actin secretion, therefore, is not a viable explanation for the loss in actin observed.

## Further Exploration into the Mechanism of ACTH-Induced Actin

Loss: Evidence for Hormonal Regulation of Actin Degradation

In addition to actin secretion, various molecular mechanisms involving changes in the rate of actin synthesis and/or degradation can be invoked to explain the selective loss of adrenocortical actin in response to ACTH. ACTH could lower the steady state level of actin either by decreasing the rate of actin synthesis, or by increasing the rate of actin degradation, or both. Two observations argue against hormone-mediated inhibition of actin synthesis as a means of lowering actin content. First, loss of actin occurs even in the presence of cycloheximide at concentrations which inhibit adrenocortical protein synthesis to greater than 80%. Second, as actin turnover proceeds very slowly, with a $t_{1/2}$ on the order of days in all cells in which it has been measured (515,516), it is unlikely that shutting off actin synthesis alone could alone promote a loss of over 40% of total actin within 12 hours after ACTH administration. These observations, together with the demonstration that actin is not secreted from the adrenocortical cell, leave a change in the rate of actin degradation as the most attractive mechanism by which ACTH promotes actin loss.

To explore the possibility that ACTH induces actin loss by inhibiting actin degradation, rat adrenocortical cells were pulse-chased with radioactive amino acid in the presence or absence of ACTH to determine the respective rates of actin degradation. Cells were pulsed with $^{35}S$-methionine to steady state labelling conditions, then placed into fresh medium lacking label, and radioactivity incorporated into cellular protein

chased in the presence or absence of 2.2nM ACTH for various times. In these experiments, labelled cells were chased in the presence of 100ng/ml cycloheximide to inhibit protein synthesis to ensure that any observed changes in the rate at which label is lost from actin solely reflects changes in actin degradation, and not changes in both actin degradation and synthesis.

To determine the time of lablling necessary to achieve steady state labelling for this experiment, adrenocortical cells were pulsed with $^{35}$S-methionine for various times as shown in Figure 43. Cells were incubated with 15uCi/ml $^{35}$S-methionine for the times indicated, then washed with PBS containing 2mM methionine, and solublized in 1N NaOH. Aliquots were counted for radioactivity to determine the total radioactivity taken up into the cells; TCA-precipitates of parallel aliquots were counted to determine total radioactivity incorporated into cellular protein. Examination of the time course of label incorporation into rat adrenocortical cells reveals that steady state labelling in achieved sometime after 8 hours of labelling. Figure 43 also shows that cycloheximide treatment during labelling inhibits at least 80% of the total protein synthesis in these cells. This confirms other reports of the effect of this agent on adrenocortical cell protein synthesis (518).

To determine the rate of actin degradation in ACTH-treated and untreated adrenocortical cells, cells were labelled with $^{35}$S-methionine for 24 hours to ahcieve steady state labelling, reincubated for various times in complete medium containing 100ng/ml cycloheximide and 2mM methionine, then solublized in SDS

lysis buffer and analyzed by SDS-PAGE. Figure 44 documents the composite result of several experiments. Each point represents the mean of several densitometric evaluations of autoradiograms of the actin band in cell lysates run on SDS gels. Over the 48 hour chase period studied, total trichloroacetic acid-insoluble radioactivity dropped linearly 30-50% in both populations of cells. It is quite evident that radioactivity is lost from actin in ACTH-treated cells at a faster rate than in untreated cells, strongly suggesting that ACTH promotes an increase in the rate at which actin is degraded. The plresence of cycloheximide during the chase period ensures that loss of radioactivity from this band reflects true actin degradation, and again demonstrates that the effect of ACTH on actin content is not dependent on the synthesis of new protein.

Evidence for Lysosomal Involvement in ACTH-Induced Actin Loss

ACTH appears to induce actin loss by catalyzing actin degradation, but how is this effect brought about? To approach this question, we explored the possibility that lysosomal enzymes might be involved in hormone-induced actin degradation. The lysosome is the major protein-degradating organelle in the cell (414), and lysosomal proteases have been implicated in the control of general cellular protein degradation (520) as well as in specific degradation of internalized surface receptors (521). The loss of 35% of the total adrenocortical actin accounts for a loss of over 2% of the total cellular protein. This is an enormous amount of protein to be disposed of; therefore, the

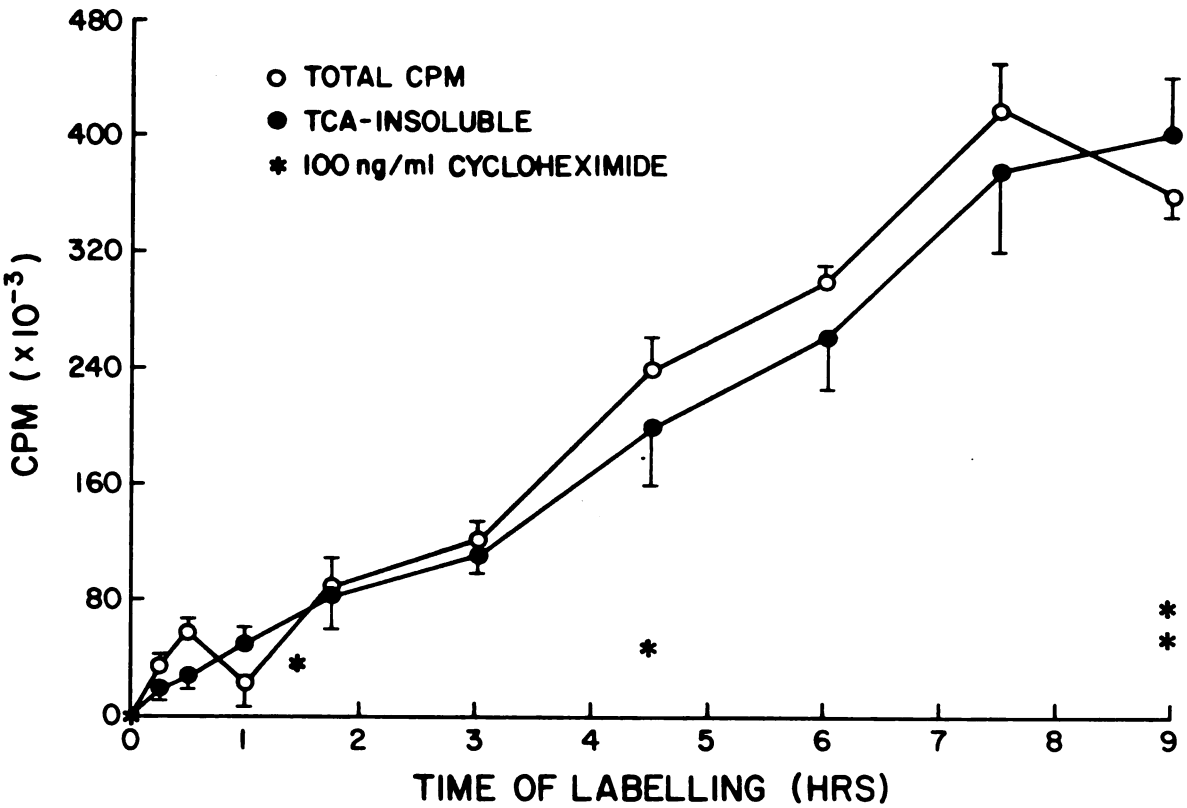



Figure 43. Time Course of Incorporation of $^{35}$S-methionine into Rat Adrenocortical Cells in Culture. Cells (150K/well) were cultured for 7 days, then incubated in medium containing 15uCi/ml of $^{35}$S-methionine for the times indicated. Cells were then washed 2X with medium containing 2mM methionine, and lysed in SDS solublizing buffer. Lysates were heated to 90°C for 5 mins., then insoluble material removed by centrifugation at 1500 x g for 15 mins. Total cellular radioactivity was determined on triplicate 10ul-aliquots of the lysate; radioactivity within protein was estimated from TCA precipitates of other 10ul-aliquots of lyastes precipitated in the presence of 0.9mg BSA.
* level of incorporation into cells which have been labelled for the times indicated in the presence of 100ng/ml cycloheximide.

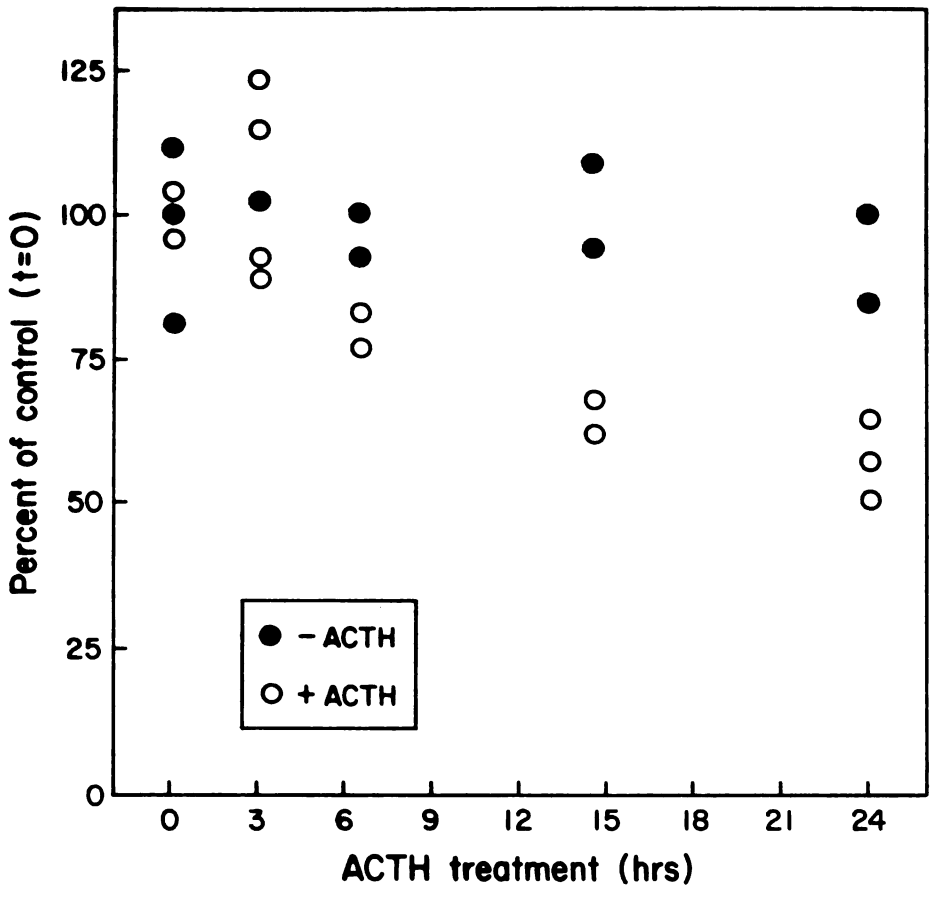



Figure 44. Evidence for an ACTH-induced Increase in the Rate of Actin Degradation. Densitometric analysis of autoradiograms of labelled rat adrenocortical cells. Cells (200K/well) were labelled with $^{35}$S-methionine for 24 hrs., then chased with cold medium containing 100ng/ml cycloheximide in the presence (open circles) or absence (closed circles) of 2.2nM ACTH for the times indicated. Cells were lysed in SDS solublizing buffer and run on 7% polyacrylamide gels. Gels were dried onto filter paper and autoradiographed for 7-10 days at -70°C. After developing, bands on autoradiograms corresponding to actin were scanned using a Joyce-Loebel scanning densitometer, and peak areas quantitated by hand planimetry. Each data point is the mean of three separate determinations, each made in triplicate, in three separate experiments.

| Treatment | Dose | Corticosterone production* | |
|---|---|---|---|
| | | After drug treatment | After return to control medium |
| Basal (No ACTH) | | 2.63±0.42 | 2.23± 1.01 |
| ACTH | 22 nM | 12.19±3.40 | 16.16 ± 7.27 |
| Chloroquine | 0.1 μM | 20.42±8.77 | 25.85± 12.86 |
| | 1.0 | 16.84±2.64 | 20.20± 1.14 |
| | 10 | 12.74±3.40 | 13.08± 2.62 |
| | 50 | 1.62 ± 1.34 | 4.95± 4.49 |
| $NH_4Cl$ | 1.0mM | 10.09 ± 2.78 | 14.19± 5.10 |
| | 10 | 11.64 ± 2.39 | 15.44± 3.37 |
| | 25 | 13.73 ± 3.28 | 15.48± 2.51 |
| | 50 | 8.12 ± 2.19 | 6.10± 2.83 |
| Leupeptin + pepstatin | 25 μM | 13.76 ± 4.03 | 16.93± 3.37 |
| | 50 | 9.41 ± 2.91 | 13.35± 4.70 |
| | 100 | 13.86 ± 2.32 | 20.88± 1.00 |
| | 200 | 10.63 ± 1.61 | 17.05± 3.22 |

* ng corticosterone/100K cells/hour in the presence of 2.2nM ACTH

Table IV. Effect of Lysosomotropic Agents on Adrenocortical Steroidogenesis. Cells (110K/well) were treated with various agents for 31 hours, then stimulated with 2.2nM ACTH for 1.5 hours in the presence of inhibitors, and aliquots of media assayed for corticosterone production by RIA. Cells were then washed 2X with fresh medium, and incubated for 2.5 days in medium alone. At this time, ACTH stimulation was repeated in the absence of inhibitors, and media again assayed for corticosterone production. All values reported are the mean ± S.D. of four separate determinations.

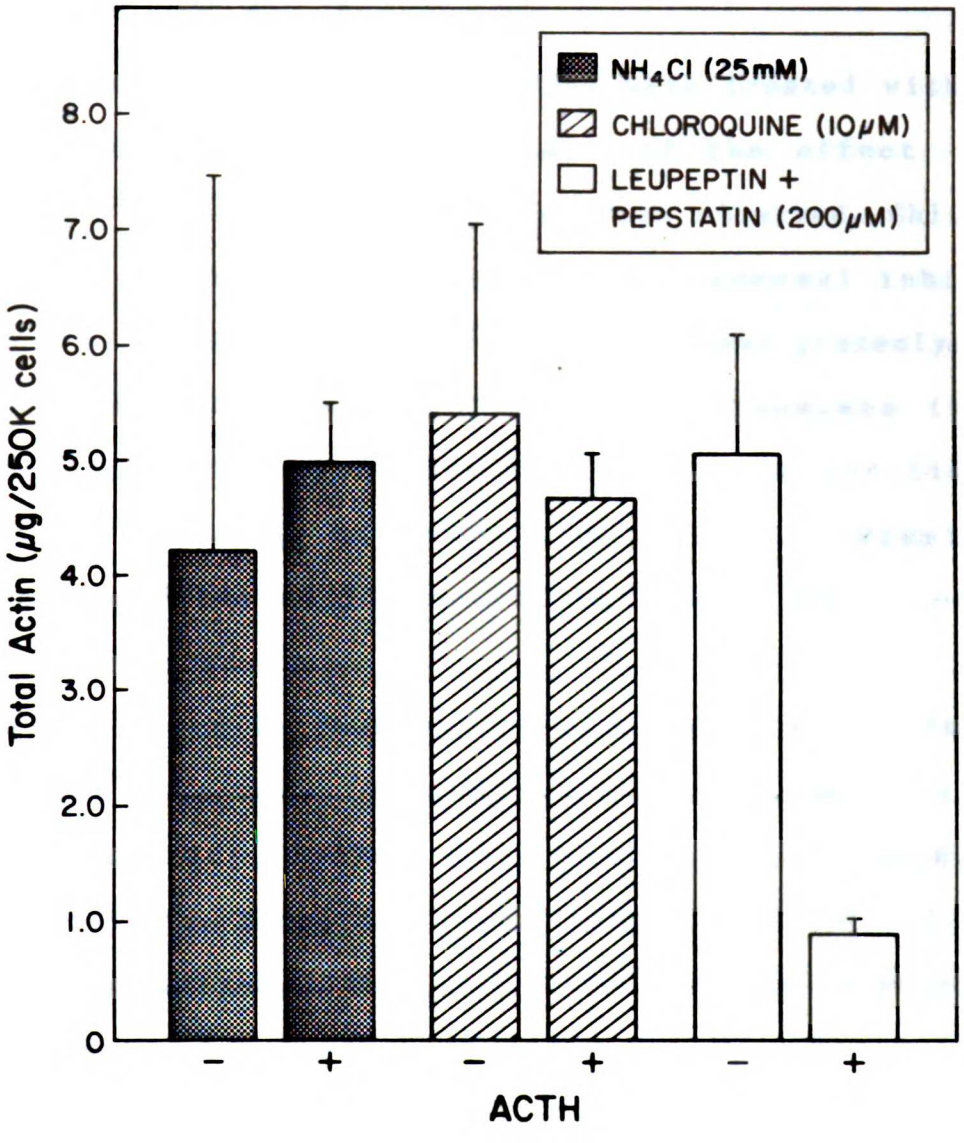



Figure 45. Inhibition of ACTH-Induced Actin Loss by the Lysosomotropic Agents Chloroquine and Ammonium Chloride, and Lack of Effect of Leupeptin and Pepstatin. Cells were cultured for 7 days, then treated with the various agents indicated for 30 hrs. Cells were washed 2X with iced PBS and lysed for actin determination by DNase immunoprecipitation. The data represents three experiments; each bar graph represents the mean ± S.D. of nine separate actin determinations, each determination in triplicate.

disposal of this much protein must involve some major intracellular degradative system(s). For these reasons, experiments were initiated to detect any lysosomal involvement in the course of actin degradation.

To this end, adrenocortical cells were treated with agents which inhibit lysosomal proteases, and the effect of this inhibition on ACTH-mediated actin loss was observed. Chloroquine and ammonium chloride were selected as lysosomal inhibitors. These drugs are specific inhibitors of lysosomal proteolysis, and have been used extensively to implicate lysosomes in known cellular degradative processes (521, 522). In addition, two peptidic intracellular protease inhibitors, leupeptin and pepstatin, were tested in concert for their effect on ACTH-induced actin degradation.

To allay concerns that any observable effect of these agents on actin degradation might be due to general damage to cellular integrity, rather than from specific inhibition of lysosomal activity, these drugs were tested for their ability to impair a major intracellular activity, namely, adrenocortical steroidogenesis. Rat adrenocortical cells were incubated with various doses of each drug for 24 hours, then incubated with 2.2nM ACTH in the presence of these drugs, and the media analyzed for stimulation of corticosterone production. Cells were then placed in fresh medium for 2 days, reincubated with ACTH alone, and the media reassayed for ACTH stimulation of steroidogenesis. Table IV shows that although chloroquine and ammonium chloride at 50mM irreversibly inhibit steroidogenesis, lower doses of these

agents have no effect on adrenocortical steroidogenesis. Leupeptin and pepstatin, at all concentrations tested, also have no effect on corticosterone production. The observation that these agents, at the lower doses tested, have no effect on adrenocortical steroidogenesis makes in unlikely that any inhibition of actin degradation observed at these doses would be the result of nonspecific cell toxicity.

Figure 45 shows the effect of these lysosomotropic agents on ACTH-induced actin degradation. Cells were treated with drugs at the doses indicated for 24 hrs. in the presence (+) or absence (-) of 2.2nM ACTH, then solublized and assayed for total actin content by the DNase immunoprecipitation assay. None of the drugs alone had any effect on actin levels in unstimulated cells. However, chloroquine or ammonium chloride, at concentrations that had no effect on adrenocortical steroidogenesis, completely blocked actin loss induced by ACTH. On the other hand, leupeptin and pepstatin in concert had no inhibitory effect on hormone-induced actin loss. These results strongly support the hypothesis that hormone-inducible actin degradation is lysosomally mediated.

# CHAPTER 6

# DISCUSSION

The results presented in this dissertation offer strong experimental support for the hypothesis that ACTH regulates adrenocortical cell shape through effects on cytoskeletal content and organization. In this final chapter, I would like to discuss several aspects of these findings, and would like to present some literature evidence that hormones regulate other important cellular activities such as cell growth and differentiation through effects on the cytoskeleton.

## Hormonal Control of Cell Shape and Cytoskeleton

The observation reported here that chronic ACTH treatment promotes a change in rat adrenocortical cell shape is not unique, and has been reported previously by several investigators. In an early study of the inhibitory effects of ACTH on replication of normal rat adrenocortical cells in culture, Ramachandran and Suyama (440) noted that prolonged stimulation of cells with ACTH initiates cell retraction. This observation has also been reported elsewhere in functional studies on the effects of ACTH antiserum on adrenal growth (452), and in early electron microscopic investigations of morphological transformations in adrenocortical cell ultrastructure in response to ACTH (424,524). While optimizing culture conditions for growth and maintainence of isolated rat adrenocortical cells, Rybak and Ramachandran (411) have found that cells plated in plastic culture dishes

markedly retract in response to physiological concentrations of ACTH. In a study of ACTH inhibition of DNA synthesis in isolated rat adrenocortical cells, McPherson and Ramachandran (438) have reported that ACTH-induced cell retraction occurs at the onset of inhibition of DNA synthesis.

Moreover, the effect of ACTH on adrenocortical cell shape is not confined to the rat adrenocortical cell. Similar retraction has been observed in cultured cells isolated from fetal human adrenals (434) and in mouse tumor Y-1 adrenocortical cells (438,477,486).

Hormonal regulation of cell shape is not unique to the adrenocortical cell; a similar response to chronic administration of other hormones has been observed in a host of nonadrenal cells. Lutropin and dibutyryl-cAMP influence the morphology of bovine luteal cells in culture, and promote a shift from an elongated, fibroblastic to polygonal, epithelial morphology which is fully reversible upon hormone removal (525). Follitropin induces retraction of Sertoli cells in culture (526). Human epidermoid carcinoma cells treated with epidermal growth factor retract from the surface of tissue culture plates, become more rounded and pile up to form multilayered colones (527). Chronic thrombin stimulation of human platelets produces diverse morphological changes in these cells which include a transformation from smooth discs to spheres with long thin protrusions (487). Other hormonally-induced changes in cell shape have been reported for the trophic hormones TSH (528), norepinephrine (529) and nerve growth factor (530).

In addition to alterations in gross cell shape, chronic stimulation with other hormones produces changes in cell morphology similar to those described for the adrenocortical cell in response to ACTH. For instance, epidermal growth factor (EGF) promotes rapid membrane rufflings in pheochromocytoma cells (531), and in cultured mouse epithelial cells in association with increased pinocytosis (531). EGF also induces long-term membrane ruffling and pinocytotic activity in cultured glial cells (531). Interferon, in addition to its effects on cell proliferation of human carcinoma cells, produces dramatic alterations in cell shape, and membrane mobility and rigidity, upon chronic administration (532). Thrombin induces blebbing of the plasma membrane in platelets during changes in cell shape (533). A similar blebbing has been described both in adrenal cells chronically treated with ACTH in vivo (424), and in Y-1 cells given ACTH or dibutyryl cyclic AMP, coincident with cell rounding (477). These reports attest to a general phenomenon of hormonal control over cell shape, and indirectly supports the notion that cell shape plays a key role in the underlying control mechanisms of cellular activities common to hormone-sensitive cells.

We have further shown that dramatic changes in the organization of the adrenocortical cytoskeleton accomany ACTH-induced cell rounding. Chronic administration of ACTH to isolated rat adrenocortical cells in culture promotes a complete loss of actin-containing stress cables, as viewed by immunofluorescence staining for actin. In well-retracted cells, actin appears to be distributed randomly throughout the cell either as monomeric

actin or in small oligomers of actin. As with hormone-induced cell retraction, the effect of ACTH on adrenocortical microfilament organization is hormone-specific, qualitatively dose-dependent, and reversible upon hormone removal. Hormone-induced loss of adrenocortical stress cables follows a time course which matches that of hormone-induced cell retraction.

Two previous studies have documented the effects of ACTH on the rat adrenocortical cell cytoskeleton reported here. In an investigation of the effects of cycloheximide on ACTH-induced ultrastructural differentiation in cultured fetal rat adrenocortical cell from the zona glomerulosa and zona fasciculata, Kahri (412) reported that electron microscopic evaluation of ACTH-treated cells revealed a significant reduction in the number of cytoskeletal filaments which by size and appearance appeared to correspond to actin microfilaments. In contrast to changes in inner mitochondrial membrane and smooth surfaced endoplasmic reticulum, this loss of microfilaments occurred in the presence of cycloheximide, suggesting that such cytoskeletal changes are not dependent on the synthesis of new protein(s). This conclusion is in total agreement with similar observations made here on cell shape responses in isolated rat adrenocortical cells. Slavinski-Turley and Auersperg (524) reported identical results in cultured rat adrenocortical cells. Here, stimulation of of cells for three days with micromolar doses of ACTH induced cell rounding and transformations in mitochondrial ultrastructure as well as a marked reduction in the number of "cytofilaments", i.e., microfilaments in the cytoplasm.

All of these changes were clearly evident after 24 hours of ACTH treatment, and persisted for at least 5 days in the continued presence of the hormone. Again these results are consistent with our findings. The results presented here, however, are the first reported attempt to use immunofluorescent staining of actin-containing structures to visualize the changes in rat adrenocortical cytoskeletal organization (loss of stress cables) in response to ACTH stimulation.

A similar loss of actin-containing cytoskeletal filaments has been observed in adrenocortical cells from other animals. Rainey et al. (347) have reported an identical hormone-induced loss of stress cables in isolated fetal human adrenocortical cells. They found that cultured cells treated with 1uM ACTH for as little as eight hours showed signs of retraction; after 48 hours of stimulation, they had fully retracted. At the electron microscopic level, it was clear that this hormone-induced cell retraction involved loss of microfilaments; this was corroborated by fluorescent staining for actin-containing filaments using NBD-phallacidin. In ACTH-treated human cells, actin localization revealed a diffuse, unorganized staining localized primarily around the nucleus and in the cytoplasmic processes, precisely the situation described here in the rat adrenocortical cell.

In the same way that ACTH-induced adrenocortical cell rounding is one example of a more general phenomenon of hormonal regulation of cell shape, so too is the effect of ACTH on the organization of the adrenocortical cytoskeleton. The literature abounds with reports in which hormonal stimulation leads to

alterations in intracellular actin distribution and assembly. For example, in the case of the human platelet, Carlsson et al. ( ) have used the DNase I inhibition assay to show that thrombin activation of these cells leads to a rapid polymerization of intracellular actin, coincident with transformation of platelets from smooth discs to spiky spheres, and the formation of plasma membrane protrusions. Immunofluorescent staining for actin-containing structures (487) has confirmed that thrombin stimulation of human platelets results in formation of actin filament bundles within these membrane protrusions, suggesting that polymerized actin bundles is responsible for membrane protrusion. Pribluda et al. (534) have confirmed this finding using a fluorescent modification of the DNase I inhibition. Swanston-Flatt et al. (535) have taken a similar approach to demonstrate that glucose stimulation of insulin secretion leads to both changes in cell morphology and a concommittant increase in the fraction of filamentous actin. In the case of interferon, Wang et al. (532) have decorated actin filaments with heavy meromyosin to demonstrate that interferon stimulation of HeLa-$S_3$ cells and human fibroblasts induces a striking change in actin polymerization and assembly that results in the formation of a dense submembranous meshwork of microfilaments and the appearance of large cytoplasmic actin fibers. These authors have postulated that such hormone-induced alterations in microfilament organization are involved in the underlying molecular mechansism responsible for decreased in lateral mobility and inhibition of surface capping observed with interferon stimulation. In

epidermal carcinoma cells, EGF induces both cell retraction and a striking change in the distribution of actin and alpha-actinin, observed by electron microscopy as a transient decrease in organized microfilament bundles and an accumulation of actin at the cell periphery (527). As with ACTH-induced loss of adrenocortical microfilaments, the effects of EGF on the distribution of actin-containing filaments are specific to this class of cytoskeletal structures; this hormone has no effect on the organization of microtubules or intermediate filaments. Morphological changes induced by TSH in thyroid cells have been correlated with a reorganization of microfilaments (528). As with ACTH-induced loss of adrenocortical microfilaments, the effect of TSH on the thyroid cytoskeleton can be mimicked by cAMP analogs (536). In Sertoli cells, FSH disorganizes microfilaments coincident with changes in cell morphology (537). The widespread coicidence of hormonal effects on cytoskeletal organization and on cell shape strongly suggests that the cytoskeleton is intimately involved in, and may mediate, hormonal regulation of cell shape.

## Evidence for Cytoskeletal Control of Cell Shape

Much experimental evidence has been collected which supports the notion that cytoskeletal structures such as microfilaments and microtubules regulate cell shape in cultured cells. Evidence for this hypothesis comes from several different approaches to the study of the cytoskeleton: studies in which the effects of agents which selectively alter cytoskeletal filaments are

correlated with changes in cell shape; investigations of the capping phenomenon and other events in which movements of surface proteins can be correlated with redistribution of cytoskeletal components; solublization studies in which the shape of isolated cytoskeletons have been compared to the shape of the intact cells from which they were prepared; from observations of the effects of viral transformation on cell shape. All of these studies are consistent with electron micrographic evidence for a physical association of cytoskeletal structures with the plasma membrane, an interaction which is believed to be intimately involved in defining cell shape.

Colchicine and the vinka alkaloids have been extensively used to implicate microtubular involvement in cell shape in numerous cultured cells. For example, in the case of cultured lens epithelial cells, it has been noted that colchicine and related microtubule-depolymerizing drugs inhibit the transformation of cell shape from rounded to elongated morphology (538). This inhibition coincides with cessation of terminal cellular differentiation and disruption of axially arragned microtubules in these cells, suggesting that microtubule organization are important for both of these processes in the lens cell. In isolated polymorphonuclear leukocytes, microtubule-disrupting agents such as colchicine, nocodazole, and diamide all lead to profound changes in cell shape (249). These drugs produce a characteristic protuberane of the plasma membrane at one pole of the cell which becomes more highly folded and noticeably smoother in appearance than in other regions of the membrane.

Formation of this outpocketing has been shown to be strictly dependent upon disruption of membrane-associated microtubules. In the case of the nonmammalian erythrocyte, development and maintainence of a flattened cell shape has been correlated with the integrity of a peripheral ring of microtubules called the marginal band (539). Destruction of this band by cold or colchicine treatment results in loss of microtubules and transformation from discoid to spherical shape. Removal of cold or colchicine restores both the marginal band and the discoid shape of the cell (539). Platelets also have a microtubule ring located at the rim of the cell underlying the plasma membrane, and exhibit a similar sensitivity to colchicine (540). Cilia and flagella, both of which contain elaborate microtubule-based cytoskeletons, are resorbed before mitosis and reappear after cell division (541), and can regenerate after amputation (542); in both instances, regrowth involves formation of new microtubule structures and can be prevented by treatment with colchicine and related drugs (542). Here again, changes in cell shape can be correlated with changes in microtubule organization.

Similar drug studies using cytochalasin B and other microfilament-disruping agents have specifically implicated these cytoskeletal filaments in the control of cell shape in other cells. Most nonmotile cells, when plated onto an artificial surface, quickly flatten onto the surface after initial attachment (339). With time in culture, the cells assume characteristic asymmetrical shapes coincident with the appearance of prominents actin stress cables in the cytoplasm. The

arrangement of these cables in cultured cells often reflects the general shape of the cells (19), implying that the organization of stress cables is in some way related to cell shape. Treatment of flattened, cultured cells with cytochalasin B (438,486) or one of its analogs (243) typically induces rounding of the cell body and retraction off the substratum, in a manner identical to that reported here with the rat adrenocortical cell. Moreover, treatment of cells with cytochalasin B and its analogs is specific to microfilaments, as this treatment has no effect on microtubule distribution (243). Cell rounding in response to cytochalasin B cannot be a consequence of general cell toxicity, for example through energy deprivation, for analogs such as dihydrocytochalasin B which can mimick the effect of cytochalasin B cell shape have no effect on other cellular activities such as sugar and thymidine transport (438). These results again imply an important role for the cytoskeleton in the maintainence of cell shape.

The response of the cytoskeleton to hormonal stimulation in hormone-sensitive cells may itself be a specialized case of the ways in which cells respond to their external environment. The physical association of the cytoskeletal components with membrane-associated surface proteins during the process of receptor capping, offers one clear case in which cells respond to external stimuli by alterations in intracellular cytoskeletal structures. As detailed in Chapter 1, there is abundant microscopic and biochemical evidence to suggest that microfilaments and microtubules interact directly, or indirectly

through other proteins, with proteins embedded in the plasma membrane. Much of this evidence rests on the use of selective inhibitors of these filaments. In the case of concanavalin A and Ig immunoglobulin receptor capping, microfilaments and/or microtubules have been implicated in the regulation of receptor mobility, patching, capping, and internalization of receptors (543). In other cases involving hormone surface receptors, studies using the cytochalasins and colchicine have shown that cytoskeletal filaments are involved in linking hormone-sensitive adenylate cyclase to surface in numerous hormone-sensitive cells (543), suggesting that the cytoskeleton may function in the transduction of external signals in cells where hormone action is thought to be mediated through this nucleotide. Ash et al. (383) have used indirect immunofluorescence to stain for several surface proteins - $beta_2$-macroglobulin, aminopeptidase, and the Na+,K+-ATPase - on the surface of human fibroblasts to analyze the distribution of these proteins relative to underlying intracellular microfilaments. They found that antibody-induced clustering of each surface protein was associated with a redistribution of microfilaments, suggesting that upon clustering, these integral proteins can form transmembrane linkages to actin-containing filaments. In numerous cases of hormone-sensitive cells, drugs such as colchicine (543) or cytochalasin B (544) have been shown to inhibit capping of specific receptors. In all these cases, binding of ligand to surface receptor, and subsequent receptor aggregation, induces dramatic changes in the underlying cytoskeleton.

Further evidence for a cytoskeletal response to external stimuli comes from a study on anchorage-dependent rat embryo cells in culture (545). These cells lose actin-containing stress cables when treated for limited times with plasmin or other proteolytic enzymes. This result strongly suggests that cytoskeletal components interact with surface proteins which receive and transmit external stimuli to the cell. If ACTH alter cytoskeletal organization in the rat adrenocortical cell to mediate its actions on other cellular activities, e.g., on long-term steroidogenesis or on DNA synthesis, then the observation presented here that ACTH has a profound effect on adrenocortical cytoskeletal architecture is entirely consistent with this hypothesis.

The mammalian erythrocyte offers a clear example in which the three-dimensional organization of the cytoskeleton directly reflects the shape of the intact cell. Mammalian erythrocytes do not contain microtubules; instead their extensive cytoskeleton consists of a complex web of interconnecting actin filaments and actinassociated proteins known as the spectrin-actin lattice (19). In addition to actin, this matrix contains spectrin and a number of other membrane-associated proteins (see Chapter 1, page 97). Erythrocyte ghosts, isolated by hypotonic lysis of intact cells, have been shown to retain this lattice (317); moreover, this lattice retains the general shape of both the erythrocyte ghose and the intact red blood cell (316). Factors such as deoxygenation, warm temperature ($37^{0}$C), and slight acidity, which promote a change in shape from spheroid to discoid morphology,

are the same factors which promote microfilament polymerization in these cells (546). Furthermore, cytoskeletons isolated from diseased red blood cells which display abnormal shapes, such as from irreversibly sickled erythrocytes (547) and from the elliptical erythrocytes of patients with hereditary elliptocytosis (548), retain the abnormal shapes of the cells from which they are extracted. Analysis of cytoskeletons from red blood cells from rare human blood disorders in which the erythrocytes are unusually fragile due to chemical abnormalities in spectrin (317) or in band 3 (316) reveal that the same anomolies in cell shape evident in the intact cells are retained in these cytoskeletons, implicating the spectrin-actin lattice in the maintainence of erythrocyte shape.

A similar correspondence between cytoskeletal and cell shape is observed in Triton-resistant cytoskeletons prepared from nonerythroid cells. In the case of human fibroblast (385) or of the ascites tumor cell (389), for example, the shape of the cytoskeleton prepared by detergent extraction closely resembles the shape of the intact cell. Moreover, nuclear matrices derived from mitotic cells have been shown to reflect the shapes of the nuclei of these cells at different stages of cell division (see Chapter 1, page 62). In the case of the adrenocortical cell, electron microscopic analysis of cytoskeletons isolated from cells treated with or without ACTH (549) have clearly shown that the shape of the resulting cytoskeleton closely resembles the shape of the intact cell. Examination of Figures 1 and 10 in this dissertation confirms this observation. In these pictures, it is

evident that the general appearance of microfilaments in ACTH-treated and untreated cells reflects the overall shape of the cells from which they are derived.

Further evidence for cytoskeletal regulation of cell shape comes from studies of cellular transformation. In addition to alterations in the control of cell growth and function, viral transformation of normal cultured cells leads to drastic changes in cell shape and morphology. Transformed cells typically display changes in plasma membrane morphology, e.g., excessive membrane blebbing (44), as well as plasma membrane-related abnormalities including increased transport of metabolites (44), and altered secretion of plasminogen activator (550). Many established cell lines, including Swiss 3T3 cells (551), Balb/c mouse cells (399), and normal rat kidney (398) cells, grow in culture as flattened, elongated cells. When transformed with a variety of viral agents, these cells characteristically round up and lift off the surface to which they are attached (399). Concomittant with this change in cell shape is a complete loss of actin stress cables, shown both by light and immunofluorescence microscopy (398,399). Normal cells transformed in vitro by oncogenic viruses and untransformed cells infected with paramyoxoviruses contain fewer numbers of microfilaments (231). Other studies have shown that virus-transformed fibroblasts lose microtubules as well as actin stress cables (389). In an extensive comparison of normal and transformed cells, Weber et. al. (553) produced cells which were either fully or partially transformed by a number of criteria. By staining these cells by immunofluorescence for the presence of

actin stress cables, they were able to demonstrate a correlation between changes in cell shape and the state of stress cables: fully transformed cells, which were round and loosely attached, completely lacked stress cables, whereas the partial transformants, which displayed varying degrees of rounding, showed varying degrees of stress cable loss. Nontransformed cells contained numerous stress cables. Other fully transformed cells in this series partially reverted back to normal phenotypes during the course of their experiments; these cells regained stress cables as well as a flattened morphology. Thus, alterations in microfilament organization during transformation paralleled changes in cell shape, again offering indirect evidence for a cause-effect relationship between these events.

## The Relationship Between Cell Shape and Microfilament Organization

In the case of the rat adrenocortical cell, ACTH-induced adrenocortical cell retraction parallels ACTH-initiated microfilament depolymerizaiton in several respects. Both events are hormone-specific and occur on the order of hours after ACTH administration. Both events are independent of protein and RNA synthesis indicating that cell retraction and reversal, as well as disassembly and reassembly of microfilaments, utilize pre-existing cell components and do not require the synthesis of new protein(s).

However, the question of whether microfilament disassembly is a prerequisite for ACTH-induced changes in cell shape is still

unanswered. The observation reported here in the rat adrenocortical cell that loss of stress cables in response to ACTH and exogenous cyclic AMP always accompanies changes in cell shape suggests that these events are somehow coupled in the cell. The observation that cytochalasin B can produce cell retraction and loss of stress cables in the absence of ACTH or cAMP further suggests that microfilament disassembly may mediate cell rounding. Thus, there is little doubt that actin stress cables are intimately involved in defining cell shape. However, we have no proof at this time that the cell shape response to ACTH is a a consequence of hormone-induced stress cable depolymerization. As we did not attempt a detailed time course of the effect of ACTH on microfilament organization, we cannot at this time comment on the precise timing of these events, let alone conclude that loss of stress cables precedes cell rounding. In fact, there is some evidence in other systems that stress cables are not necessary for changes in cell shape to occur. For example, there are cases in which changes in cell shape have been observed in cells which completely lack stress cables (220,317). Moreover, Tomasek et al. (439) have shown that corneal and fibroma fibroblasts grown on collagen matrices demonstrate changes in cell shape upon attachment the without formation of stress cables which normally appear when these cells are cultured on glass surfaces. In light of these observations, it is premature to conclude that alterations in the adrenocortical cytoskeleton are responsible for the cell rounding observed in response to hormonal stimulation.

## Cell Shape, the Cytoskeleton, and Adrenocortical Steroidogenesis

The observation that adrenocortical steroidogenesis is altered by the same agents (ACTH and cytochalasin B) which induce changes in cell shape strongly suggests some important relationship between these events. Moreover, the observation made here that these same agents both initiate microfilament disassembly in this cell suggests the exciting possibility that these cytoskeketal structures mediate the actions of ACTH on cell shape and on steroid production. In fact, the literature abounds with studies, both on these cells and on other hormone-sensitive cells, which implicate cytoskeletal components in the hormonal regulation of cellular activities. Much of the biochemical evidence for this hypothesis comes from studies on the effects of specific cytoskeletal-disrupting agents on biological function. In the case of the adrenocortical cell, for example, it has been known for some time that agents such as cytochalasin B which disrupt microfilaments also inhibit ACTH-stimulated steroidogenesis (438). In a series of studies on the effect of this agent on movement of cholesterol in adrenal cells in vivo and in isolated adrenocortical cells, Crivello et al. (553) have reported that cytochalasin B, at concentrations which effect complete disassembly of microfilaments, inhibits ACTH-induced increases in corticosterone production. This result has been fully confirmed by McPherson and Ramachandran (438) using isolated normal rat adrenocortical cells in culture. They found that 10uM cytochalasin B, a concentration used in the present studies to induce cell retraction, produced a slight but

significant inhibition of ACTH-stimulated steroidogenesis. Cytochalasin D, a much less potent inhibitor than cytochalasin B of glucose uptake in other cells, also promoted cell rounding and inhibited hormone-stimulated steroidogenesis, suggesting that the effect of cytochalasin B on adrenal steroidogenesis is not due to nonspecific cellular damage due to energy depletion.

In an extensive analysis of the effect of cytochalasin B on basal and ACTH-stimulated rates of steroid production in Y-1 tumor cells in vitro, Mattson and Kowal (486) have shown that this agent has profound effects on adrenocortical cytoskeletal organization and on steroid production. At low doses of cytochalasin B (below 10ug/ml), cytochalasin B either stimulates a slight increase in basal steroid levels or has no effect on this production. However, higher doses are progressively inhibitory. When added to cells in the presence of ACTH at doses of 10-100ug/ml, cytochalasin B produces progressively increasing inhibition of the ACTH response. At 50-100ug/ml cytochalasin B, this inhibition reaches 80%. All inhibitions are completely reversible upon drug removal. At all cytochalasin B doses, morphological changes are evident; however, at low doses (1-3ug/ml), membrane alterations dominate, whereas at higher doses (10-100ug/ml), disruption of microfilament networks and loss of stress cables are most prominent. These authors have proposed that loss of stress fibers enhances the uptake of lipoproteins, providing additional substrate for steroidogenesis, and that at higher doses of the drug, production of large quantities of disordered microfilaments (due to disassembly of stress cables)

inhibits ACTH-stimulated steroidogenesis by preventing movement of lipoprotein substrate to sites of steroid biosynthesis.

Other investigations of the mechanism of cytochalasin B inhibition of adrenal steroidogenesis have provided support for a cytoskeletal role in the regulation of steroidogenesis by demonstrating that cytochalasin B specifically inhibits transport of cholesterol to the mitochondria without effecting entry of this substrate into the cell or further processing of cholesterol once it has been cleaved to pregnenolone (554). Again, this effect of cytochalasin B is somewhat specific to the steroid biosynthetic pathway in that this agent had no effect on total protein synthesis or on ATP concentration.

Hall et al. (255) have taken a different experimental approach to demonstrate that microfilaments are somehow involved in the regulation of adrenal steroidogenesis. They have shown that actin antibodies introduced into intact, fully functional Y-1 cells via liposomes can block increases steroid production in response to ACTH or to dibutyryl cyclic AMP. Moreover, this inhibition is localized to events involving the transport of cholesterol to the mitochondrial side-chain cleavage enzyme, again suggesting that microfilaments are specifically involved in the transport of cholesterol to the site of the rate-limiting step in steroid biosynthesis.

A study by Mrotek and Hall (556) has presented further evidence that steroidogenesis specifically involves microfilaments. Here, it was shown that colchicine administered to Y-1 cells has no effect either on basal or ACTH-stimulated

steroidogenesis. Other inhibitors of microtubules ($D_2o$, podophyllotoxin, griseofulvin, and vinblastine) also failed to inhibit steroidogenesis except under conditions in which non-specific effects were probable. On the other hand, halothane, which promotes disassembly of both microfilaments and microtubules, did decrease the steroidogenic response to ACTH. These results strongly suggest that microtubules, unlike microfilaments, are not involved in the response of these cells to ACTH. If ACTH stimulation of steroidogenesis involves changes in microfilament organization in the adrenocortical cell, then our results that cytochalasin B, but not colchicine, effects a disappearance of actin stress cables is consistent with the hypothesis that these filaments mediate hormonal stimulation of adrenal steroidogenesis.

The observation that cytochalasin B inhibits adrenocortical steroidogenesis as well as disrupting microfilaments is not an isolated case; similar effects have been observed on other cellular activities in other hormone-sensitive cells. For example, addition of cytochalasin B to prelabelled mouse thryoid glands interferes with the release of radioactive iodine derived from thyroglobulin, and blocks colloid enocytosis, suggesting that microfilaments are involved in these processes in this cell (244). Work by Zor et al. (557) using cytochalasin B and colchicine to study the effects of these agents on lutropin stimulation of ovarian steroidogenesis has suggested that microfilaments, but not microtubules, are intimately involved in the process of LH regulation of this activity. Prentki et al.

(558) have tested phalloidin and cytochalasin D on isolated rat hepatocytes to see whether these agents, which differentially alter microfilament assembly might have any effect on cellular morphology and function. They found that both drugs produced a modification of cell shape, characterized by protrusions bulging from the cytoplasm. In phalloidin-treated hepatocytes, actin microfilaments were found in abundance at the base of each protrusion; this accumulation of filaments was absent in cytochalasin D-treated cells. Moreover, both agents inhibited the release of triglycerides, an index of very low density lipoprotein secretion, in these cells. Using a series of phalloidin analogs, a correlation was observed between the potency of these analogs to stabilize F-actin in vitro, and the extent to which they altered cell shape and triglyceride secretion, again suggesting a relationship between microfilament status and functionally viability.

Other studies employing a similar approach have implicated microtubules in the secretion processes of many organs, including the thyroid gland (244), pancreatic islets (559), and the adrenal medulla (560). The pioneering work of Greengard et al. (561) has clearly demonstrated that colchicine and vinca alkaloids markedly potentiate the stimulatory effect of prostaglandin E, catecholamines and histamine of cAMP production in human leukocytes. Lumicolchicine, on the other hand, which does not induce microtubule disassembly, failed to enhance hormonal action (562), while various other drugs which could disrupt these filaments mimicked the effect of colchicine on microtubule

integrity (562). One recent study by Hagmann and Fishman (563) on isolated macrophages clearly suggests a correlation between cell shape and hormonal sensitivity. Freshly isolated macrophages are round in shape and demonstrate a significant response to PGE or to isoproterenol in terms of cAMP production. However, upon treatment with chemotactic factors, these cells change shape, flatten out, and lose all sensitivity to these hormones. Treatment with colchicine restores macrophage morphology back to a spheroid shape as well as hormone sensitivity, suggesting that in these cells, hormone sensitivity is a function of cell shape which, in turn, is a function of microtubule organization.

Results presented in this dissertation demonstrate several striking parallels between ACTH stimulation of adrenocortical steroidogenesis and ACTH-induced cell retraction. Both events are hormone-specific, yet can be mimicked by exogenous cAMP which theoretically bypasses the ACTH receptor. On the other hand, $ACTH_{7-38}$, which is an antagonist of ACTH in terms of adrenal steroidogenesis, is unable to evoke a change in cell shape. Both responses to ACTH are dependent on the dose of hormone, and are completely reversible upon hormone removal. Cell retraction and stimulation of steroidogenesis both occur in the physiological range of ACTH doses, though changes in cell shape appear, at least at the light microscopic level, to occur at the higher end of this spectrum. These similarities in shape and steroidogenic response to ACTH strongly suggest that these events are coordinated in the adrenal cell, perhaps mediated through a common intracellular pathway or by some shared "second

messenger." One likely candidate is the cytoskeleton. Such a hypothesis for cytoskeletal control of hormone action is particularly attractive and exciting in light of recent findings that surface receptors for aceytlcholine (564) and for nerve growth factor (565) specifically bind to actin filaments in cytoskeletons isolated from hormone-sensitive cells.

Though there is little doubt that the loss of actin stress cables is at least in part responsible for the dramatic change in adrenocortical cell shape evident in response to ACTH, the precise relationship between these events remains elusive. Moreover, if hormone-induced cell retraction simply reflects microfilament disassembly, then several observations rule out a simple cause-and-effect relationship between hormone-induced cell retraction and activation of steroidogenesis. First, the time course of ACTH-induced loss of microfilaments is quite different from that of ACTH-stimulated corticosterone production. While maximal stimulation of corticosterone production occurs within minutes after ACTH administration, ACTH-induced cell rounding becomes evident only after hours of hormone treatment. Thus, cell retraction occurs long after the maximal activation of steroidogenic response has been achieved. Second, Rainey et al. (347) have presented evidence that the steroidogenic and retraction responses to ACTH are separable events. They have studied the effects of ACTH on adrenal steroidogenesis and on cell retraction in cultured bovine adrenal and human fetal adrenal cells, and have shown that while both cell types respond similarly to ACTH in terms of increased steroid production, only

the human cells retract in response to hormonal stimulation. Fluorescent staining of actin in these cells prior to and after ACTH treatment further reveals a loss of actin stress cables in the human cells but not in the bovine cells. These observations clearly indicate that the steroidogenic response to ACTH in cultured bovine cells is not dependent on changes in cytoskeletal organization or changes in cell shape. The fact that these events can be dissociated strongly argues against a simple cause-and-effect relationship between these events.

We have observed a similar disparity of responses in cultured rabbit adrenocortical cells. Though these cells are responsive to ACTH in terms of steroidogenesis, they do not change shape upon chronic exposure to ACTH (unpublished observation). Furthermore, we have noted that isolated rat adrenocortical cells cultured on extracellular matrices (ECM) of corneal endothelial cells do not retract in response to ACTH, even though they are as sensitive to ACTH in terms of steroidogenesis as the same cells grown on plastic. This observation again argues that cell retraction is not a prerequisite for steroidogenic sensitivity to ACTH.

Taken together, these observations argue against an absolute requirement for changes in adrenocortical cell shape in the hormonal regulation of adrenal steroidogenesis. Whether ACTH stimulation of steroidogenesis requires alterations in microfilament assembly, on the other hand, cannot at present be evaluated. This will require a more detailed investigation of ACTH action on microfilament assembly and distribution, in

particular, a more detailed analysis of microfilament assembly at early times after ACTH administration to observe possible rapid hormonal effects on these structures.

## Adenylate Cyclase, Cyclic AMP, and the Adrenocortical Cytoskeleton

The observation that cyclic AMP can induce adrenocortical cell rounding and stress cable disassembly in the absence of ACTH suggests that this nucleotide might mediate the effects of the hormone on these events. It is well established that in this system, as well as in other hormone-sensitive cells, hormonal stimulation results in activation of hormone-sensitive adenylate cyclase and, as a consequence, increased rates of cAMP production (415). In fact it was the action of ACTH on the adrenocortical cell which Sutherland and others (453) to propose that cAMP is a universal "second messenger" which mediates the effects of hormones on diverse cellular activities. The observation presented here that cAMP alone can mimick ACTH-induced changes in cytoskeletal organization and cell shape also suggests that this nucleotide may mediate these responses to ACTH. If cAMP indeed mediates ACTH stimulation of steroidogenesis in this cell, then perhaps cytoskeletal microfilaments in turn mediate the effect of cAMP on this biological activity.

Such a model requires some interaction of the cytoskeleton with the machinery responsible for cAMP production. There is, in fact, considerable experimental evidence to suggest such an interaction of adenylate cyclase with intracellular cytoskeletal

components. In some hormone-sensitive cells in culture, for example, agents which disrupt cytoskeletal filaments can also alter the cAMP response of these cells to appropriate hormones. Cytochalasin B has been shown to enhance the stimulatory effect of isoproterenol, $PGE_1$, and cholera toxin on cAMP production. Colchicine and anti-tubulin serum abolish the stimulatory effect of PGE on cAMP production in cultured Graafian follicles (557). Cytochalasin B or anti-actin antiserum inhibit the stimulatory effect of LH on cAMP production in these cells, but neither agent has an effect on PGE stimulation, suggesting that microfilaments may be involved in LH-induced, but not PGE-induced, biological activities. These results further suggest that different cytoskeletal filaments may mediate different cellular responses to different hormones.

Furthermore, both microfilaments and microtubules have been implicated in the control of hormone desensitization (543). Zor et al. (566) have demonstrated that chronic exposure of cultured Graafian follicles to $PGE_2$ results in loss of the cAMP response to fresh hormone. This desensitization could be prevented by addition to the medium of agents which stabilize microtubules ($D_2O$ or Li+) as well as by those which stabilize microfilaments (phalloidin). Moreover, the spontaneous recovery of responsiveness to $PGE_2$ upon incubation of refractory follicles in hormone-free medium could be prevented by addition to the medium of cytochalasin B, myosin subfragment HMM-S1, or of anti-actin antiserum, all of which destabilize microfilaments. These authors have proposed that both types of cytoskeletal filaments are

involved in hormone-induced desensitization. A similar involvement of microfilaments and microtubules has been observed in Ehrlich ascites tumor cells in response to catecholamines (567), and in macrophages in response to prostaglandins (563).

Recent studies have offered more direct evidence for a physical interaction of adenylate cyclase with the cytoskeleton. Sahyoun et al. (568) have obtained evidence that detergent-solublized adenylate cyclase can specifically bind to erythrocyte-derived, Triton X-100 cytoskeletal shells. This binding is saturable with respect to adenylate cyclase concentration, requires the presence of 5'-guanylyl imidodiphosphate and isoproterenol, or NaF; furthermore, this binding is destroyed by pretreating cytoskeletons with agents which release actin-containing microfilaments. Dictyostelium discoideum cells use exogenous cyclic AMP as an "acrasin" or chemoattractant during the aggregation phase of the developmental transition from a population of single amoeboid cells to a multi-cellular slug (101). Cell surface receptors have been inferred from binding studies with labelled cAMP (569). These receptors appear to be linked to directional cell mobility and to stimulation of adenylate cyclase. Wallace and Frazier (101) have photoaffinity labelled cAMP receptors in D. disoideum crude membrane preparations, and have shown that this procedure also specifically labels an AMP binding site on the plasma membrane which has been been identified as actin. Galvin et al. (392) have extended this study by showing that this cAMP chemotaxis receptor is specifically retained in detergent-insoluble cytoskeletons

extracted with Triton X-100. This evidence strongly suggests that membrane-associated actin is physically linked to surface chemotaxic receptors which in turn somehow interact with adenylate cyclase. In this cell, actin-containing cytoskeletal filaments may mediate the migratory response of the cell to cAMP.

In light of these observations, it is conceivable that cytoskeletal filaments such as microfilaments interact directly with membrane-bound adenylate cyclase in hormone-sensitive, cAMP-responsive cells to control cellular responses to hormones. Such an association might explain the effects of cAMP on the organization of cytoskeletal microfilaments, as well as the effect of cytoskeletal-disrupting drugs on hormone sensitivity, observed in many cells. In the rat adrenocortical cell, membrane-associated microfilaments interacting with adenylate cyclase might mediate the stimulation of steroidogenesis produced by ACTH. Such a model would be consistent with the observations that cAMP alone can mimick the effect of ACTH both on adrenocortical steroidogenesis and on cytoskeletal integrity.

The observation made here that NPS-ACTH can produce adrenocortical cell retraction further implies that if cAMP is the mediator of ACTH-induced changes in cell shape, then only a fraction of the cAMP potentially produced by ACTH stimulation is necessary for this induction. This conclusion is consistent with recent data on the binding of ACTH to its adrenocortical receptor (419) which suggests that only a fraction of cAMP produced as a consequence of ACTH binding is required to effect maximal stimulation of adrenal steroidogenesis. Resolution of the precise

role of intracellular cAMP on ACTH-induced alterations in cell shape and cytoskeletal organization, and the relationship between these events and adrenal steroidogenesis, awaits further investigation.

ACTH-Induced Actin Loss

Several different experimental approaches have been taken in this dissertation work to demonstrate that ACTH induces a change in the content, as well as in the distribution, of adrenocortical actin. The loss of a significant proportion of total actin in cultured rat adrenocortical cells, originally noted in SDS-PAGE gels of cell lysates from treated and untreated cells, has been confirmed using both an enzymatic assay and a modified radioimmunoassay based on the binding of actin to the pancreatic enzyme deoxyribonuclease I. The loss of actin measured by either of these assays is not a consequence of loss of cells or inhibition of cell proliferation, for chronic hormonal stimulation of up to 48 hours does not result in any significant difference in either total cell protein or DNA relative to untreated cells. The effect of ACTH on actin content appears to be a specific effect on this protein, as evaluation of other adrenocortical proteins from ACTH-treated cells on SDS-PAGE gels (Figures 11-14) does not reveal any consistent change in the contents of these proteins.

The observation that ACTH induces changes in adrenocortical actin content adds one more instance of hormonal regulation of cytoskeletal protein content to a growing list of reported

examples which together indicate that cytoskeletal content, as well as cytoskeletal organization, may be under hormonal control. In the course of differentiation of cultured 3T3-F442A mouse preadipocytes into mature fat cells in response to insulin or dexamethasone, preadipocytes undergo a dramatic change in cell shape from a flat, fibroblastic form to a nearly spherical morphology (570). Spiegelmann and Farmer (571) and Spiegelman and Green (570) have shown that accompanying this morphological change is a decrease in the level of total actin to approximately 20% that in untreated preadipocytes. This drop in total actin is accompanied by a loss of thick actin fibers from the cells, as observed by immunofluorescence staining of actin. Sidhu (510) has further confirmed this drop in 3T3-L1 preadipocytes by quantitating total actin contents in preadipocytes and fully differentiated adipocytes by the DNase I inhibition assay. He has shown that differentiation leads to a loss of 83% of the total actin. Actin content in cultured Drosophila melanogaster Kc 0% cells is also subject to change in the course of morphological modifications in response to ecdysteroid hormones (489). In these cells, ecdysterone treatment induces a 225% increase in total actin levels; this increase accompanies a shift in actin from G-actin to F-actin, and a concomittant appearance of actin stress cables.

Actin content is also subject to alterations during the course of cellular differentiation in other cells. Le Stourgeon et. al. (572) have observed increases in the levels of actin in Physarum polycepum during the early stages of sporulation.

Prashad et al. (573) have analyzed differentiating mouse neuroblastoma cells and have reported an increase in actin content during neurite formation. During the encystment stage of Acanthamoeba castellanii development, actin content drops coincident with a parallel decrease in the relative rate of actin synthesis (513). Later stages of development in this organism are characterized by a further loss of total actin concomittant with complete cessation of actin synthesis. In the case of bovine lens cell differentiation in culture, the appearance of long lens fibres, i.e., actin stress cables, which accompanies the morphological transformation of cells from a spherical to elongated shape, is in turn accompanied by a slight by significant increase in the total amount of actin relative to total cellular protein (615). These results testify to the widespread hormonal control of actin content; furthermore, they imply an underlying importance of the cytoskeleton in the expression and regulation of cellular activities.

The use of the DNase I inhibition and immunoprecipitation assays for the measurement of adrenocortical actin have precident in past attempts to quantitate cellular actins. Until recently, SDS-PAGE analysis of cell lysates has been the method of choice for the quantitation of cellular actin. Scanning densitometry of SDS gels of cellular extracts has been used to determine absolute actin levels in isolated fibroblasts (512), in cultured macrophages (519), in sea urchin eggs (514), and in 3T3 mouse cells (570), as well as in homogenates of chick embryo brain (69). With the development of the DNase I inhibition assay of

Blikstad et al. (456), this assay has been used extensively to analyze both actin content and state of assembly in numerous cells, including 3T3-L1 preadipocytes and adipocytes (510), human platelets (456), lymphocytes (456), and HeLa cells (456). The development by Snabes et al. (459) of an immunoassay based on actin binding to DNase I has confirmed previous estimates of actin contents obtained using very different methods. For example, their estimate of total liver actin (1.2% of total protein) agree very well with the Gordan et al. (574) estimate of liver actin obtained by purifying actin. The immunoassay has the distinct advantage over both inhibition assay and SDS-PAGE densitometry in that it is much more sensitive and, at least in our hands, more reproducible than these other techniques.

As with cell retraction and microfilament disassembly, the loss of adrenocortical actin in response to ACTH is hormone-dependent, specific to ACTH, and can be mimicked by cAMP analogs in the absence of ACTH. The observation that cytochalasin B, but not colchicine, can alone produce actin loss similar in magnitude to that with ACTH suggests that hormonally-induced actin loss may be mediated through the action of this nucleotide. Though a rigorous analysis of the time course of ACTH action on actin content was not attempted, it is evident that the drop in total actin occurs at least coincidently with the change in cell shape and the loss of stress cables. Figure 37 shows that a significant proportion of the total actin is already lost after 10 hours of ACTH treatment, a time when cell retraction is first evident.

It may be that actin loss occurs very rapidly upon hormone

treatment. If this is the case, then stimulation of steroidogenesis might also involve this loss of actin. Alternatively, actin loss may simply be the consequence of stress cable depolymerization. It is tempting to conclude on teleological grounds that actin loss is a consequence of actin depolymerization, for disassembly of stress cables and depolymerization of actin microfilaments surely results in a large increase in intracellular G-actin which the cell must deal with. Unless this actin is used for other purposes, the cell would be inclined to dispose of it. Obviously, future studies must be initiated to define the precise relationship of ACTH-induced actin loss to shape, cytoskeletal, and steroidogenic responses to hormone stimulation.

Though the correspondence of ACTH-induced actin loss resembles ACTH-induced cell retraction and microfilament disassembly, and suggests that these events may be causally related, the dose reponse of ACTH action on actin content reveals a discrepancy between the effect of ACTH on actin content, and on cell shape and on cytoskeletal status. Dose response curves relating ACTH concentration to actin loss (Figures 13 and 36) suggest that actin loss occurs at concentrations far below those at which cell rounding is evident. For example, at 22pM ACTH, a dose which has no obvious effect on cell shape at the light microscopic level, actin content has already dropped to a level achieved with higher doses of ACTH which do promote cell retraction. In fact, the sensitivity of actin levels to ACTH is in the same range as that of ACTH stimulation of steroidogenesis

(415), i.e., in the picomolar range, again suggesting an intimate relationship between actin content and steroidogenic capacity.

In contrast to both changes in cell shape and microfilament organization, ACTH-induced actin loss does not appear to be reversible (Figure 38). Even 96 hours after hormone removal, a full 48 hours after fully retracted cells have completely reflattened and stress cables have reappeared, actin content remains below that level found in cells never treated with ACTH. We have no explanation at this time why actin content does not return to control levels within four days after hormone removal. Perhaps the fact that actin is a cellular protein with a half-life on the order of days accounts for the very slow replenishment of actin content after hormone removal. This result indicates that even with a loss of 50% of total actin, the adrenocortical cell is still able to reassemble stress cables after removal of hormonal stimulation. Moreover, the observation that retraction and reflattening of the adrenocortical cell, as well as disassembly and reassembly of stress cables, are independent of protein and RNA synthesis (figures 4 and 10) implies that these events utilize pre-existing cellular components and thus do not require the synthesis of new protein. The most likely explanation for the ability of the cell to reflatten in the absence of newly synthesized actin is that actin not lost upon ACTH stimulation is recruited for the construction of new stress cables.

ACTH promotes the loss of 40-60% of the total cellular actin as measured by the DNase immunoprecipitation assay. Repeated

cycles of ACTH stimulation and homone removal do not result in further actin loss (unpublished observation, our laboratory). This implies that the actin lost upon hormone stimulation is in some way different from that actin which is not lost, and suggests that actin might reside in the cells in different pools which are differentially sensitive to hormone-induced degradation. There is much evidence that nonmuscle actins are heterogeneous in chemical form, e.g., different isoionic species, and that more than one actin type may reside in a single cell (575). There is even recent evidence that different nonmuscle actins, even those within one cell, may be coded for by different genes (394). Whether the different species of actin within a cell are involved in different biological functions is not yet known; therefore, the question of whether actin tagged for destruction is chemically distinct from actin not destroyed upon ACTH stimulation is an open question which deserves future attention.

The concept of actin pools is well established. The observation that actin filaments are present in different spatial arrangements in different regions of the cell - as stress cables running near the plasma membrane, as a fine mesh of filaments in membrane ruffles and lamellipodia, as closely bundled filaments in the contractile ring of dividing cells - is consistent with the concept of different intracellular actin pools. Measurements of unpolymerized actin pools in nonmuscle cells have shown that G-actin typically constitues a significant fraction, often upwards of 50%, of the total cellular actin. The DNase I inhibition assay of Blikstad et al. (456) has been very useful in

determining the sizes of unpolymerized and polymerized actin pools. For example, Blikstad et al. (456) have found that monomeric actin constitutes 59-70% of the total actin in human platelets, 62% in human lymphocytes, and 59% of the total actin in HeLa cells. Using this assay, we have determined that monomeric actin constitutes 67.87 $\pm$ 8.95% (15 separate determinations) of the total actin in cultured rat adrenocortical cells. This agrees well with estimates of unpolymerized actin in bovine adrenal homogenates measured by differential centrifugation of F-actin (576). Whether actin can be shuttled from one pool to another in response to new cellular demands is not yet known; however, recent studies using fluorescently-labelled actin microinjected into living fibroblastic cells (258) and into intact cardiac myocytes (219) clearly demonstrates that actin introduced into cells rapidly redistributes from initial membrane sites to specialized regions of cell attachment (adhesion plagues) and into specific cytoskeletal structures, i.e., stress cables, suggesting a redistribution of intracellular actin as well as a shift from monomeric to polymeric form. Perhaps in the case of the rat adrenocortical cell, ACTH treatment results in loss of one pool, e.g., polymeric actin, in the cell. If so, then perhaps stress cable reformation upon hormone removal is the consequence of recruitment of G-actin from the remaining monomer pool. Such a model is consistent with other observed shifts in monomer-polymer actin pools, for example, in the well-documented case of the acrosome reaction in Limulus sperm where unpolymerized actin is explosively polymerized in

response to calcium (79).

The effect of cytochalasin B on hormone-induced adrenocortical cell retraction and actin loss deserves further comment. Cytochalasin B can mimick ACTH in producing cell rounding, microfilament disassembly, and actin loss. This observation suggests that these events are causally related. Cytochalasin B, like ACTH, produces cell rounding and loss of stress cables is a dose-dependent manner. Moreover, the effect of both ACTH and cytochalasin B on adrenocortical cell morphology is reversible upon hormone or drug removal (486). Cytochalasin B also has a profound inhibitory effect on ACTH stimulation of adrenal steroidogenesis (486). At the dose of cytochalasin B used in these studies (10uM), the inhibitory effect of this agent on adrenal steroidogenesis is also completely reversible upon drug washout (486). Cytochalasin B does not inhibit glucose transport in rat adrenocortical cells as it does in other cells (438), suggesting that the effects of this drug on adrenocortical cell function and morphology are specific, and not due to general cellular damage. Mattson et al. (486) have shown that cytochalasin B produces many of the changes in membrane morphology also induced by ACTH. In light of the similarities in effects of ACTH and cytochalasin B on adrenocortical morphology, and in light of the known property of this drug to promote actin filament depolymerization in a dose-dependent fashion (243), it is tempting to conclude that microfilaments are also involved in the mechanism of action of ACTH on adrenal steroidogenesis.

However, Mattson et al. (486) have pointed out a discrepancy

between the effects of cytochalasin B and ACTH on adrenocortical cell shape. They have shown that the time course of action of ACTH on the shape of Y-1 adrenal cells is quite different from that of cytochalasin B. While ACTH-induced cell retraction is first evident after six hours of hormone administration, retraction due to cytochalasin B is seen within 30 minutes. We have observed identical results with normal rat adrenocortical cells. Moreover, cytochalasin B has other effects on adrenocortical cell morphology, e.g., specific effects on cell membrane ultrastructure, which are distinct from those brought about by ACTH (486). Therefore, conclusions concerning ACTH mechanism of action drawn from the effects of cytochalasin B on adrenocortical cell morphology and function should be made with caution.

One interesting observation concerning ACTH-induced cell retraction is that cell retraction due to hormonal stimulation diminishes with time of incubation. While cell rounding persists for up to 60 hours in the continued presence of ACTH, further stimulation cannot maintain this effect, and with time, cells return to a flattened, asymmetrical shape even in the presence of hormone. This is in contrast to cytochalasin B-induced cell rounding, which persists for as long as the drug is present (486). Moreover, adrenocortical cells maintained in culture for several weeks do not respond to ACTH by changing shape, while cytochalasin B produces retraction in these cells (unpublished observation). However, prolonged treatment of such cells with ACTH results in cell retraction, on a time course which parallels

resumption of steroidogenic sensitivity (413). Lack of ACTH sensitivity in long-term cultures may be the result of loss of steroidogenic capacity due to loss of steroidogenic enzymes (411) or due to loss of ACTH receptors (419), while loss of sensitivity over 60 hours of hormone stimulation may be due to hormone degradation. These results suggest that ACTH and cytochalasin B may affect cell shape through a common pathway involving cytoskeletal structure. The fact that cytochalasin B can override insensitivity in long-term cultures implies that this drug can circumvent the ACTH receptor in producing its effects on the adrenocortical cytoskeleton.

Finally, we are left with the paradox that while ACTH stimulates adrenal steroidogenesis, cytochalasin B inhibits this process. As the relationship between cytoskeletal integrity and steroidogenic potential is not yet clear, we can only conclude that if cytoskeletal microfilaments are involved in regulating adreonocortical steroidogenesis, then the precise effect of ACTH on microfilament disassembly must be fundamentally different from that of cytochalasin-induced depolymerization.

## The Mechanism of ACTH-Induced Actin Loss

The final experiments presented in this dissertation have revealed an underlying molecular mechanism for ACTH-induced adrenocortical actin loss. The observation that actin loss in response to ACTH can be produced in the absence of protein synthesis (Figure 44) eliminates the possibility that actin loss in a result of changes in actin synthesis. As actin is not found

in the medium of hormone-treated cells, secretion as a means of actin loss is not a viable alternative explanation. Pulse-chase experiments on ACTH-treated and untreated cells (Figure 44) have revealed that actin loss is a consequence of increased actin degradation. Moreover, this degradation appears to be lysosomally mediated, as inhibition of lysosomal proteases inhibits ACTH-induced actin loss (Figure 45).

The observation made here that ACTH alters the rate of actin degradation is consistent with findings in other cells that actin content is modulated by changes in actin synthesis and/or degradation. For example, Spiegelman and Farmer (572) have shown that the drop in actin content in 3T3-L1 preadipocytes during dexamethasone-induced differentiation can be accounted for by a concommitant decrease in actin synthesis. In Acanthamoeba castellanii, loss of actin during encystment is also the result of decreased actin synthesis (513). Ecdysterone stimulation of cultured Drosophila melanogaster cells leads to a specific stimulation of actin synthesis as monitored by pulse-labelling (489). DuBois et al. (577) have shown that in bovine adrenocortical cells, chronic ACTH treatment results in an increase in the synthetic rates of several proteins, one of which is actin. Virally transformed fibroblasts often show a reduction of actin synthesis in addition to actin filament redistribution and cell rounding (389). In the case of 3T3 cells transformed with SV40 virus, actin and tubulin synthetic rates are altered such that the total amount of both proteins decreases relative to uninfected cells (221). Changes in actin degradation have also

been reported. For example, Rubinstein et al. (578) have shown that during myoblast formation of myotubes, the half-life of actin in myoblasts increases from 2.5-3 days to approximately 6 days without a change in the rate of actin synthesis, revealing a decrease in actin degradation in the course of cellular differentiation.

The observation that lysosomotropic agents can prevent hormone-induced actin loss implicates lysosomes in the mechanism of actin degradation. Cloroquine and ammonium chloride are weak bases which are known to concentrate in lysosomes and to inhibit lysosmal protease actin by elevating intralysosomal pH (579,580). They have been used extensively in studies on surface receptors to demonstrate lysosomal involvement in the degradation of internalized receptors (581,582). The observed inhibition of actin loss in response to ACTH argues for a key role of these proteases in hormone-mediated actin degradation.

The lack of effet of leupeptin and pepstatin further suggests that hormone-induced actin degradation is the specific concern of lysosomal enzymes. Though these agents are known to inhibit various intracellular proteases, there is no evidence that these peptides inhibit lysosomal degradative functions, or that these agents even enter lysosomes (583). Based on the assumption that these drugs inhibit extralysosomal proteases without any effect on lysosomal enzymes, the motive for using leupeptin and pepstatin in these experiments was to act as a negative control for the effects of chloroquine and ammonium chloride. The obseved lack of effect of these agents on ACTH-

induced actin loss is, therefore, indirect confirmation that actin degradation is mediated through lysosomal proteases.

If lysosomal proteases are responsible for increased actin degradation, then actin tagged for destruction must be brought into contact with these proteases upon hormone administration. As lysosomal proteases are confined within lysosomes, there must be a hormone-induced association of actin with lysosomes, i.e., movement of actin to lysosomes, or vice versa, in response to ACTH. Though there is no direct biochemical evidence that lysosomes interact with cytoskeletal structures to regulate their organization, there is abundant microscopic evidence that suggests that these organelles physically associate. For example, electron microscopic evaluation of polymorphonuclear leukocytes has clearly demonstrated physical association of microfilaments with lysosomes (584). Other electron microscopic studies have demonstrated elaborate direct associates of microtubules (585), and microtubules and microfilaments (213), with phospholipid vesicles and other "membranous organelles" which resemble lysosomal organelles in appearance. One study of ACTH-induced redistribution of acid phosphatase-positive "residual bodies", i.e., lysosomes, in adrenal tumor cells has even provided electron microscopic evidence for hormone-induced aggregation of lysosomes with microtubules in these cells (586). The authors of this study concluded that microtubules play a key role in the translocation of lysosomes to the adrenocortical plasma membrane in response to ACTH.

Other studies employing colchicine have specifically

implicated microtubules in the control of saltatory movements of lysosomes in isolated squid axons (586,587), and in the endocytotic activities involving lysosomes of cultured cells (588). Szego et al. (592) have provided immunological evidence that estrogen induces a redistribution of lysosomes in the preputial gland when given to ovariectomized rats. These observations support the hypothesis that cytoskeletal filaments interact with lysosomes to control various cellular activities.

Finally, in light of the observation that ACTH-induced actin degradation occurs over the course of hours (Figure 43) and that actin has a half-life on the order of days (578,589), lysosomal participation in actin degradation is consistent with an earlier proposal that lysosomal proteases function specifically to degrade long-lived ($t_{1/2} \geq 24$ hrs.), but not short-lived, proteins (590). Taken together, these results make plausible the hypothesis that ACTH promotes lysosomal association with the adrenocortical cytoskeleton as an initial step in hormone-induced actin degradation.

Future Directions

The distinguishing feature of ACTH-induced changes in adrenocortical cell shape and cytoskeletal organization is that it requires chronic hormone stimulation, on the order of hours, for these effects to be observed, and that the time course of these hormone-induced events parallels that for other known trophic actions of ACTH, including inhibition of DNA synthesis (438), induction of steroidogenic enzymes (413), and alterations in mitochondrial ultrastructure (412,424). This coincidence of events suggests the intriguing possibility that such hormonally-regulated events are coordinated in time and space within the adrenocortical cell. The goal of future research on the mechanism of ACTH action of adrenocortical cell function will be to precisely define the relationship between the adrenocortical cell shape and these other cellular activities, and in particular to investigate the role of the cytoskeleton in the regulation of adrenocortical cell function.

Though it is clear that ACTH catalyzes adrenocortical actin degradation, the precise molecular mechanism by which the cell regulates actin levels is not at all evident. In some systems in which actin levels are subject to hormonal control, it has been shown that changes in actin content, associated with alterations in the rate of actin synthesis, are due to changes in the rate at which $mRNA_{actin}$ is translated (571,591). This observation suggests that actin content might be ultimately controlled at the mRNA level. Though we have not explored the precise relationship between adrenocortical actin organization and actin content, it

is possible that hormone-induced actin loss is initiated by microfilament disassembly. Perhaps the signal for actin destruction is the increase in monomer actin pool size as a result of extensive microfilament depolymerization. If this is the case, G-actin then regulates its own content in this cells. Such a hypothesis has precedence in the regulation of microtubule content where it appears that the level of monomer tubulin regulates its own synthesis by feedback at the level of the $mRNA_{tubulin}$ (593). Hormonal regulation of actin content and organization, therefore, is another potentially interesting direction for future research.

The Cytoskeleton and the Control of Cell Growth

It has long been recognized that cell growth and division is under hormonal control. In many hormone-sensitive cells, including the rat adrenocortical cell, the same external stimuli which alter cell growth also affect changes in cell shape. In many cultured cells, the process of cell division itself involves cell rounding during cytokinesis. Virally transformed cells in culture characteristically lose anchorage-dependent growth controls, and proliferate freely in suspension as detached, rounded cells, whereas their nontransformed counterparts require firm substrate attachment for cell growth. These observations suggest that cell shape may be an important determinant in the regulation of cell growth.

The hypothesis that control of cell growth involves cell shape has been given strong experiment support from the work of

Folkman and Moscona (594). In a series of fascinating experiments, they made use of the observation that normal cultured cells, when plated in dishes coated with varying concentrations of the hydrophilic hydrogel poly(2-hydroxyethylmethacrylate), or poly(HEMA), assume different shapes depending on the concentration of the poly(HEMA). They found an inverse relationship between the extent of cell rounding and the dilution of poly(HEMA): the higher the concentration of poly(HEMA), the rounder the cell. Poly(HEMA) was used, then, to "fix" normally flattened cells in various shapes covering the full spectrum of morphologies from extremely flat and asymmetrical to essentially spherical. When sister cells held in different shapes were assayed for DNA synthesis by pulse-labelling with $^{3}$H-thymidine, it was discovered that DNA synthesis was tightly coupled to cell shape. DNA synthesis increased in direct proportion to the dilution of poly(HEMA), suggesting that cell flattening was a prerequisite for cell growth in these cells. Moreover, they found that the same cells after viral transformation lost anchorage-dependent growth controls, suggesting an even more fundamental role of cell shape in the expression of "normal" growth.

Savion et al. (591) have reported similar results with confluent primary cultures of adult bovine aortic endothelial, corneal endothelial, and fetal bovine heart endothelial cells. These cells form a monolayer of highly flattened, closely apposed, nonoverlapping cells which show a 6-fold decrease in DNA synthesis upon flattening, again suggesting shape control over

cell growth.

Ben Ze'ev and Raz (595) have further explored shape regulation of cell growth by demonstrating that DNA replication and cytokinesis are separable events, and that both of these events are sensitive to cell-surface contact with the substratum. They have shown that when metatstatic cells (3T3 mouse fibroblasts, epithelial monkey kidney cells and B16 melanoma cells) are plated on dishes coated with high concentrations of poly(HEMA), or grown in suspension culture in methyl cellulose, all cell types cease dividing yet continue to synthesized DNA. As a result, colonies of large, multinucleated cells form. However, when multinucleated cells are replated on tissue culture plates, normal cell division resumes by an early extrusion of the extra nuclei. These results suggest a differential sensitivity of cytokinesis and karyokinesis to cell-surface contact with the substrate.

Ben-Ze'ev (596) has further investigated the involvement of cell shape in control of cellular macromolecular metabolism. Again using poly(HEMA)-coated dishes, he has shown that when monkey epithelial cells are forced to assume a spherical shape, both DNA and RNA synthesis are inhibited by more than 90%, while the level of protein synthesis is reduced to a lesser extent, sugggesting that nuclear events and cytoplasmic events, while both sensitive to changes in cell shape, are differentially affected by changes in shape. Ben-Ze'ev et al. (597) have extended these findings to 3T6 mouse fibroblasts. By growing these cells in long-term suspension culture, DNA, RNA and protein

synthesis systems are inhibited. Upon replating cells on dishes coated with thin layers of poly(HEMA), they found that while protein synthesis resumes soon after contact with the substratum, RNA and DNA synthesis requires extensive cell spreading. It appears that contact of a limited portion of the plasma membrane with the solid culture dish surface is sufficient to reactivate protein synthesis. Moreover, though reactivation of DNA and RNA synthesis is sensitive to the shape of the replated cell, which is dictated by the poly(HEMA) concentration, protein synthesis is not. Thus, cell surface contact and cell shape give rise to different regulatory responses.

The precise locus of cell shape control over cell growth is not known; however, one candidate is the extracellular substratum itself. Gospodarowicz et al. (598) have shown that the nature of the extracellular matrix can greatly influence the response of cells to external stimuli. For example, changes in cell shape alter the sensitivity of cells to hormonal stimulation. Corneal epithelial cells adopt a flattened configuration when maintained in vito on plastic. In this state, the cells are very sensitive to fibroblast growth factor (FGF) but not to epidermal growth factor (EGF) in terms of growth. However, the same cells grown on collagen become tall and columnar, and respond primarity to EGF. Heeuptle et al. (599) have found a similar effect on other important cellular activities in isolated rabbit mammary cells. They have shown that hormonal stimulation of lipogenesis is dependent on the state of attachment to the substratum. Cells grown within collagen gels are flat in shape; these cells can

synthesize DNA but are totally unresponsive to prolactin in terms of lipogenesis or milk protein production. However, those cells in direct contact with the medium, become cuboidal and develop sensitivity to prolactin. Thus, the shape of the mammary cell in some way regulates the sensitivity of the cell to external stimuli which in turn control major intracellular activities such as synthesis, storage, and secretion of fat and milk proteins.

Another locus for cytoskeletal control of cell growth and differentition may be at the level of the cytoplasmic protein synthetic machinery itself. Recent biochemical, immunological and morphological investigations have revealed that many of the components involved in protein synthesis specifically interact with cytoskeletal filaments. Ribosomes and polyribosomes have been found specifically retained in isolated, detergent-resistant cytoskeletons in close physical contact with cytoskeletal filaments (600). Such associations appear to be mediated through mRNA (601). Treatment of cytoskeletons with cytochalasin B abolishes many of these interactions, implicating microfilaments as the cytoskeletal components involved. Other biochemical and microscopic data reveals involvement of microtubules and intermediate filaments as well (600). Several groups have presented data that suggests that only a specific population of mRNA, i.e., only actively translated mRNA, is found in a cytoskeletal-bound form (602). Specific translation components, including 5'-terminal cap binding protein (CBP) and the initiation factors eIF-2 and eIF-4A, have been located on cytoskeletal filaments in isolated cytoskeletons (603). Finally,

Van Venrooij et al. (604) have demonstrated that changes in the mRNA population associated with the cytoskeleton correlate with changes in protein synthesis, suggesting that cytoskeletal components might serve as regulators of protein synthesis.

The concept that the cytoskeleton is involved in the regulation of cell growth has some experimental support from studies in which cytoskeletal-disrupting drugs have been used to assay the response of cell growth to changes in cytoskeletal organization. Crossin and Carney (605) have used drugs which disrupt microtubules to show that these agents can alone initiate DNA synthesis in serum-free cultures of nonproliferating fibroblast-like cells. They found that colchicine stimulates thymidine incorporation in these cells up to 75% of that achieved by growth-promoting hormones; furthermore, this effect specifically involved microtubules as lumicolchicine, which has no effect on microtubule integrity, had no effect on DNA synthesis. Indirect immunofluorescent staining for tubulin further revealed a correlation between the extent of microtubule depolymerization and the degree to which colchicine stimulated DNA synthesis. Taxol, a drug which stabilizes cytoplasmic microtubules, blocked colchicine stimulation of DNA synthesis, indicating that microtubule depolymerization itself is necessary for this activation. These results demonstrate that microtubule depolymerization is sufficient to initiate DNA synthesis, and suggest that microtubule depolymerization is required in hormonal initiation of cell proliferation in these cells.

Cytochalssin B and other microfilament-disrupting agents

have been likewise used to implicate microfilaments in the control of cell growth. Cytochalasin B inhibits DNA synthesis in many cell types in culture, including the adrenocortical cell (438). In 3T3 fibroblasts, dihydrocytochalasin B has been shown to inhibit initiation of DNA synthesis by serum growth factors, coincident with cell rounding and loss of actin microfilament bundles (243). In the adrenocortical cell, the effects of cytochalasin B on cell growth, and on cell shape and cytoskeletal architecture, are strikingly similar to those produced by ACTH. Both cytochalasin B and ACTH inhibit DNA synthesis in normal rat adrenocortical cells (438). We have shown here that both cytochalasin B and ACTH also induce cell rounding and loss of actin content and actin stress cables in these cells. In light of other reports that cytochalasin B specifically disrupts actin-containing microfilaments in situ when administered to living cells (175), it is not far-fetched to consider that microfilaments as key regulatory elements in the control of adrenocortical growth.

Though cAMP can mimick ACTH effects in many of its trophic actions on the adrenocortical cell, its precise role in these events is still unclear. Studies with NPS-ACTH (406) suggest that only a fraction of the cAMP produced in reponse to ACTH actually mediates the steroidogenesis. If this is the case, then the majority of cAMP produced upon ACTH stimulation is left unaccounted for. This observation suggests another function(s) for cAMP in the adrenocortical cell. One possibility is the control of cell growth. In other cells, such as S49 lymphoma

cells and neuroblastoma cells (606,607), administration of exogenous cAMP causes inhibition of cell growth and cell death. In the mouse adrenal tumor Y-1 cell, high doses of cAMP cause growth arrest and cell death. In a study involving a series of cAMP-resistant Y-1 variants, Masui et al. (477) have demonstrated that resistance to cAMP-induced inhibition of proliferation is coupled to resistance to cAMP-induced morphological changes, suggesting that cell morphology is somehow related to cAMP sensitivity in the control of cell growth. In light of the observation that cAMP has effects on cell morphology and cell growth, and can alter adrenal steroidogenesis in the absence of ACTH, one challenge of future research will be to explore whether this nucleotide is a common "second messenger" in mediating the effects of ACTH on adrenocortical cell growth and function through the cytoskeleton.

## Cell Shape and Cellular Transformation

A rapidly growing area of intensive research is the field of cellular transformation. From studies on transformed cell lines in culture, it is apparent that cellular transformation, in particular viral transformation, involves complex and diverse changes in cellular functions. Some of the hallmark characteristics of transformation include the loss of normal growth control involving contact and density inhibition, loss of specific cell surface constituents such as fibronectin, and changes in cell morphology which include cell rounding and loss of cytoskeletal stress cables (44). Immunofluorescent staining of

actin-containing structures in transformed and nontransformed cells reveals dramatic changes in filament organization (384). Other studies have shown that changes in actin content often accompany loss of actin microfilaments (608). In light of observations that membrane-associated fibronectin can interact with actin and influence the distribution of intracellular microfilaments (339), and that loss of surface fibronectin often accompanies transformation (609), perhaps alterations in cytoskeleton organization are responsible for the decrease ability of transformed cells to adhere tightly to artificial surfaces.

Several recent studies have presented evidence for a cytoskeletal involvement in virally-induced cellular protein phosphorylation (610). Several of the proteins phosphorylated in response to viral infection have been identified as cytoskeletal proteins. Sefton et al. (611) have shown that viral transformation in cells leads to phosphorylation of vinmentin, a protein localized to adhesion plaques in cultured cells and involved in cell attachment. A second cytoskeletal protein phosphorylated under these conditions is vinculin, another protein involved in cell attachment. Phosphorylation of this protein has been correlated with cell rounding, suggesting that these events are somehow coupled to one another. It has been proposed that phosphorylation of these proteins may mediate the expression of reduced cell adherence in response to viral infection (611). Moreover, it is known from in vitro work on microtubule assembly that phosphorylation of tubulin and of

microtubule-associated proteins regulates microtubule assembly (117). Steinberg (119) has presented evidence in S49 lymphoma cells that newly synthesized actin is phosphorylated in response to cAMP. In the adrenocortical cell, cAMP-dependent protein kinases have been identified that specifically phosphorylate several intracellular proteins in response to ACTH (417). Perhaps some of these proteins will turn out to be cytoskeletal proteins. If this is the case, then cAMP-dependent phosphorylation of cytoskeletal filaments such as actin may mediate the effects of ACTH on adrenocortical cell shape, and perhaps on cell function as well.

The hypothesis that the cytoskeleton mediates some of the effects of viral transformation has received indirect experimental support from several directions. First, there is some evidence that viral translation factors, including viral mRNA, are physically associated with cytoskeletal filaments (600). Several laboratories have demonstrated that the appearance of viral proteins in HeLa cells infected with poliovirus (602), human KB cells infected with adenovirus (604), and BSC-1 cells infected with SV40 (612) is correlated with the appearance of viral mRNA associated with cytoskeletal filaments. Second, viral transformation has been shown to result in the appearance of cytoskeletal components on the surface of cells (609). For example, Bachvaroff et al. (382) have shown that leukemia cells and lymphocytes tranformed by Epstein-Barr virus display actin on the outer surface of the plasma membrane; actin is not found on the nontransformed counterparts. Third, immunofluorescent

staining of the product of the src gene, $pp60^{src}$, in RSV-transformed cells (611) reveals a co-localization of this protein kinase with vinculin in regions of close contact with the substratum. These observations together argue for an important role(s) of the cytoskeleton in the expression of cellular transformation.

Finally, these results take on greater significance in the face of recent discoveries that hormone receptors such as the EGF receptor share similar protein kinase activity with that of $pp60^{src}$ (614). Binding of EGF to its receptor on human A431 tumor cells induces rapid phosphorylation of tyrosine in cellular proteins (613). EGF binding also induces marked cell rounding and plasma membrane blebbing, suggesting that these events may be coupled. The fact that vinculin is phosphorylated on tyrosine residues in response to RSV virus (611) offers indirect support for the hypothesis of cytoskeletal control over cell shape. It may turn out that hormones and viruses utilize common intracellular pathways in the regulation of biological activities. The cytoskeleton may be one such locus for control. Thus, another important avenue for future research on the adrenocortical cell will be toward exploring whether viral-like cellular responses occur in the adrenocortical cell in the course of ACTH action, and if so, how they might relate to the mechanism of ACTH action on adrenocortical cell growth and function.

## Closing Statement

In light of these observations, the work presented in this dissertation demonstrating hormonal regulation of adrenocortical cell shape and cytoskeleton takes on added significance, for any attempt to unravel the underlying molecular mechanisms by which hormones influence cellular activities will bring us closer to an to understanding of how cell growth and differentiation are regulated in normal cells. Though the concept of the cytoskeleton as a major intracellular regulatory system is a relatively new one, there is already much evidence to support this hypothesis. The fruits of future research into the cytoskeleton will surely lead to a greater understanding of the role(s) this system plays in the overall regulation of adrenocortical cell function. I believe that the research presented in this dissertation is a step in that direction.

## References


1. F.H.C.Crick, A.F.W.Hughes (1950) Exp. Cell Res. 1,37.
2. B.Katz (1966) in Nerve, Muscle and Synapse NY:McGraw-Hill.
3. H.E.Huxley (1964) Science 164,1356.
4. H.G.Mannherz, R.S.Goody (1976) Ann. Rev. Biochem. 45,427.
5. G.Offer (1974) in Companion to Biochemistry, A.T.Bull, ed., NY:Longmann, 623-671.
6. J.H.Collins, M.Elzinga (1975) J. Biol. Chem. 250,5915.
7. D.Suck, W.Kabsch, H.G.Mannherz (1981) Proc. Nat. Acad. Sci. U.S.A. 78,4319.
8. R.Cooke (1975) J. Supramol. Struct. 3,146.
9. R.Cooke (1975) Biochemistry 14,3250.
10. Cell Motility, (1979) H.Stebbings and H.S.Hyams, eds., NY:Longman.
11. C.B.Klee, T.H.Crouch, P.G.Richman (1980) Ann. Rev. Biochem. 49,489.
12. H.E.Huxley, J.Hanson (1954) Nsature 173,973.
13. H.Rasmussen, D.B.P.Goodman (1977) Physiol. Rev. 57,421.
14. D.B.Slautterback (1963) J. Cell Biol. 18,367.
15. P.Satir (1974) Sci. Am. 231,44.
16. J.W.Sanger, J.M.Sanger (1979)Meth. Achiev. exp. Pathol. 8,110.
17. J.A.Snyder, J.R.McIntosh (1976) Ann. Rev. Biochem. 45,699.
18. K.Weber, R.Pollack, T.Bibring (1975) Proc. Nat. Acad. Sci. U.S.A. 72,459.
19. R.R.Weihing (1979) Meth. Achiev. exp. Pathol. 8,42.
20. J.B.Olmsted, G.G.Borisy (1973) Ann. Rev. Biochem. 42,507.
21. R.F.Luduena, D.O.Woodward (1973) Proc. Nat. Acad. Sci. U.S.A. 70,3594.
22. G.G.Borisy, J.M.Marcum, J.B.Olmsted, D.B.Murphy, K.A.Johnson (1975) Ann. N.Y. Acad. Sci. 253,107.
23. R.C.Weisenberg (1972) Science 177,1104.
24. S.Inoue, H.Ritter (1975) om Molecules and Cell Movement, S.Inoue and R.E.Stephens, eds., NY:Raven Press, 3-30.
25. J.M.Marcum, J.R.Dedman, B.R.Brinkley, A.R.Means (1978) Proc. Nat. Acad. Sci. U.S.A. 75,3771.
26. B.Eipper (1974) J. Biol. Chem. 249,1398.
27. K.Roberts (1972) Prog. Biophys. Mol. Biol. 28,371.
28. K.Weber, M.Osborn (1979) in Microtubules, K. Roberts and J.S. Hyams, eds., NY:Academic Press, 279-313.
29. J.R.McIntosh, S.C.Landis (1971) J. Biol. Chem. 49,468.
30. J.S.Hyams, G.G.Borisy (1978) J. Cell Biol. 78,401.
31. J.A.Connolly, V.I.Kalnins (1980) Exp. Cell Res. 127,341.
32. D.B.Murphy, G.G.Borisy (1975) Proc. Nat. Acad. Sci. U.S.A. 72,2696.
33. D.W.Cleveland, S.-Y.Hwo, M.W.Kirschner (1977) J. Mol. Biol. 116,207.
34. R.D.Sloboda, W.L.Dentler, J.L.Rosenbaum (1976) Biochemistry 15,4497.
35. J.Connolly, V.I.Kalnins, D.W.Cleveland, M.W.Kirschner (1977) Proc. Nat. Acad. Sci. U.S.A. 74,2437.
36. A.H.Lockwood (1978) Cell 13,613.
37. J.A.Weatherbee, R.B.Luftig, R.R.Weihing (1978) J. Biol. Chem. 78,47.

38. J.C.Bulinske, G.G.Borisy (1979) Proc. Nat. Acad. Sci. U.S.A. 76,293.
39. B.H.Gibbons, I.R.Gibbons (1973) J. Cell Sci. 13,337.
40. M.A.Sleigh (1969) Int. Rev. Cytol. 25,31.
41. G.B.Witman, R.Fay, J.Plummer (1976) in Cell Motility, R.D.Goldman, T.Pollard, J.Rosenbaum,eds., NY:Cold Spring Harbor Publications.
42. P.A.Simpson, J.A.Spudich (1980) Proc. Nat. Acad. Sci. U.S.A. 77,4610.
43. D.F.Albertini, E.Anderson (9177) J. Cell Biol. 73,111.
44. Molecular Biology of the Cell (1983) B.Alberts, D.Bray, J.Lewis, M.Raff, K.Roberts, J.D.Watson, eds., NY:Garland Publishing, Inc.
45. R.W.Linck (1976) J. Cell Sci. 20,405.
46. R.E.Stephens (1975) in Molecules and Cell Movement, S.Inoue and R.E.Stephens, eds., NY:Raven Press.
47. I.R.Gibbons, E.Fronk, B.H.Gibbons, K.Ogawa (1976) in Cell Motility, R.Goldman, ed., NY:Cold Spring Harbo Publications.
48. F.D.Warren, D.R.Mitchell (1980) Int. Rev. Cytol. 66,1.
49. J. Wehland, M.Osborn, K.Weber (1977) Proc. Nat. Acad. Sci. U.S.A. 74,5613.
50. R.Cooke, L.Murdoch (1973) Biochemistry 12,3927.
51. P.Satir, J.Wais-Steider, S.Leduska, A.Nash, J.Avolio (1981) Cell Motility 1303.
52. F.D.Warner, P.Satir (1974) J. Cell Biol. 63,35.
53. Cell Motility (1979) H.S.Stebbings and J.S.Hyams, eds. NY:Longman.
54. B.H.Gibbons, I.R.Gibbons (1972) J. Cell Biol. 54,75.
55. K.Summers, I.R.Gibbons (1973) J. Cell Biol. 58,618.
56. E.Lazarides, K.Weber (1974) Proc. Nat. Acad. Sci. 71,2268.
57. J.A.Weatherbee (1981) Int. Rev. Cytol. 12, Academic Press, Inc.
58. L.A.Staehelin, B.E.Hull (1978) Sci. Am. 238,141.
59. J.M.Sanger, J.W.Sanger (1980) J. Cell Biol. 86,568.
60. Cell Motility 91979) H.S.Stebbings and J.S.Hyams, eds. NY:Longman.
61. J.A.Weatherbee (1981) Int. Rev. Cytol.12 Academic Press, Inc.
62. Cell Motility (9179) H.S.Stebbings and J.S.Hyams, eds. NY:Longman.
63. T.D.Pollard (1981) J. Cell Biol. 91,156s.
64. C.C.Wang, J.T.Yang (1976) Biochemistry 15,3007.
65. F.Oosawa, S.Asakuna (9175) in Thermodynamics of the Polymerization of Protein, NY:Academic Press.
66. D.G.Uyemura, S.S.Brown, J.A.Spudich (1978) 253,9088.
67. R.Lu, M.Elzing (1976) in Cell Motility, R.Goldman,ed., Cold Spring Harbor Laboratory, Cold Spring Harbor,NY, 487-492.
68. J.Vandekerhove, K.Weber (1978) J. Mol. Biol. 126,783.
69. D.Gordon, J.Boyer, E.Korn 91977) J. Biol. Chem. 252,8300.
70. J.L.Morgan, C.R.Holliday, B.S.Spooner (1980) Proc. Nat. Acad. Sci. 77 2069.
71. U. Grosche-Stewart (1980) Int. Rev. Cytol. 65,193.
72. Cell Motility (1979) H.S.Stebbings and J.S.Hyams, eds., NY:Longmann.
73. L.G.Tilney (1976) J. Cell Biol. 69,73.
74. S.C.Mockrin, J.A.Spudich (1976) Proc. Nat. Acad. Sci.

73,2321.
75. E.Lazarides (1975) J. Cell Biol. 65,549.
76. R.A.Badley, C.W.Llyod, A.Woods, L.Carruthers, C.Alcock, P.A.Reese (1978) Exp. Cell Res. 117,231.
77. J.Bryan, R.E.Kane (1978) J. Mol. Biol. 125,207.
78. D.J.DeRosier, K.T.Edds (1980) Exp. Cell Res. 126,490.
79. L.G.Tilney (1975) J. Cell Biol. 64,289.
80. M.Schliwa (1981) Cell 25,587.
81. T.D.Pollard (1982) in Cell and Muscle Motility, vol. 2, R.M.Dowben and J.W.Shay, eds., NY:Plenum Press.
82. J.H.Hartwig, T.P.Stossel (1975) J. Biol. Chem. 250,5696.
83. C.C.Wang (1977) Biochemistry 16,1857.
84. N.Mimura, A.Asano (1979) Nature 282,44.
85. Bretcher, K.Weber (1980) Cell 20,839.
86. K.Sobue, Y.Muramoto, M.Fujita, S.Kakiuchi (1981) Proc. Nat. Acad. Sci. 78,5652.
87. Isenberg (1980) Nature 288,455.
88. Hasegawa (1980) Biochemistry 19,2677.
89. Yin, T.P.Stossel (1980) J. Biol. Chem. 255,9490.
90. K.J.Yamamoto, J.Pardee, J.Reidler, L.Stryer, J.Spudich (1982) J. Cell Biol. 95,711.
91. L.Carlsson (1977) J. Mol. Biol. 115,483.
92. L.G.Tilney, E.M.Bonder, L.M.Coluccio, M.S.Mooseker (1983) J. Cell Biol. 97,112.
93. G.R.Holmes, D.E.Goll, A.Suzudi (1971) Biochem. Biophys. Acta 253,240.
94. J.V.Small, G.Rinnerthaler, H.Hinssen (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,599.
95. B.Geiger, S.J.Singer (1979) Cell 16,213.
96. E.Lazarides (1975) J. Histo. Cytochem. 23,507.
97. Z.Avnur, J.V.Small, B.Geiger (1983) J. Cell Biol. 96,1622.
98. V.Fowler, D.L.Taylor (1980) J. Cell Biol. 85,361.
99. V.M.Fowler, E.J.Luna, W.R.Hargreaves, D.L.Taylor, D.Branton (1981) J. Cell Biol. 88,388.
100. J.J.Tomasek, E.D.Hay, K.Fumiwara (1982) Dev. Biol. 92,107.
101. L.G.Tilney, L.A.Jaffe (1980) J. Cell Biol. 87,771.
102. D.T.Woodrum, S.A.Rich, T.D.Pollard (1975) J. Cell Biol. 67,231.
103. S.L.Brenner, E.D.Korn (1980) J. Biol. Chem. 255,841.
104. E.M.Bonder, M.S.Mooseker (1983) J. Cell Biol. 96,1097.
105. T.D.Pollard, M.S.Mooseder (1981) J. Cell Biol. 88,654.
106. A.Wegner (1976) J. Mol. Biol. 1088,139.
107. M.W.Kirschner (1980) J. Cell Biol. 86,330.
108. I. Blikstad, F.Markey, L.Carlsson, T.Persson, U.Lindberg (1978) Cell 15,935.
109. L.M.Coluccio, L.G.Tilney J. Cell Biol. 97,1629.
110. S.W.Craig, T.D.Pollard (1982) Trends Biochem. Sci. 7,88.
111. E.D.orn (1982) Physiol. Rev. 62,672.
112. Harris, Weeds (1978) FEBS Lett. 90,84.
113. W.Birchmeier, T.A.Liebermann, B.A.Imhof, T.E.Kreis (1982) Cold Spring Harbor Symposia on Quantitative Biology 46,755.
114. L.G.Tilney (1978) J. Cell Biol. 77,551.
115. R.L.Margolis, L.Wilson (1978) Cell 13,1.
116. D.B.Murphy, K.A.Johnson, G.G.Borisy (1977) J. Mol. Biol. 117,35.

117. L.Jameson, T.Frey, B.Zeeberg, F.Dalldorf, M.Caplow (1980) Biochemistry 19,2472.
118. R.DSloboda, S.A.Rudolph, J.L.Rosenbaum, P.Greengard (1975) Proc. Nat. Acad. Sci. 72,177.
119. R.A.Steinberg (1980) Proc. Nat. Acad. Sci. 77,910.
120. D.J.DeRosier, L.G.Tilney (1982) Cold Spring Harbor Symposia on Quantitative Biology 46,525.
121. J.A.Spudich, L.A.Amos (1979) J. Mol. Biol. 129,319.
122. S.Inoue (1981) J. Cell Biol. 91,132s.
123. J.D.DeRosier, E.Mandelkow, A.Silliman, L.Tilney, R.Kane (1977) J. Mol. Biol. 113,679.
124. J.D.DeRosier, R.Censullo (1981) J. Mol. Biol. 146,77.
125. M.S.Mooseker (1976) J Cell Biol. 71,417.
126. C.L.Howe, M.S.Mooseker, T.A.Graves (1980) J. Cell Biol. 85,916.
127. A.Bretscher, K.Weber (1978) Exp. Cell REs. 116,397.
128. A.Bretscher, K.Weber (1978) J. Cell Biol. 79,839.
129. M.S.Mooseker, L.G.Tilney (1975) J. Cell Biol. 67,725.
130. P.T.Matsudaira, D.R.Burgess (1975) J. Cell Biol. 84,667.
131. A.Bretscher, K.Weber (1979) Proc. Nat. Acad. Sci. 76,2321.
132. B.Geiger, K.T.Tokuyasu, S.J.Singer (1979) Proc. Nat. Acad. Sci. 76,2833.
133. A.Bretscher, K.Weber (1980) Cell 20,839.
134. A.Bretscher, K.Weber (1980) J. Cell Biol. 86,335.
135. R.D.Goldman, ElLazarides, R.Pollack, K.Weber (1975) Exp. Cell Res. 90,33.
136. N. McNutt, L.A.Culp, P.H.Black (1971) J. Cell Biol. 50,691.
137. M.Ambercrombie, G.A.Dunn (1975) Exp. Cell Res. 92,57.
138. J.P.Revel, K.Wolken (1973) Exp. Cell Res. 78,1.
139. I.K.Buckley (1975) Tissue Cell 7,51.
140. J.E.M.Heaysman, S.M.Pegrum (1973) Exp. Cell Res. 78,71.
141. M.Ambercrombie, J.E.M.Heaysman, S.M.Pegrum (1971) Exp. Cell Res. 67,359.
142. J.W.Sanger,J.M.Sanger, B.M.Jockusch (1983) J. Cell Biol. 96,961.
143. K.Funiwara, T.D.Pollard (1976) J. Cell Biol. 71,848.
144. E.Lazarides (1976) J. Cell Biol. 68,202.
145. K.Wang, J.F.Ash, S.J.Singer (1975) Proc. Nat. Acad. Sci. 72,4483.
146. W.E.Gordon (1978) Exp. Cell Res. 117,253.
147. T.E.Dreis, W.Birchmeier (1980) Cell 22, 555.
148. J.E.Schollmeyer,L.Furcht,D.E.Goll,R.M.Robson M.H.Stromer (1976) Cold Spring Harbor Conference on Cell Proliferation 3,361, NY:Cold Spring Harbor Laboratory.
149. G.Isenberg, P.C.Rathke, N.Hulsmann, N.W.Franke (1976) Cell Tissue Res. 166,427.
150. R.D.Goldman, J.A.Schloss, S.M.Starger (1976) in Cell Motility, R.D.Goldman, ed., Book A, 217-245.
151. M.Ambercrombie, J.Heaysman, S.Pegnum (1971) Exp. Cell Res. 67,359.
152. N.K.Wessells, B.Spooner, N.Lunduena (1973) in Locomotion in Tissue Cells, 53-82, Elsevier, Amsterdam.
153. E.P.Reaven, S.Axline (1973) J. Cell Biol. 59,12.
154. R.D.Goldman, M.-J.Yerna, J.Schloss (1976) J. Supramol.

Struct.5,155.
155. M.H.Heggeness, K.Wang, S.Singer (1977) Proc. Nat. Acad. Sci. 74,3883.
156. R.R.Weihing (1982) Cold Spring Harbor Conference on Cell Proliferation. vol. 3, 671-684, NY:Cold Spring Harbor Laboratory.
157. K.Mariyama, S.Ebashi (1965) J. Biochem. 58,13.
158. T.P.Stossel, J.Hartwig (1976) J. Cell Biol. 68,602.
159. H.Maruta, E.D.Korn (1977) J. Biol. Chem. 252,399.
160. K.Wang, S.Singer (1977) Proc. Nat. Acad. Sci. 74,2021.
161. J.H.Hartwig, T.Stossel (1976) J. Cell Biol. 71,295.
162. R.R.Weihing (1977) J. Cell Biol. 75,95.
163. R.Niederman, P.Amrein, J.Hrtwig (1983) J. Cell Biol. 96,1400.
164. K.kMaruyama, M.Kaibara,R.Fukada (1974) Biochem. Biophys. Acta 271,20.
165. D.L.Taylor, J.Condeelis (1979) Int. Rev. Cytol. 56,57.
166. S.MacLean-Fletcher, T.Pollard (1980) J. Cell Biol. 85,414.
167. H.L.Yin, T.Stossel (1979) Nature 281,583.
168. N.Mimura, A.Asano (1979) 282,44.
169. K.Wang, J.Singer (1977) Proc. Nat. Acad. Sci. 74,2021.
170. E.A.Brotschi, E.Korn (1979) J. Biol. Chem. 254, 9982.
171. J.Bryan, R.Kane (1978) J. Mol. Biol. 125,207.
172. H.Maruta, E.Korn (1977) J. Biol. Chem. 252,399.
173. L.M.Griffith, R.Pollard (1982) J. Biol. Chem. 257, 9143.
174. J.H.Hartwig, T.Stossel (1979) J. Mol. Biol. 134,539.
175. S.MacLean-Fletcher, T.Pollard (1980) Cell 20,329.
176. H.L.Yin, J.Hartwig, K.Maruyama, T.Stossel (1981) J. Biol. Chem. 256,9693.
177. G.Isenberg, U.Aebi, T.Pollard (1980) Nature 288,455.
178. J.S.Condeelis, D.Taylor (1977) J. Cell Biol. 74,901.
179. Maruta, Korn (1977) J. Biol. Chem. 252,399.
180. T.D.Pollard (1981) J. Biol. Chem. 256,7666.
181. K.T.Edds (1977) J. Cell Biol. 73,479.
182. K.T.Edds (1977) Exp. Cell Res. 108,452.
183. D.F.Albertini, E.Anderson (1977) J. Cell Biol. 73,111.
184. J.H.Hartwig, T.Stossel (1981) J. Mol. Biol. 145,563.
185. B.E.Hull, L.Staehlin (1979) J. Cell Biol. 85,916.
186. R.Rosewald, S.Newman, M.Karnovsky (1976) J. Cell Biol. 70,541.
187. L.G.Tilney, S.Hatano, H.Ishidawa, M.Mooseker (1973) J. Cell Biol. 59,109.
188. L.G.Tilney, J.Clain, M.S.Tilney (1979) J.Cell Biol. 81,229.
189. L.G.Tilney, D.Kiehart, C.Sardet, M.Tilney (1978) J. Cell Biol. 77,536.
190. D.J.DeRosier, E.Mandelkow, A.Sillman, L.Tilney, R.Kane (1977) J. Mol. Biol. 113,679.
191. E.Lazarides (1980) Nature 283,249.
192. K.Weber, M.Osborn (1982) Path. Res. Pract. 175, 128.
193. B.H.Anderton (1981) J. Muscle Res. Cell Motility 2,141.
194. K.Ishikawa, C.Kuroda, K.Ogata (1969) Biochem. Biophys. Acta 179, 316.
195. W.W.Franke, E.Schmid, M.Osborn, K.Weber (1978) Proc. Nat. Acad. Sci. 75,5034.
196. M.W.Klumkowsky (1981) Nature 291,249.

197. W.J.Nelson, P.Traub (1981) Eur. J. Biochem. 116,51.
198. D.T.Mayer, A.Gulick (1942) J. Biol. Chem. 146,433.
199. S.H.Kaufman, D.Coffey, J.Shaper (1981) Exp. Cell Res. 132,121.
200. J.H.Shalper, D.Pardoll, S.Kaufman, E.Barrack, B.Vogelstein, D.Coffey (1979) Advances in Enzyme Regulation 17,213.
201. K.R.Shelton, D.C.Cochran (1978) Biochemistry 17,1212.
202. R.Berezney, B.Hughes (1977) J. Cell Biol. 75,406a.
203. G.Herlan, F.Wunderlich (1976) Cytobiologie 13,291.
204. I.L.Cameron, S.Prescott (1963) Exp. Cell Res. 30,609.
205. L.D.Dodge, P.Mancini, F.Davis, P.Heyword (1977) J. Cell Biol.72,194.
206. F.Wunderlich, R.Berezney, H.Kleining (1976) in Biological Membranes, D.Chalpman, D.Wallach, eds., NY:Academic Press.
207. F.Jacob, S.Brenner, F.Cuzin (1963) Cold Spring Harbor Symposia on Quantitative Biology 28,329.
208. R.Berezney, S.Coffey (1975) Science 189,291.
209. J.R.Paulson, U.Laemmli (1977) Cell 12,817.
210. G.Herlan, R.Quevedo, F.Wunderlich (1978) Exp. Cell Res. 29,129.
211. W.W.Chin, J.Maizel,Jr. (1977) Virology 76,79.
212. M.Schliwa (1979) Exp. Cell REs. 118,323.
213. N.Hirokawa (1982) J. Cell Biol. 94,129.
214. J.F.Reiger (1966) J. Ultrastruct. Res. 14,602.
215. L.M.Griffith, R.Pollard (1978) J. Cell Biol. 79,958.
216. G.S.Bloom, R.Valle (1983) J. Cell Biol. 96,1523.
217. K.Wang, J.Feramisco, J.Ash (1982) in Methods in Enzymology 85,514.
218. P.Cooke (1976) J. Cell Biol. 68,539.
219. S.D.Glacy (1983) J. Cell Biol. 97,1207.
220. R.O.Hynes, A.Destree (1978) Cell 15,875.
221. F.Mullen, E.Lazarides, J.Elias (1976) Clin. Immunol. Immunopathol. 5, 416.
222. B.Geiger, S.Singer (1980) Proc. Nat. Acad. Sci. 77,4769.
223. G.Utter, P.Biberfeld, R.Norberg, R.THorstensson, A.Fagreaus Exp. Cell Res. 114,127.
224. J.B.Olmsted, J.Marcum, K.Johnson, C.Allen, G.Borisy J. Supramolec. Struct. 2,429.
225. C.Allen, G.Borisy (1974) J. Mol. Biol. 90,381.
226. R.E.Stephens (1970) J. Mol. Biol. 47,353.
227. J.Tannenbaum, S.Tannenbaum, G.Godman (1977) J. Cell. Physiol.ds ddd91,225.
228. A.Helenius, K.Simons (1975) Biochem. Biophys. Acta 415,29.
229. S.J.Singer (1974) Ann. Rev. Biochem. 43, 805.
230. P.B.Moore, C.Ownby, K.Carraway (1978) Exp. Cell Res. 115,230.
231. M.F.Mescher, M.Jose, S.Balk (1981) Nature 289,139.
232. Aldrich Technical Information Sheet, Aldrich Chemical Co.
233. N.K.Wessellls (1971) Science 171,135.
234. A.C.Allison, PL.Davies, S.DePetris (1971) Nature New Biol. 232,153.
235. B.S.Spooner, K.Yamada, N.Wessells (1971) J.Cell Biol.49,595.
236. A.Dipaspluale (1975) Exp. Cell Res. 95,425.
237. J.V.Small, G.Rinnerthaler, H.Hinssen (1982) Cold Spring Harbor Symposia on Quantitative Biology 46,599.

238. S.J.Altas, S.Lin (1978) J. Cell Biol. 76,360.
239. R.R.Weihing (1976) J. Cell Biol. 72,459.
240. A.Krishan (1972) J. Cell Biol. 54,657.
241. R.F.Kletzien, J.Perdue, A.Springer (1972) J. Biol. Chem. 247,2964.
242. M.A.McPherson, J.Ramachandran (1980) J. Cell Biol. 86,129.
243. P.F.Maness, R.Walsh,Jr. (1982) Cell 30,253.
244. J.A.Williams, J.Wolff (1971) Biochem. Biophysic. Res. Commun. 44,422.
245. M.A.McPherson, J.Schofield (1972) FEBS Lett. 24,45.
246. R.J.Halsam (1972) Biochem. J. 127,34P.
247. H.Faulstich, A.Shafer, M.Weckauf (1977) Hoppe-Scyler's Z. Physiol. Chem. 358,181.
248. F.Lutz, H.Glossmann, M.Frimmer (1972) Arch. Pharmakol. 273,341.
249. J.M.Olver, R.Berlin (1979) Symposia of the Society for Exp. Biology 33,277.
250. D.C.Beebe, D.Feagans, E.Blanchette-Mackie, M.Nau (1979) Science 206,836.
251. S.B.Mizel, L.Wilson (1972) Biochemistry 11,2573.
252. W.A.Creasy, K.Bensch, S.Malawista (1979) Biochem. Pharmacol. 20,1579.
253. S.E.Malawista, H.Sato, K.Bensch (1968) Science 160,770.
254. P.S.Lin, D.Wallach, S.Tsai (1973) Proc.Nat.Acad.Sci. 70,2492.
255. L.G.Tilney, J.Gibbins (1969) J. Cell Biol. 41,227.
256. L.G.Tilney, R.Cardell,Jr. (1970) J. Cell Biol. 47,408.
257. D.L.Taylor, Y.-L.Wang (1980) Nature 284,405.
258. S.D.Glacy (1983) J. Cell Biol. 96,1164.
259. J.Weilhland, K.Weber (1980) Exp. Cell Res. 127,397.
260. R.Burridge, F.Feramisco (1980) Cell 19,587.
261. P.Wadsworth,R.Sloboda (1983) J. Cell Biol. 97,1249.
262. J.M.Wiehland, M.Osborn, K.Weber (1980) Eur. J. Cell Biol. 21,188.
263. D.Rungger, E.Rungger-Brnandle, C.Chaponier, G.Gabbiani (1979) Nautre 282,320.
264. D.P.Kiehart, I.Mabuchi, S.Inoue (1982) J. Cell Biol. 94,165.
265. J.Lin, J.R.Feraminsco (1981) Cell 24,185.
266. T.Matusaka (1976) M. Ultrastruct. Res. 54,318.
267. L.E.Westrun, E.G.Gray (1976) Brain Res. 105,547.
268. D.S.Smith, U.Jarlfors, B.Cameron (1975) Ann. N.Y. Acad. Sci. 253,472.
269. E.G.Gray (1978) Proc. R.Soc. London Ser.B 203,219.
270. P.Satir, A.Stuart (1965) J. Cell Biol. 244,277.
271. R.E.Ostlund, J.Leung, D.Kipnis (1977) J. Cell Biology 73,78.
272. M.H.Ellisman, J.Rach, L.Staehelin, K.porter (1976) J. Cell Biol. 68,752.
273. M.Schliwa (1975) J. Ultrastruct. Res. 52,377.
274. J.M.Lauweryns, J.Baert, W.Deloecker (1975) Cell Tissue Res. 163,111.
275. L.G.Tilney (1975) in Molecules and Cell Movement, Inoue and Stephens, eds., NY:Raven Press, 339-388.
276. Y.J.Lebeux, J.Willemot (1975) Cell Tissue Res. 160,37.
277. R.L.Hayes, R.Kelley (1969) J. Morph. 127,151.
278. J.V.Schollmeyer, D.Goll, R.Robson, M.Stromer (1978) J.

Cell Biol. 59,036a.

279. J.E.Schollmeyer, L.Furcht, D.Goll, R.Robson, M.Stromer (1976) Cold Spring Harbor Conference on Cell Proliferation 3, NY:Cold Spring Harbor Laboratory, 361-388.
280. J.V.Scholmeyer, D.Goll, L.Tilney, M.Mooseker, R.Robson, M.Stromer (1974) J. Cell Biol. 63,304a.
281. H.Mohr, T.Mohri, I.Mabuchi, I.Yazaki, H.Sakae, K.Ogawa Dev. Growth Diff. 18,391.
282. P.B.Moore, D.Anderson, J.Huggins, K.Carraway (1976) Biochem. Biophys. Res. Comm. 72,288.
283. D.G.Taylor, R.Mapp, N.Crawford (1975) Biochem. Soc. Trans. 3,161.
284. E.J.Luna, J.Fowler, J.Swanson, D.Branton, D.Taylor (1981) J. Cell Biol. 88,396.
285. V.M.Fowler, H.Pollard (1982) J. Cellular Biochem. 18,295.
286. J.S.Becker, J.Oliver, R.Berlin (1975) Nature 254, 152.
287. P.Sherline K.Schiavone (1977) Science 198,1038.
288. N.Zisapel, M.Levi, I.Gozs (1980) J. Neorochem. 34,26.
289. I.Gozes, C.Richter-Lnadsberg FEBS Lett. 95,169.
290. R.E.Stephens (1980) Fed.Proc.Fed.Am.Soc.Exp.Biol. 39,2163.
291. T.D.Pollard, E.Korn (1973) J. Biol. Chem. 24,448.
292. J.Condeelis (1979) J. Cell Biol. 80,751.
293. O.S.Kochhar (1979) Exp. Cell Res. 118,191.
294. W.A.Davies, T.Stossel J. Cell Biol. 75,941.
295. E.Gruenstein, A.Rich, R.Weihing (1975) J. Cell Biol.64,223.
296. A.L.Blitz, R.Fine (1974) Proc. Nat. Acad. Sci. 71,4472.
297. J.Tannenbaum, R.Rich )1979) Anal. Biochem. 95,236.
298. L.J.Wallace, W.Frazier (1979) J. Biol. Chem. 254,109.
299. F.F.Schreiner, E.Unanue (1976) Adv. Immunol. 24,37.
300. D.A.Williams .Boxer, J.Oliver, R.Baehner (1977) Nature 267,255.
301. G.L.Nicolson (1976) Biochem. Biophys. Acta 457,57.
302. D.F.Albertini, J.Clark (1975) Proc. Nat. Acad. Sci. 72,4976.
303. K.-G.Sundqvist, A.Ehrnst (1976) Nature 264,226.
304. J.Lotem, I.VClodavsky, L.Sachs (1976) Exp. Cell Res. 101,323.
305. L.Y.W.Bourguignon, K.Tokuyasu, S.Singer (1978) J. Cell. Physiol.95,239.
306. G.Gabbiani, C.Chaponnier, A.Zumbe, P.Vassalli (1977) Nature 269,697.
307. I.I.Singer (9179) Exp. Cell Res. 122,251.
308. J.Flanagan, G.Koch (9178) Nature 273,278.
309. M.S.Bretscher (1976) Nauture 260,21.
310. G.L.Nicolson, G.Poste (9176) N. Engl. J. Med. 295,197.
311. R.D.Berlin, J.Oliver (1978) J. Cell Biol. 77,197.
312. R.D.Berlin, J.Caron, J.Oliver (1979) in Microtubules, K.Roberts, J.S.Hyams, eds., NY:Academic Press, 443-485.
313. J.Braun, K.Fujiwara, T.Pollard, E.Unanue (1978) J. Cell Biol. 79,409.
314. K.Weber, P.Rathke, M.Osborn, W.Franke (1976) Exp. Cell Res. 102,285.
315. G.L.E.Koch, M.Smith (1978) Nature 273,274.
316. D.Branton, C.Cohen, J.Tyler (1981) Cell 24,24.

317. V.Bennett (1982) J. Cell. Biochem. 18,49.
318. T.L.Steck (1974) J. Cell Biol. 62,1.
319. S.E.Lux, K.John, MlKarnovsky (1976) J. Clin. Invest. 58,955.
320. J.Avruch G.Fairbanks (1974) Biochemistry 13,5507.
321. G.Hiller, K.Weber (1977) Nature 266,181.
322. M.S.Bretscher (1971) J. Mol. Biol.58,775.
323. V.Bennet, D.Branton (1977) J. Biol. Chem. 252,2753.
324. J.Yu, F.Goodman (1979) Proc. Nat. Acad. Sci. 76,2340.
325. J.Tyler, W.Hargreaves, D.Branton (1979) Proc. Nat. Acad. Sci. 76,5192.
326. W.Hargreaves, KnN.Giedd, A.Verkleij, D.Branton (1980) J. Biol. Chem. 255,11965.
327. L.G.Tilney, P.Detmers (1975) J. Cell Biol. 66,508.
328. S.L.Brenner, E.kKorn (1979) J. Biol. Chem. 254,8620.
329. C.M.Cohen, C.Korsgren (1980) Biochem. Biophys. Res. Comm. 97,1429.
330. C.M.Cohen, S.F.Foley (1980) J. Cell Biol. 86,694.
331. E.Ungewickell, P.Bennet, R.Calvert, V.Ohanian, W.Gratzer (1979) Nature 280,811.
332. C.M.Cohen, P.Jackson, D.Branton (1978) J. Supramol. Struct. 9,113.
333. V.M.Fowler, E.Luna, W.Hargreaves, D.Taylor, D.Branton (1981) J. Cell Biol. 88,388.
334. S.L.Brenner, E.Korn (1980) J. Biol. Cehm. 255,1670.
335.J.C.Pinder, E.Undewickell, R.Calvert, E.Morris, W.Gratzer (1979) FEBS Lett. 104,396.
336. M.P.Sheetz, S.Lux (1980) J. Biol. Chem 255,11512.
337. P.Helper (1980) J. Cell Biol. 86,490.
338. D.Shelton, J.Wyatt, J.Heuser (1979) J. Cell Biol. 83,2749.
339. R.O.Hynes, A.Destree, D.Wagner (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,659.
340. B.Geiger, K.Tokujasu, A.Dutton, S.Singer (1980) Proc. Nat. Acad. Sci. 77,4127.
341. C.S.Izzard, L.Lochner (1976) J. Cell Sci. 21,129,1976.
342. J.P.Heath, G.Dunn (1978) J.Cell Sci. 29,197.
343. J.G.Heletjaris, P.Lombardi, L.Glasgow (1976) J. Cell Biol. 69,407.
344. S.G.Azline, E.Reaven (1974) J. Cell Biol. 62,647.
345. P.Bornstein, D.Duksin, G.Balian, J.Davidson, E.Crouch (1978) Ann. N.Y. Acad. Sci. 312,93.
346. M.C.Willingham, I.Pastan (1975) J. Cell Biol. 67,146.
347. W.E.Rainey, P.Hornsby, J.Shay (1983) Endocrinology 113,48.
348. Z.Avnur, J.Small, B.Geiger (1983) J. Cell Biol. 96,1622.
349. B.Geiger (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,671.
350. W.E.Gordon (1978) Exp. Cell Res. 117,253.
351. P.Bornstein, D.Duskin, G.Balian, J.Davidson, E.Crouch (1978) Ann. N.Y. Acad. Sci. 312, 93.
352. I.U.Ali, R.Hynes (1977) Biochem. Biophys. Acta 471,16.
353. R.O.Hynes (1982) in Cell Biology of Extracellular Matrix E.D.Hay, ed. NY:Plenum Press, 335-378.
354. K.M.Yamada, K.Olden (1978) Nature 275,1798.
355. C.H.Fox, M.H.Cottler-Fox, K.Yamada (1980) Exp. Cell Res. 130,77.
356. R.O.Hynes (1981) Cell Surf. Rev. 7,97.

357. I.I.Singer, Pardiso (1981) Cell 24, 481.
358. R.O.Hynes, A.Destree (1978) Cell 15,875.
359. Z.Avnur, B.Geiger (1981) Cell 25,121.
360. A.C.Taylor, E.Robbins (1963) Dev.Biol. 7,660.
361. M.Abercrombie, J.Heaysman, S.Pegrum (1970) Exp. Cell Res. 60,437.
362. G.Albrecht-Buehler (1976) J. Cell Biol. 69,275.
363. C.S.Izzard, L.Lochner (1980) J. Cell Sci. 42,81.
364. L.R.Lochner, C.Izzard (1973) J. Cell Biol. 59,199a.
365. J.V.Small, J.Cellis, G.Isenberg (1980) in Transfer of Cell Constituents into Eukaryotic Cells, J.E.Celis, ed., NY: Plenum Press.
366. B.Geiger (1979) Cell 18,193.
367. M.H.Heggeness, K.Wang, S.Singer (1977) Proc. Nat. Acad. Sci. 74,3883.
368. A.Bretscher, K.Weber (1980) J. Cell Biol. 86,335.
369. K.Weber, U.Groschel-Stewort (1974) Proc. Nat. Acad. Sci. 71,4561.
370. M.DeBrabander, J.DeMey, R.VandeVeire, F.Aerts, G.Geuens (1977) Cell Biol. Int. Rep. 1,453.
371. A.K.Harris (1973) in Locomotion of Tissue Cells, Ciba Foundation Symp. 1972, No. 14.
372. M.Abercrombie, J.Heaysman, S.Pegrum (1971) Exp. Cell Res. 67,359.
373. D.Bray (1973) Nature 244,93.
374. V.M.Ingram (1969) Nature 222,641.
375. S.O.Mast (9131) Protoplasma 14,321.
376. K.Wang, S.Singer (1977) Proc. Nat. Acad. Sci. 74,2021.
377. T.D.Pollard (1976) J. Cell Biol. 68,579.
378. K.Sobue, K.Kanda,J.Adachi,S.Kakiuchi (1983) Proc. Nat. Acad. Sci. 80,6868.
379. S.Higashi-Fujume (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,69.
380. H.E.Huxley (1973) Nature 243,445.
381. A.N.Bhisey, J.Freed (1971) Exp.Cell Res. ,430.
382. R.J.Bachvaroff, F.Miller, R.Rapaport (1980) Proc. Nat. Acad. Sci. 77,4979.
383. J.F.Ash, D.Lovard, S.Singer (1977) Proc. Nat. Acad. Sci. 74,5584.
384. I.U.Ali, V.Mautner, R.Lanza, R.Hynes (1977) Cell 11,115.
385. S.Brown, W.Levinson, J.Spudich (1976) J. Supramol. Struct. 5,119.
386. S.Maclean-Fletcher, T.Pollard (1980) Biochem. Biophys. Res. Comm. 96,18.
387. S.A.Mulhun, E.Eisinberg (1976) Biochemistry 15,5702.
388. R.Pollack in Cell and Muscle Motility, Vol. 2, R.M.Dowbin, ed., NY:Plenum Press, 1-13.
389. P.Bannasch, H.Zerban, D.Mayer (1982) Path. Res. Pract. 175,196.
390. M.Osborn, N.Geisler, G.Shaw, G.Sharp, K.Weber (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,413.
391. T.D.Pollard, U.Eabi, J.Cooper, W.Fowler, P.Tseng (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,513.
392. N.J.Galvin, Dk.Stockhausen, B.Meyers-Hutchins, W.Frazier (1984) J. Cell Biol. 98,548.

393. D.Charlemagne, K.Schwartz, S.Bernard, A.Paraf (1979) J. Immunol. Methods 29,145.
394. S.L.Tobin, E.Zulauf, F.Sanchez, E.Craig, B.McCarthy (1980) Cell 19,121.
395. E.A.Fryberg, K.Kindle, N.Davidson, A.Sodju (1980) Cell 19, 365.
396. J.L.Lessard, D.Carlton, D.Rein, R.Akeson (1979) Anal. Biochem. 94,140.
397. L.Jameson, M.Caplow (1981) Proc. Nat. Acad. Sci. 78, 3413.
398. R.W.Rubin, R.Warren, D.Lukeman E.Clements (1978) J. Cell Biol. 78,28.
399. K.Weber, J.Glenney,Jr. (1982) in Cold Spring Harbor Symposia on Quantitative Biology 46,541.
400. G.F.Schreiner, E.Unanue (1975) Ann. N.Y. Acad. Sci. 253,37.
401. L.Y.W.Bourguignon, M.Nagpal, K.Balazovich, V.Gueriero, A.Means (1982) J. Cell Biol. 95,793.
402. G.A.Nelson, M.Andrews, M.Karnovsky (1982) J. Cell Biol. 95,771.
403. A.R.Means, J.Dedman (1980) Mol. and Cell. Endo. 19,215.
404. E.M.Bonder, M.Mooseder (1983) J. Cell Biol. 96,1097.
405. J.W.Sanger, J.M.Sanger (1979) Meth. Achiev. exp. Pathol. 8,110.
406. J.Ramachandran(1973) in Hormonal Proteinsand Peptides, vol. II, C.H.Li, ed., 1.
407. J.Ramachandran, S.Farmer, S.Liles, C.H.Li (1972)
408. J.Ramachandran, D.Chung, C.H.Li (1965) J.Amer.Chem.Soc. 87,2696.
409. B.Dixon, L.Wietkamp (1962) Biochem. J. 84,462.
410. K.Hoffman, W.Wingender, F.Finn (1970) Proc. Nat. Acad. Sci. U.S.A. 67, 829.
411. S.Rybak, J.Ramachandran (1981) In Vitro 17, 599.
412. A.Kahri (1971) Anat. Rec. 171,53.
413. S.Liles, J.Ramachandran (1977) Biochem. Biophys. Res. Commun. 79,226.
414. S.Liles, doctoral dissertation, UCSF.
415. S.Sheela Rani, G.Keri, J.Ramachandran (1983) Endocrinology 112, 315.
416. J.Ramachandran, Y.Kong, S.Liles (1976) Acta Endocrinologica 82,587.
417. L.Garren, G.Gill, H.Masui, G.Walton (1971) Recent Prog. Horm. Res. 27, 433.
418. R.Haynes,Jr., S.Koritz, F.Peron(1959)J. Biol. Chem. 234,1421.
419. D.Buckley, J.Ramachandran (1981) Proc. Nat. Acad. Sci. U.S.A. 78,7431.
420. U.Zor (1983) Endocrine Reviews 4,1.
421. L.Garren, W.Davis, R.Crocco, R.Ney (1966) Science 152,1386.
422. K.Asano, B.Harding (1976) Endocrinology 99, 977.
423. T.Kido, T.Kimura (1979) J. Biol. Chem. 254, 11806.
424. A.Kahri (1968) J. Cell Biol. 36, 181.
425. A.Dazord, D.Gallet, J.Saez (1978) Biochem. J. 176,233.
426. A.Dazord, D.Gallet, J.Saez (1977) Biochem. Biophys. Res. Commun. 76, 1238.
427. E.Podesta, A.Milani, H.Steffen, R.Neher (1979) Proc. Nat. Acad. Sci. U.S.A. 67,5187.

428. R.Rubin, B.Sheid, R.McCauley, S.Laychock (1974) Endocrinology 95,370.
429. S.Laychock, R.Rubin (1974) Steroids 24, 177.
430. M.Nakamura, M.Watanuki, P.Hall(1978)Mol. Cell. Endocrinology 12, 209.
431. G.Gill, L.Garren (1970) Eur. J. Biochem. 72,335.
432. G.Beckett, G.Boyd (1977) Eur. J. Biochem. 72,223.
433. G.Bhargva, E.Schwartz, S.Koritz (1978) Proc. Soc. Exp. Biol. Med. 158, 183.
434. W.Rainey, P.Hornsby, J.Shay (1983) Endocrinology 113, 48.
435. R.Lefkowitz, J.Roth, W.Pricer, I.Pastan (1970) Proc. Nat. Acad. Sci. U.S.A. 65, 745.
436. R.Farese, W.Reddy (1963) Biochem. Biophys. Acta 76,145.
437. M.Dallman, W.Engeland, J.Shinsako (1976) Am. J. Physiol. 231, 408.
438. M.McPherson, J.Ramanchandran (1980) J. Cell Biol. 86,129.
439. H.Masui, L.Garren (1971) Proc. Nat. Acad. Sci. U.S.A. 68,3206.
440. J.Ramachandran, A.Suyama (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 113.
441. D.Cater, M.Stack-Dunne (1975) J. Endocrinol. 12,174.
442. L.Morrow, B.Burrow, R.Mulrow (1967) Endocrinology 80,883.
443. A.deNicola, M.Clayman, R.Johnstone (1968) Endocrinology 82,436.
444. C.H.Li, J.Meienhofer, E.Schnabel, D.Chung, T.Lo, J. Ramachandran (1960) J. Amer. Chem. Soc. 82,168.
445. C.H.Li, E.Schnabel, D.Chung, T.Lo (1961) Nature 189,143.
446. G.Robison, E.Sutherland, E.Butcher (1971) "Cyclic AMP", Academic Press, N.Y.
447. D.Rudman, M.DiGirolamo, M.Malkin, L.Garcia (1965) in Handbook of Physiology, Williams and Wilkins, eds., Baltimore, 533.
448. W.Olophsen, J.Ramachandran (personal communication)
449. D.Buckley, J.Hagman, J.Ramachandran (1981) Endocrinology 109,10.
450. C.Rubin, O.Rosen (1975) Ann. Res. Biochem. 44,831.
451. B.Ross (1973) Endocrinology 93,1287.
452. J.Rao, J.Long, J.Ramachandran (1978) Endocrinology 102, 371.
453. E.Sutherland, W.Rall (1958) J. Biol. Chem. 232, 1065.
454. J.Harper, G.Brookes (1975) J. Cyclic Nucleotide Res.1,207.
455. U.Laemmli (1970) Nature (London) 227,680.
456. I.Blikstad, F.Markey, L.Carlsson, T.Persson, U.Lindberg (1978) Cell 15, 935.
457. M.Willard, M.Wiseman, J.Levine, P.Skene (1979) J. Cell Biol. 81,581.
458. P.Nakane, A.Kawaoi (1974) J. Histochem. Cytochem.22,1084.
459. M.Snabes, A.Boyd,III, R.Pardue, J.Bryan (1981) J. Biol. Chem. 256, 6291.
460. C.M.Behrens, doctoral dissertation, UCSF.
461. E.Canova-Davis, J.Ramachandran (1976) Biochemistry 15,921.
462. C.Li, D.Chung, D.Yamashiro, C.Less (1978) Proc. Nat. Acad. Sci. U.S.A. 75,4306.
463. C.Li, D.Chung, L-F.Tseng, H.Loh (1977) J.Med.Chem. 20,325.
464. O.Lowry, N.Rosebrough, A.Farr, R.Randall (1951) J. Biol. Chem. 193,265.

465. M.Bradford (1976) Anal. Biochem. 72,248.
466. G.Ceriotti (1952) J. BiolbChem. 198, 297.
467. E.Short,Jr., H.Warner, J.Koerner (1968) J. Biol. Chem. 243, 3342.
468. U.Groschel-Stewart (1980) Int. Review of Cytology 65,193.
469. T.Stossel, T.Pollard (1973) J. Biol. Chem. 248, 8288.
470. E.Lazarides (1976) J. Biol. Chem. 68, 202.
471. E.Lazarides, K.Weber (1974) Proc. Nat. Acad. Sci. 71,2268.
472. G.Gabbiani, G.Ryan, J.Lamelin, P.Vassali, G.Majno, C.Bouvier, A.Cru, E.Luscher (1977) Am.J.Pathol. 27, 473.
473. U.Groschel-Stewart, K.Unsicker (1977) Histochem. 51, 315.
474. B.Jockusch, K.Keley, R.Meyer, M.Burger (1978) Histochem. 55, 174.
475. K.Weber, P.Rathke, M.Osborn (1976) Exp. Cell Res.102,285.
476. E.Lazarides (1975) J. Histochem. Cytochem. 23, 507.
477. H.Masui, L.Reid C.Glans (1978) Exp. Cell Res. 117,219.
478. G.Feuer, F.Molnar, E.Pettko, F.Straub (1948) Hung. Acta Physiol. 1, 150.
479. J.Pardee, J.Spudich (1982) in Methods in Enzymology, vol. 85, Academic Press, N.Y., 164.
480. S.MacLean-Fletcher, T.Pollard (1980) Biochem. Biophys. Res. Commun. 96, 18.
481. S.Moore (1972) in Proc. of Peptides, 3rd American Peptide Symposium, J. Meienhofer, ed., Boston, 629.
482. E.Lazarides, U.Lindberg (1974) Proc. Nat. Acad. Sci. U.S.A. 71, 4742.
483. W.Fish (1975) in Methods in Membrane Biology, E.Korn, ed., Plenum Press, N.Y., 189.
484. K.Burridge, J.Feramisco (1980) Cell 19, 587.
485. S.Brown, W.Levinson, J.Spudich (1976) J. Supramolecular Structure 5, 119.
486. P.Mattson, J.Kowal (1982) Endocrinology 111, 1632.
487. L.Carlsson, F.Markey, I.Blikstad, T.Persson, U.Lindberg (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 6376.
488. H.Bloemendal (1981) Expt. Cell Res. 135, 454.
489. J.Couderc, A.Cadic, M.Sobrier, B.Dastugue (1982) Biochem. Biophys. Res. Commun. 188, 1982.
490. H.Mannherz, J.Barrington-Leigh, R.Leberman, H.Pfang (1975) FEBS Letters 60, 34.
491. K.Zechel (1980) Eur. J. Biochem. 110, 337.
492. W.Moyle, Y.Kong, J.Ramachandran (1973) J. Biol. Chem. 248, 2409.
493. R.Cheitlin, J.Ramachandran (1981) J. Biol. Chem. 256,3156.
494. M.Willard, M.Wiseman, J.Levine, P.Skere (1979) J. Cell Biol. 81, 581.
495. J.Morgan, C.Holladay, B.Spooner (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 2069.
496. J.Vanderderckhove, K.Weber (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 1106.
497. J.lessard, K.Carlton, D.Rein, R.Akeson (1979) Anal. Biochem. 94, 140.
498. D.Charlemagne, K.Schwartz, S.Bernard, A.Paraf (1979) J. Immunol. Methods 29, 145.
499. P.Kurki 91978) Clinical Immunology and Immunopathology 11,328.

500. P.Suck, W.Kabsch, H.Mannherz (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 4319.
501. S.Hitchcock, L.Carlsson, U.Lindberg (1976) Cell 7, 531.
502. D.Uyemura, S.Brown, J.Spudich (1978) 253, 9088.
503. K.Zechel (1980) Eur. J. Biochem. 110, 348.
504. in Selected Methods in Cellular Immunology (1980) B. Mischell, S.Shiigi, eds. W.H. Freedman, San Francisco.
505. G.Kronvall, U.Seal, J.Finstad (1970) J. Immunol. 104, 140.
506. E.Wang, A.Goldberg (1978) J.Histochem.Cytochem. 26,745.
507.D.Cleveland,M.Lopata, P.Sherline, M.Kirschner (1981) Cell 25, 537.
508. F.Karlsson, C.Grunfeld, C.Kahn, J.Roth (1979) Endocrinology 104, 1383.
509. B.Spiegelman, H.Green (1980) J. Biol. Chem. 255, 8811.
510. R.Sidhu (1979) J. Biol. Chem. 254, 11111.
511. Y.Kato, K.Komiya, H.Sasaki, T.Hashimoto (1980) J. Chromatog. 190, 297.
512. D.Bray, C.Thomas (1975) Biochem. J. 147, 221.
513. H.Jantzen (1981) Dev. Biol. 82, 113.
514. A.Spudich, J.Spudich (1979) J. Cell Biol. 82, 212.
515. N.Rubinstein, J.Chi, H.Holtzer (1976) Exp. Cell Res.97,387.
516. R.Fine, L.Taylor (1976) Exp. Cell Res. 102, 162.
517. R.Bachvaroff, F.Rapaport (1979) Transplant. Proc. 12,205.
518. J.Kowal (1970) Endocrinology 87, 951.
519. J.Hartwig, T.Stossel (1975) J. Biol. Chem. 250, 5696.
520. H.Segal, J.Winkler, M.Miyagi (1974) J. Biol. Chem. 249,6364.
521. M.Costlow, A.Hample (1982) J. Biol. Chem. 257 9330.
522. M.Krupp, M.Lane (1982) J. Biol. Chem. 257, 1372.
523. R.Hynes (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 3170.
524. E.Slavinski-Turley, N.Auersperg (1978) J. Endocrinology 78, 426.
525. D.Gospadarowicz, F.Gospodarowicz (1975) Endocrinology 96, 458.
526. M.O'Hare, A.Neville (1973) J. Endocrinology 56, 529.
527. J.Schlessinger, B.Geiger (1981) Exp.Cell Res. 134, 273.
528. D.Tramontano, A.Avivi, F.Ambesi-Impiombato, L.Bank, B.Geiger, J.Schlessinger (1982) Exp. Cell Res. 137, 269.
529. N.Batu, M.Cowden, S.Schwartz (1977) J.Pharm.Therap. 210,627.
530. D.Schubert, M.LaCorbere, C.Whitlock, W.Stallarp (1978) Nature 273, 1987.
531. U.Keski-Oja, B.Wetzel (1981) J. Cell Biol. 90, 537.
532. E.Wang, L.Pfeffer, I.Tam (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 6281.
533. C.Gitler, V.Pribluda, F.Laub, A.Rotman (1980) in Platelets: Cellular Response Mechanisms and their Biological Significance, A.Rotman, ed., J.Wiley and Sons.
534. V.Pribluda, R.Laub, A.Rotman (1981) Eur.J.Biochem. 116,293.
535. S.Swanston-Flatt, L.Carlsson, E.Gylfe (1980) FEBS Lett. 117, 299.
536. T.Lawrence, R.Ginzberg, N.Gilula, W.Beers (1979) J. Cell Biol. 80, 21.
537. A.Amsterdam, M.Knecht, K.Catt (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 3000.
538. J.Piatigorsky, H.Webster, M.Wollberg (1972) J. Cell Biol. 55, 82.

539. L.Barrett, R.Dawson (1974) Devl. Biol. 36, 72.
540. B.Bertolini, G.Monaco (1976) J.Ultrastruc.Res. 54, 59.
541. S.Famm (1964) J. Exp. Zool. 164, 163.
542. J.Rosenbaum, F.Child (1976) J. Cell Biol. 34, 345.
543. U.Zor (1983) Endocrine Reviews 4, 1.
544. Y.Henis, M.Elson (1981) Proc.Nat.Acad.Sci. U.S.A. 78,1072.
545. R.Pollack, D.Rifkin (1975) Cell 6, 495.
546. J.Snyder, J.McIntosh (1976) Ann. Rev. Biochem. 45, 699.
547. F.Lutz, H.Glossman, M.Frimmer (1972) Arch. Pharmokol. 273, 341.
548. M.Tomaselli, S.Lux (1977) Clin. Res. 25, 519a.
549. J.Mrotek, W.Rainey, T.Sawada, R.Lynch, M.Mattson, I.Lacko (1982) in Cell and Muscle Motility, R.Dowben, J.Shay, eds., Plenum Press, N.Y.
550. R.Pollack, R.Risser, S.Conlon, D.Rifkin (1968) Proc. Nat. Acad. Sci. U.S.A. 60, 126.
551. G.Todaro, H.Green, B.Goldberg (1963) Proc. Nat. Acad. Sci. U.S.A. 51, 66.
552. K.Weber, J.Glenney,Jr. (1982) Cold Spring Harbor Symposium on Quantitative Biology 46, 542.
553. J.Crivello, C.Jefcoate (1980) J. Biol. Chem. 39, 363.
554. J.Mrotek, P.Hall (1975) Biochem. Biophys. Res. Comm. 64,891.
555. P.Hall, C.Charponnier, M.Nakamura, G.Gabbiani (1979) J. Biol. Chem. 254, 9080.
556. J.Mrotek, P.Hall (1977) Biochemistry 16, 3177.
557. U.Zor, B.Strulovici, H.Linder (1978) Biochem. Biophys. Res. Commun. 80, 983.
558. M.Prentki, C.Chaponnier, B.Jearnen, C.Gabbiani (1979) J. Cell Biol. 81, 592.
559. P.Lacy, S.Howeel, D.Young, C.Fink (1968) Nature 219, 1177.
560. A.Poisner, J.Bernstein (1971) J.Pharmacol.Exp.Ther. 177,102.
561. S.Rudolph, L.Hegstrand, P.Greengard, S.Malawista (1979) Mol. Pharmacol. 16, 805.
562. S.Rudolph, P.Greengard (1979) Proc. Nat. Acad. Sci. U.S.A. 74, 3404.
563. J.Hagmann, P.Fishman (1980) J. Biol. Chem. 255, 2659.
564. S.Nasi, O.Grillo L.Naldini, P.Marchisio, P.Calissano (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 820.
565. J.Prives, A.Fulton, S.Penman, M.Daniels, C.Christian (1982) J. Cell Biol. 92, 231.
566. U.Zor, B.Strulovici, S.Lamprecht, A.Amsterdam, A.Oplatka, H.Linder (1979) Prostaglandins 18, 869.
567. T.Kurokawa, H.Durumada, M.Yodota, M.Yoshida, S.Ishibashi (1979) Biochem. Biophys. Acta 632, 138.
568. N.Sahyoun, H.Levine,III, G.Hebdon, R.Hemadaii, P.Cuaterecases (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 2359.
569. W.Roos, G.Gerisch (1976) FEBS Lett. 68, 170.
570. B.Spiegelman, H.Green (1980) J. Biol. Chem. 255, 8811.
571. B.Spiegelman, S.Farmer (19 ) Cell
572. W.LeStourgeon, A.Forer, Y.Yang, J.Bertram, H.Rusch (1975) Biochem. Biophys. Acta 379, 529.
573. N.Prashad, B.Wischmeyer, C.Evetts, F.Baskin, R.Rosenberg (1977) Cell Differ. 6, 147.
574. D.Gordon,E.Eisenberg,E.Korn (1976) J.Biol.Chem. 251,4778.

575. E.Gruenstein,A.Rich,R.Weihing (1975) J.Cell Biol. 64,223.
576. J.Phillips, A.Slater (1975) FEBS. Lett. 556, 327.
577. R.Dubois, E.Simpson, J.Tuckey, J.Lambeth, M.Waterman (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 1028.
578. N.Rubinstein, J.Chi, H.Holtzer (1976) Exp. Cell Res. 97,387.
579. B.Drazin, M.Trowbridge (1982) J. Cell Biol. 90, 11988.
580. B.Poole, S.Ohkuma (1981) J. Cell Biol. 90, 665.
581. M.Krupp, M.Lane (1982) J. Biol. Chem. 257, 1372.
582. M.Costlow, A.Hample (1982) J. Biol. Chem. 257, 9330.
583. P.Libby, S.Bursztajn, A.Goldberg (1980) Cell 19, 481.
584. P.Morre, H.Bank, N.Brisie, S.Spicer (1976) J. Cell Biol. 76, 659.
585. J.Carron, R.Berlin (1979) J. Cell Biol. 81, 665.
586. P.Mattson, S.Lorenz, R.Benis, J.Kowal (1983) J. Cell Biol. 97 104a.
587. J.Freed, M.Lebowitz (1970) J. Cell Biol. 45, 334.
588. A.Bhisey, J.Freed (1971) Exp. Cell Res. 64, 430.
589. R.Fine, L.Taylor (1976) Exp. Cell Res. 102, 162.
590. N.Neef, G.DeMartino, A.Goldberg (1979) J. Cell PLhysiol. 110, 439.
591. N.Savion, I.Vlodavsky, G.Grennburg, D.Gospodarowicz (1982) J. Cell Physiol. 110, 129.
592. C.Szego, M.Nazarono, D.Porter (1977) J. Cell Biol. 73, 354.
593. D.Cleveland, M.Lopata, P.Sherline, M.Kirschner (1981) Cell 25, 537.
594. J.Folkman, A.Moscona (1978) Nature 273, 345.
595. A.Ben-Ze'ev, A.Raz (1981) Cell 26, 107.
596. A.Ben-Ze'ev (1983) J. Cell Physiol. 114, 145.
597. A.Ben-Ze'ev, S.Farmer, S.Penman (1979) Cell
598. D.Gospodarowicz, G.Grennburg, C.Birdwell (1978) Cancer Res.38, 4155.
599. M-T.Haeuptle, Y.Suard, E.Bogenmann, H.Reggio, L.Racine, J-P.Kaehenbuhl (1983) J. Cell Biol. 96, 1425.
600. P.Nielsen, S.Goelz, H.Traschel (1983) Cell Biol. Inter. Reports 7, 245.
601. R.Lenk, S.Penman (1979) Cell 16, 289.
602. M.Cervera, G.Dreyfuss, S.Penman (1981) Cell 23, 113.
603. N.Sonenberg, H.Traschel (1982) Current Topics in Cell Regulation 21, 65.
604. W.van Venrooij, P.Sillekens, C.Ekelen, R.Reinders (1981) Exp. Cell Res. 135, 79.
605. K.Crossin, D.Carney (1981) Cell 23, 61.
606.P.Coffino,H.Bourne,G.Tomkins(1975) J.CellPhysiol. 85,603.
607. R.Simantov, L.Sachs (1975) J. Biol. Chem. 250, 3236.
608. J.Oliver (1978) Am. J. Pahol. 93, 1978.
609. R.Hynes (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 3170.
610. B.Sefton, T.Hunter, E,Nigg, S.Singer, G.Walter (1982) Cold Spring Harbor Symposium on Quantitative Biology 46, 939.
611. B.Sefton, T.Hunter, F.Beemon, W.Eckhart (1980) Cell 20,807.
612. A.Ben-Ze'ev, M.Horowitz, H.Skolnik, R.Abulafia, O.Laub, Y.Aloni (1981) Virology 111, 475.
613. T.Hunter, J.Cooper (1981) Cell 24, 741.
614. L.King,Jr., G.Carpenter, S.Cohen(1980)Biochemistry 19,

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