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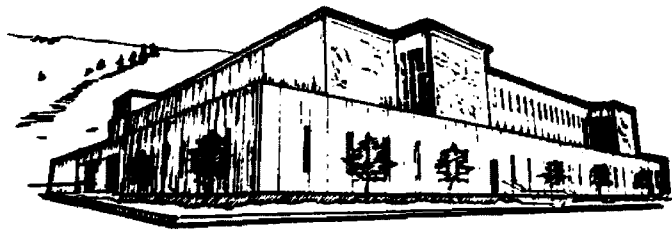
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University of  
**Montana**



**Adrenocorticotropin-Induced Increase in P-450<sub>17 $\alpha$</sub>  Protein  
Levels Corresponding to Stimulation of 17 $\alpha$ -Hydroxylase  
Activity**

by

**Michael L. Chouinard**

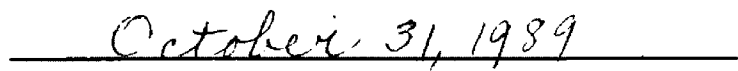
**B. A., The College of Saint Thomas, 1984**

**Presented in partial fulfillment of the requirements  
for the degree of  
Master of Science  
University of Montana  
1988**

**Approved by**

  
Chairman, Board of Examiners

  
Dean, Graduate School

  
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Adrenocorticotropin-Induced Increase in Cytochrome P-450<sub>17 $\alpha$</sub>  Protein Corresponding to Increases In 17 $\alpha$ -Hydroxylase Activity (75 pp.)

Director: Dr. H. Richard Fevold 

Adrenocorticotropin (ACTH) has previously been shown to effect an increase in rabbit adrenal microsomal 17 $\alpha$ -hydroxylase activity. In the present studies adrenal microsomes were prepared from control and 4-day ACTH-stimulated male New Zealand white rabbits and assayed for increases in both immunoreactive cytochrome P-450<sub>17 $\alpha$</sub>  protein (P-450<sub>17 $\alpha$</sub> ) using anti-porcine P-450<sub>17 $\alpha$</sub> -IgG, and 17 $\alpha$ -hydroxylase activity. Both immunoreactive P-450<sub>17 $\alpha$</sub>  content and 17 $\alpha$ -hydroxylase activity increased approximately four fold. Immunoreactive P-450<sub>C21</sub> content detected with anti-bovine P-450<sub>C21</sub>-IgG, showed only a slight 1.3 fold increase in ACTH stimulated rabbits. Inhibition studies showed that anti-P-450<sub>17 $\alpha$</sub>  IgG antibody inhibited 17 $\alpha$ -hydroxylase to less than 20% of the ACTH stimulated activity. A slight inhibition of 17 $\alpha$ -hydroxylation was obtained with anti-bovine P-450<sub>21</sub> IgG and preimmune rabbit IgG did not show any inhibition, thus demonstrating the specificity of the heterologous antibodies. Rabbit P-450<sub>17 $\alpha$</sub>  and P-450<sub>C21</sub> migrate as 52 and 53 kDa proteins respectively, under denaturing gel conditions. Cross-reactivity was also observed between the antibodies and the respective guinea pig proteins. It was concluded that the increase in rabbit adrenal immunoreactive cytochrome P-450<sub>17 $\alpha$</sub> , after ACTH-stimulation, is sufficient to account for the observed increase in enzyme activity and that there is sufficient similarity between porcine, guinea pig and rabbit P-450<sub>17 $\alpha$</sub>  and between bovine, guinea pig and rabbit P-450<sub>C21</sub> for immunological cross reaction.

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## Introduction

### The Adrenal

The adrenal glands are often "kidney bean" shaped structures that lie embedded in fat at the superior poles of each of the two kidneys in higher vertebrates. The anatomy of each adrenal is composed of two distinct regions, an inner reddish-brown core called the medulla and an outer pinkish-yellow colored cortex. The two regions function as separate glands with the medulla as part of the autonomic nervous system.

The secretory cells within the medulla are derived in the embryo from the neural crests of the ectoderm and are analogous to post-ganglionic neurons (1). Preganglionic sympathetic nerve fibers pass, without synapsing, from the intermediolateral horn cells of the spinal cord, through the sympathetic chains, through the splanchnic nerve into the medulla. There they end on the cells that secrete epinephrine (adrenalin) and norepinephrine (noradrenaline) (1,2). The medulla is relatively small compared to the surrounding cortex which in some species constitutes 90% of the total gland (2).

The cortex is derived from the mesodermal glandular tissue and is composed of three zones of cells. The outer region of cortex is called the zona glomerulosa and is the smallest of the three regions. The middle or center layer of

cortical cells is called the zona fasciculata and in humans it represents 75% of the total cortex. The inner-most region of the cortex is the zona reticularis, the region directly surrounding the medulla (2,3).

The function of the adrenal cortex is the production and secretion of steroid hormones, termed corticosteroids. The cortex produces at least 50 different steroids, some of which are biologically active hormones of different classes. The three main classes of steroids found in the adrenal are the glucocorticoids, the mineralocorticoids and the sex hormones (the androgens, estrogens, and the progestins). This later group is produced in extremely small quantities in the adrenal of most species (3).

These hormones can also be grouped by structure as well as function. The C<sub>21</sub>, 11-oxygenated corticosteroids, such as cortisol and corticosterone, produced in the zona fasciculata and reticularis, regulate carbohydrate and protein metabolism and also exhibit antiinflammatory activity. The C<sub>21</sub> steroids that lack oxygen at position C-11 have effects on electrolyte and water metabolism. These include 11-deoxycorticosterone (DOC) and 11-deoxycortisol. Aldosterone is a C<sub>21</sub>-steroid with an aldehyde grouping at position 18 and oxygen at C-11 and it affects electrolyte metabolism as well. The C<sub>19</sub> androgens, C<sub>18</sub> estrogens and C<sub>21</sub> progestins are sex hormones responsible for a variety of anabolic and phenotypic regulatory effects (1,3,4). The

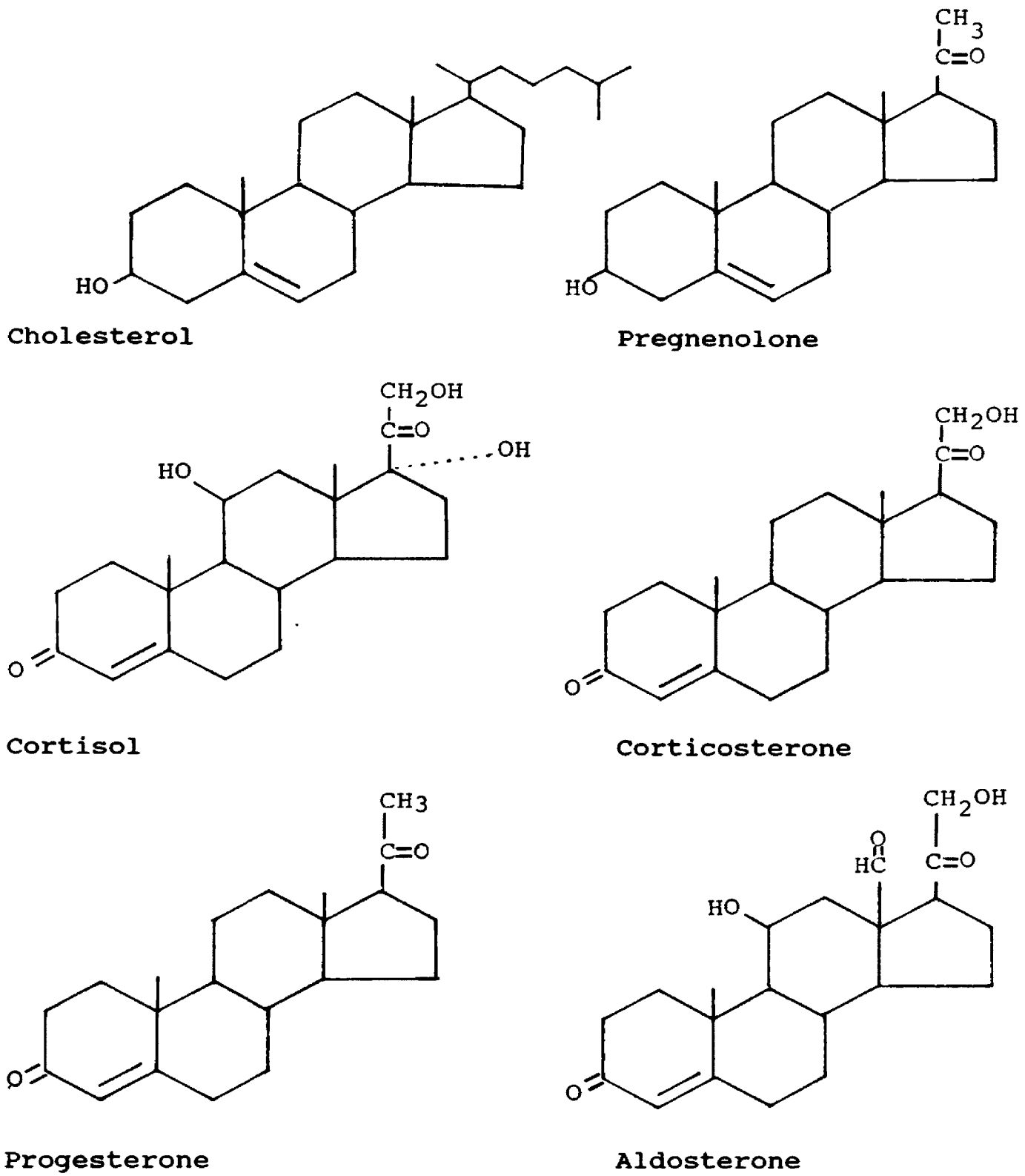
structures of some important steroids are shown in Figure 1.

The precursor of all steroid hormones is cholesterol, most of which is derived from plasma cholesterol esters. Cholesterol esters are supplied to the adrenal cells by circulating, low density lipoproteins (LDL's) and high density lipoproteins (HDL's) which are taken up by a receptor mediated "clathrin coated pit internalization mechanism" (2). Lysozymes degrade internalized LDL's and release free cholesterol which is stored as esters in fat droplets that are in equilibrium with a small pool of free cholesterol. The enzyme cholesterol ester esterase regulates this equilibrium. Only free cholesterol is used as a substrate in hormone synthesis and a small amount of this is synthesized in the adrenal directly from acetate (3).

### Cytochrome P-450

Conversion of cholesterol to pregnenolone is the rate-limiting step (RLS) in adrenal steroid hormone synthesis. This reaction occurs in the adrenal inner mitochondrial membrane and is carried out by the enzyme complex cholesterol desmolase or C-22,27 cytochrome P-450 side chain cleavage enzyme (P-450<sub>SCC</sub>). This enzyme is one of five different cytochrome P-450 mixed function oxygenases involved in the synthesis of corticosteroids (5).

Studies by Ryan and Engle showed that carbon monoxide



**Figure 1. Structures of Some Biologically Important Steroids**

inhibited the C-21 hydroxylation of  $17\alpha$ -hydroxyprogesterone in bovine adrenal microsomes (6,7). Following these findings came independent discoveries by Garfinkle and Klingenberg showing that liver microsomes contain a carbon monoxide binding pigment that, when complexed with CO, gives an absorbance maximum at 450 nm (8,9). The CO inhibition of several other hydroxylations in adrenal, liver and testes indicated the presence of a protein bound heme moiety involved in the oxidation of lipophilic substrates (10,11). Omura and Sato isolated this pigment and named it cytochrome P-450 due to its unique CO-binding spectrum (12,46).

This pigment is composed of two basic parts, a protein monomer ranging in MW from 43,000 Da to 60,000 Da, and a noncovalently bound heme moiety. In addition to hydrophobic and coulombic forces which bind the heme to the protein, the heme iron is attached to the protein through an iron-sulfur bond. The heme iron exists in either a penta- or hexacoordinate valence state with four of these valencies associated with the four pyrrole nitrogens of the heme. The fifth bond involves a sulfur atom from the protein moiety and the sixth ligand is thought to be an -OH from either an amino acid side chain or a water molecule. The active multimeric form of various P-450 enzymes is in most cases unknown although, for cytochrome P-450 C-22,27 side chain cleavage enzyme active forms appear as 850,000, 425,000 and 212,000 Da molecular weight proteins representing the

hexadecamer, octomer and the tetramer, respectively. "The monomeric 52,000 Da form of P-450<sub>SCC</sub> can be isolated only under denaturing conditions" (14).

In the past ten years numerous reaction specific P-450 molecules have been characterized based on enzyme activity, purified and even sequenced. These enzymes have been located in a variety of tissues and specific enzyme activities associated with pigment P-450's are seen in specific cellular locations, such as the endoplasmic reticulum (ER) and the mitochondria. Differences between P-450's appear to have developed through divergent evolution over 1.5 billion years. A common ancestral gene has yielded a P-450 superfamily of 10 gene families. The gene products of these families are enzymes involved in the oxidative metabolism of not only steroids but also fatty acids, prostaglandins, leukotrienes, biogenic amines, pheromones and plant metabolites. These enzymes also metabolize countless drugs, chemical carcinogens, mutagens and other environmental contaminants (13). In the metabolism of this diverse field of substrates the oxidative alteration usually leads to a more hydrophilic subsequent product, therefore chromatography is a useful tool in characterizing substrates and products of P-450 catalyzed reactions.

### The P450 Catalytic Mechanism

The mechanism of the P-450 enzymatic cycle begins with the



binding of a lipophilic substrate (Figure 2). The enzyme substrate complex accepts an electron donated by a reduced pyridine nucleotide. The transport of electrons from NADPH to P-450's located in the mitochondria involves a flavoprotein and a iron-sulfur protein (6,7,14). Microsomal P-450 complexes are not associated with an iron-sulfur protein and require only a single FAD and FMN bound flavoprotein for the transport of electrons from NADPH. Upon reduction of the enzyme-substrate complex, molecular oxygen is bound by the heme iron. A second electron is accepted by an electron carrier which activates the oxygen atoms to separate by an internal electron shift. Water is formed from one oxygen atom and a hydroxyl radical from the other atom. A carbon radical produced in a subsequent electron shift, combines with the hydroxyl radical to form a hydroxylated substrate. Following hydroxylation the more polar product dissociates from the hydrophobic substrate binding site. The cycle is diagrammed in Figure 2. The exact mechanism of oxygen insertion is not completely understood but this interpretation summarized by Hall (1985) is a plausible candidate (14).

### Adrenal Steroidogenic Pathways

Differences between oxidation (hydroxylation) steps lie not only in substrate specificity and modifiable sites but, also in the subcellular location of the enzyme. The  $3\beta$ -

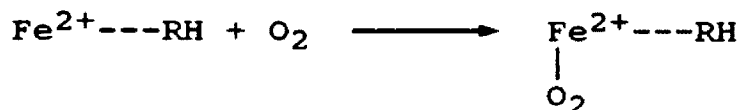
A. Step 1: Formation of the enzyme substrate complex.



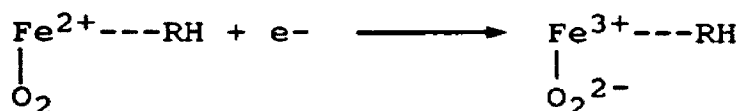
Step 2: Acceptance of the first electron.



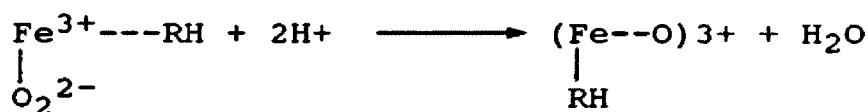
Step 3: Binding of O<sub>2</sub>



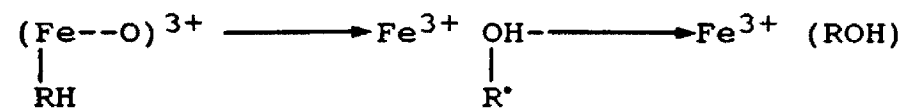
Step 4: Acceptance of the second electron.



Step 5: Oxygen cleavage.



Step 6: Internal shift of electrons.



Step 7: Dissociation of product.

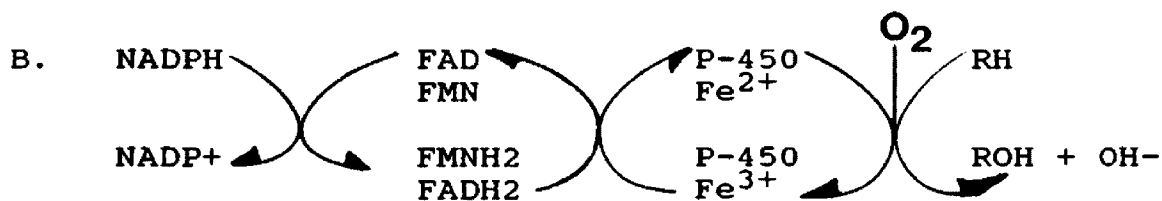


Figure 2. A. The Cytochrome P-450 Reaction Mechanism. RH represents the lipophilic steroid substrate, ROH represents the hydroxylated (oxidized) product. Fe in the two oxidized forms shown, represents the entire P-450 enzyme.

B. The Cytochrome P-450 Electron Transport System in the Endoplasmic Reticulum. In the mitochondria, an iron-sulfur protein is reduced by the flavoprotein. The electrons are then transferred to the P-450 enzyme (14).

hydroxy steroid dehydrogenase ( $3\beta$ -HSD) [E.C. 1.1.1.51] and the 3-oxo-isomerase enzyme [E.C. 5.3.3.1] are found as a complex in the ER (32,34). Together they catalyze the oxidation of the  $3\beta$ -hydroxyl and an isomerization of the <sup>5</sup> double bond of pregnenolone. The product of this reaction is progesterone, a 3 keto, <sup>4</sup> C<sub>21</sub> steroid. The cytochrome P-450  $17\alpha$ -hydroxylase (P-450<sub>17 $\alpha$</sub> ) and 21-hydroxylase (P-450<sub>21</sub>) enzymes are also found in the ER. The mitochondria of adrenocortical cells contain the cytochrome P-450 C-22,27 cholesterol side chain cleavage enzyme (P-450<sub>SCC</sub>) and the  $11\beta$ -hydroxylase (P-450<sub>11 $\beta$</sub> ) (15). The  $3\beta$ -HSD and the isomerase activities can also be found in the mitochondria (15,16). These enzymes catalyze the reactions in the metabolic pathways of adrenocorticoid biosynthesis as summarized in Figure 3. In corticosterone producing species such as rabbits, the normal biosynthetic pathway of adrenocorticoids and sex hormones begins with the conversion of cholesterol to pregnenolone in the mitochondria. Pregnenolone is transported from the mitochondria to the ER where it is converted to progesterone via the  $3\beta$ -HSD and isomerase catalyzed reactions. The arrival of pregnenolone in the ER or the formation of progesterone in the ER marks an important branchpoint in steroidogenesis. The subsequent oxidation of either of these two metabolites represents a committed step in steroid hormone synthesis. Progesterone in the ER of the gonads can become  $17\alpha$ -hydroxylated followed

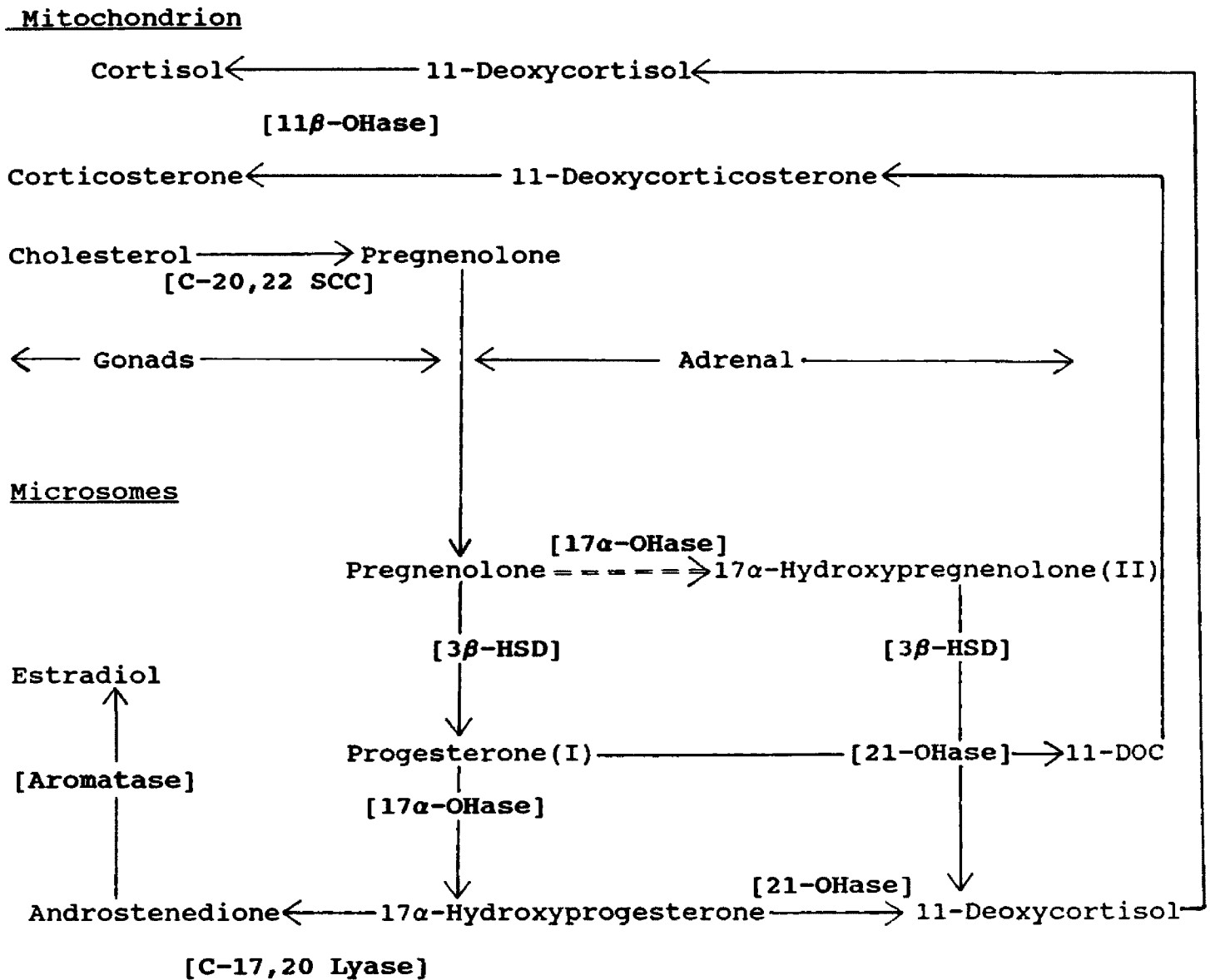


Figure 3. Pathways of Steroid Hormone Biosynthesis Within Steroidogenic Tissues. The pathway indicated by the dashed line is the mechanism of cortisol synthesis in ACTH-stimulated rabbits(II). Normal rabbits synthesize corticosterone through a progesterone intermediate (I). OHase = Hydroxylase, HSD = Hydroxysteroid dehydrogenase, +  $\Delta$ 4,5 Ketoisomerase, SCC = side chain cleavage.

by further oxidation carried out by this same enzyme in a C-20, 21 side chain cleavage reaction, to yield androstenedione. This C-17,20 lyase activity has recently been shown to be associated with the same P-450 molecule that catalyzes  $17\alpha$ -hydroxylation in the gonads as well as the adrenal (16,17). The lyase activity was found to copurify with the hydroxylase activity when isolated from bovine adrenocortical cells but regulatory control of lyase activity relative to hydroxylase activity in specific tissues seems to be membrane dependent (14,16). This C-17,20 lyase reaction represents the committed step in  $C_{19}$  and  $C_{18}$  steroid hormone synthesis and discovery of this dual enzyme activity explains observations of limited androgen secretion by the adrenals of various species (16,17). In the adrenal of corticosterone secreting species the activity of  $17\alpha$ -hydroxylase is low (18-21). Progesterone can be subjected to 21-hydroxylation and then transported to the mitochondria where P-450<sub>11</sub> can generate corticosterone via  $11\beta$ -hydroxylation. The adrenals of cortisol secreting species exhibit  $17\alpha$ -hydroxylation of pregnenolone or progesterone in the ER. This is the committed step in cortisol synthesis and is prior to oxidation by  $3\beta$ -ol-dehydrogenase, <sup>4</sup> keto isomerase and 21 hydroxylase in the ER, and by  $11\beta$ -hydroxylase in the mitochondria (16,18-21).

## Regulation of Steroidogenesis

Inhibition of enzyme activity and induction of enzyme expression are two of the most documented routes in which the activity of an enzyme may be altered. Post transcriptional and post translational modification are other possible regulatory pathways. The regulation of an enzyme often comes under the control of effector molecules. Some regulatory effectors of steroid metabolism are peptide hormones released by the posterior pituitary (adenohypophysis), including adrenocorticotropin (ACTH), follicle-stimulating hormone (FSH), leutenizing hormone (LH), and chorionic gonadotropin (CG) (22,23). These peptides stimulate protein synthesis and steroidogenesis in certain tissues through signal transduction from a membrane receptor (22,50,51). Both FSH and LH stimulate steroidogenesis in the ovary and testes along with a general increase in protein synthesis (2,22,23). ACTH acts specifically on the adrenal cortex and causes an increase in adrenal size and protein content (5,22,23).

### ACTH Regulation of Adrenal Steroidogenesis

Adrenocorticotropin (ACTH) is a polypeptide of 39 amino acid residues with a molecular weight of 4500 Da (2). This peptide is manufactured in the pituitary from a large glycoprotein precursor (MW 31,000 Da) called proopiocortin or proopioadrenocortin (1,2). Its release from the

adenohypophysis is stimulated by corticotropin releasing factor (CRF) which is secreted by the hypothalamus. Adrenocorticotropin is secreted from the adenohypophysis in response to stress. It stimulates production of glucocorticoids in the adrenal which leads to an increased rate of gluconeogenesis and supplies the organism with a source of readily useable fuel. The zona fasciculata appears to be the region most responsive to ACTH (4,24). In species such as rabbits, and some rodents, corticosterone is the glucocorticoid which is normally secreted by the adrenal. In response to ACTH stimulation the adrenal glucocorticoid output of rabbits shifts from corticosterone to cortisol, a more potent glucocorticoid (25).

ACTH stimulation of steroid hormone biosynthesis in all steroidogenic tissues, appears to occur in a biphasic manner (40). The initial event in this stimulation is the ACTH-induced increase in the supply of free cholesterol to the active site of the side chain cleavage enzyme (26). This association as stated previously, is the overall rate limiting step (RLS) in steroid hormone biosynthesis and is sensitive to inhibitors of protein synthesis (23,26,27). Furthermore this association or RLS, involves at least one type of labile protein factor, steroid activating protein (SAP) or sterol carrier proteins (SCP<sub>2</sub>) (26-28). The SAP or SCP<sub>2</sub> proteins show a dose-dependent augmentation of mitochondrial pregnenolone synthesis and they cause

cholesterol to move from the outer to the inner mitochondrial membrane (26). This is a cycloheximide sensitive event. Evidence suggests that this event may involve alterations in the ultrastructure of cells, including a change in microtubule structure (28). The binding of cholesterol to the active site of the C-22,27 SCC enzyme is the major event in phase one of this biphasic ACTH activation of steroidogenesis and effects attributed to this phenomenon are seen within minutes (26-28,49). Four events induced by ACTH contributing to the overall steroidogenic response are summarized below:

- 1.) The amount of cholesterol transferred from the blood to the adrenals is increased by ACTH. This may be attributed to an ACTH-effected increase in the available lipoprotein binding sites located on adrenocortical plasma membranes (1,2).
- 2.) ACTH enhances activity of cholesterol ester hydrolase through a cyclic adenosine monophosphate activated protein kinase (2,23).
- 3.) ACTH facilitates the interaction and binding of cholesterol to the active site of mitochondrial P-450<sub>SCC</sub> involving a labile protein factor (23,26-28).
- 4.) The specific content of P-450<sub>SCC</sub> and protein components of its associated electron transport system are increased by an ACTH/cAMP mediated induction of gene expression (2,22,23). The specific activity of certain steroid



metabolizing enzymes is stimulated (22,23,35).

The first two events are related to the RLS (event # 3) and occur quite rapidly. The fourth event represents the second phase of the biphasic ACTH stimulation of steroidogenesis, which is primarily the synthesis of cytochrome P-450, adrenodoxin and adrenodoxin reductase proteins involved in C-22,27 side chain cleavage activity. This effect is not seen until hours after ACTH stimulation (22,26-28).

#### Transduction of the ACTH Signal

The response elicited by ACTH is relayed from the membrane receptor by the second messenger, cyclic adenosine monophosphate (cAMP). This mechanism involves a guanosine triphosphate (GTP)-dependent membrane bound N-protein which functions as a complex on/off switch for adenylate cyclase. ACTH binds to its membrane receptor to form a hormone receptor complex. The receptor, in the occupied state, undergoes a conformational change that allows the GTP-dependent N-protein to bind. The GTP molecule is the major physiological regulator of adenylate cyclase (2,29). This N protein is a GTP hydrolase that is usually bound to GDP in its inactive state. Binding of the N-protein to the hormone receptor complex, causes GDP to dissociate from the N-protein. As GDP dissociates from the N-protein, GTP binds and activates the N-protein. An activated N-protein serves

to activate adenylate cyclase thereby increasing cAMP production. As GTP is hydrolyzed by the N protein the N protein becomes inactive and causes the hormone-receptor-N complex to dissociate. In the presence of high concentrations of hormone, the cycle continually regenerates active N-protein, keeping adenylate cyclase active. The cAMP in the cytosol often binds to cyclic adenosine monophosphate-dependent protein kinases (cA-PK) (2,29).

These cA-PKs usually exist in two isozymic forms. The holoenzyme is a tetramer consisting of two catalytic and two regulatory subunits. The intact tetramer is the inactive form of the species. Activation of the kinase occurs upon binding of cAMP to two sites on each of the two regulatory subunits of the kinase. This is followed by dissociation of the tetramer into individual catalytic and regulatory isozymic forms. Activated catalytic subunits phosphorylate diverse proteins and activation as well as inhibition of the substrate proteins is effected through phosphorylation. One of the proteins activated through cA-PK dependent phosphorylation is the cholesterol ester hydrolase (23,29). Catalytic subunits of cA-PK have been shown to phosphorylate DNA binding proteins and recent evidence suggests that cAMP may bind directly to a protein on DNA (30). The various aspects of the ACTH-induced steroidogenic response are diagrammed in Figure 4.

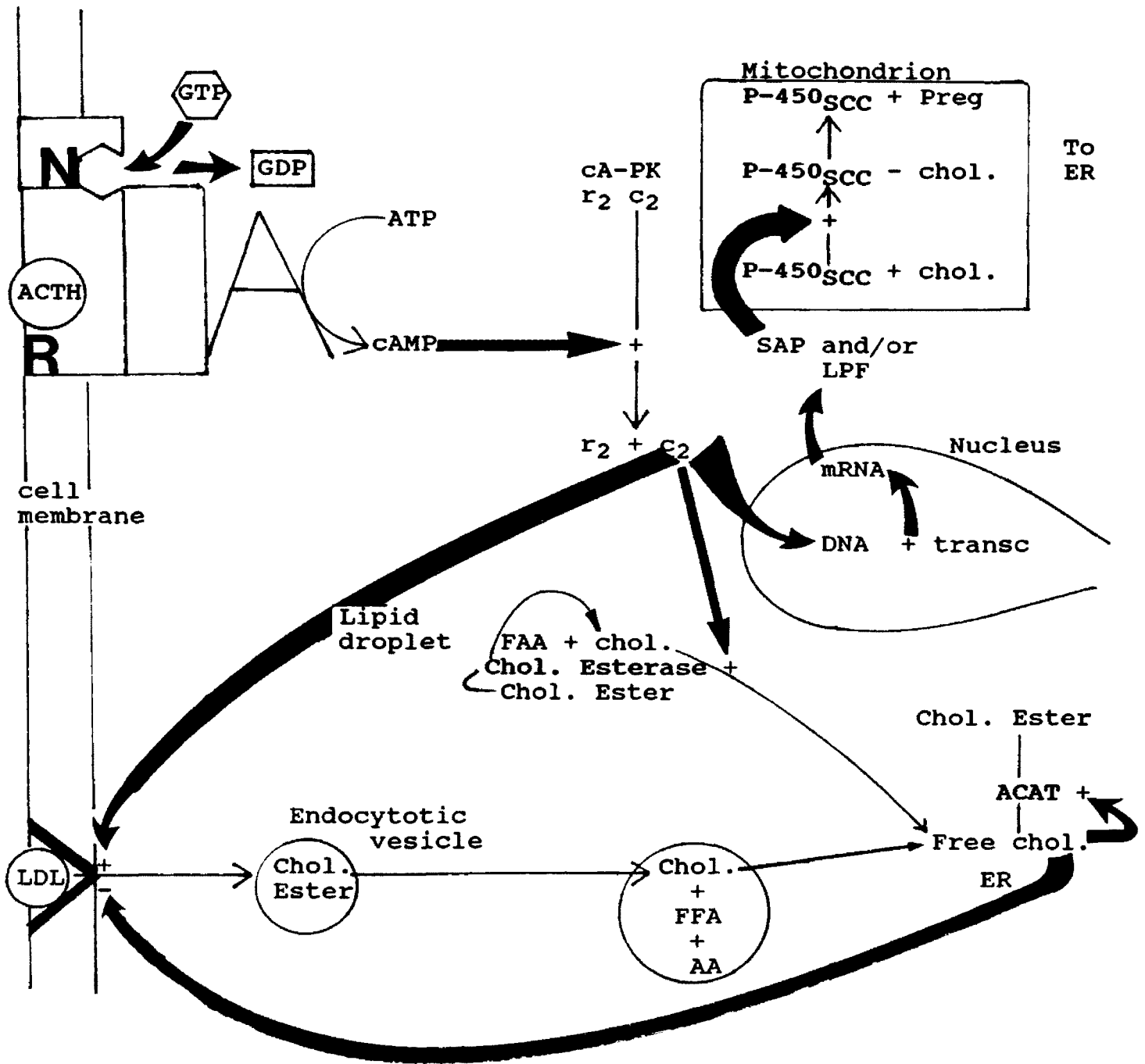


Figure 4. Mechanism of ACTH Action in Overall Steroidogenesis. Bold arrows denote regulatory pathways through inhibition, -, activation, +, or protein synthesis. Abbreviations and symbols: GTP-dependent N protein, N, ACTH receptor, R, adenylate cyclase, AC, cyclic AMP-dependent protein kinase, cA-PK with regulatory, r<sub>2</sub> and catalytic C<sub>2</sub> isozymic forms, steroid activating protein(s), SAP, labile protein factor(s), LPF, cholesterol, chol, free fatty acid, FFA, amino acid, AA, endoplasmic reticulum, ER, low density, lipoprotein, LDL, and acyl CoA: cholesterol acyl transferase, ACAT.

### Specific ACTH Regulated Steroidogenic Sites and P-450<sub>17α</sub>

The effect of ACTH on adrenal steroidogenesis described thus far has dealt only with the rate limiting step in overall steroid biosynthesis. Another important regulatory site affected by ACTH involves the P-450<sub>17α</sub> enzyme in the ER. This branchpoint enzyme is under regulatory control by both ACTH and cAMP in the adrenal (5).

ACTH stimulation of rabbits, a corticosterone producing species, causes shift in the type and amount of glucocorticoid produced by the adrenal (23,25). Studies by Fevold (1967) showed that cortisol was produced with a concomitant decrease in corticosterone production in response to ACTH treatment. Cortisol output reached a maximum level following intra-muscular injections of 50 IU of ACTH per day for 3 days. Measurable increases were obtained after just one day of ACTH treatment, ranging from nondetectable levels to 72 μg of cortisol per 100 μg of tissue (18).

Further studies by Fevold (1968,1978) showed that a specific increase in the 17α-hydroxylation of pregnenolone occurred in adrenal microsomal fractions isolated from ACTH injected rabbits and also in cultures of adrenocortical cells in the presence of ACTH (19,31). It was also shown that microsomal 21-hydroxylase activity is not affected in ACTH treated rabbits (32). Fevold, Wilson and Slanina obtained kinetic parameters for cytochrome P-450<sub>17α</sub> relative

to P-450 3 $\beta$ -HSD, in an attempt to determine an increase in the specific activity of P-450<sub>17 $\alpha$</sub>  or a lack of P-450<sub>17 $\alpha$</sub>  synthesis. Values of  $K_m$  and  $V_{max}$  were lower for 17 $\alpha$ -hydroxylase than those for P-450 3 $\beta$ -HSD, although the specific content of microsomal P-450<sub>17 $\alpha$</sub>  remained stable, as determined by substrate binding difference spectra. A specific increase in 17 $\alpha$ -hydroxylase activity was effected by ACTH with a lack of response observed for 3 $\beta$ -HSD activity (21). The results described above indicate that ACTH preferentially stimulates activity of only the P-450<sub>17 $\alpha$</sub>  microsomal enzyme of all microsomal P-450 enzymes studied in the rabbit (32). This stimulation in rabbits appeared to be brought about by a mechanism that does not involve a change in the amount of enzyme, as determined by substrate binding and CO-difference spectra (19,21). This conclusion may be misleading due to the amounts of various P-450 enzymes and their affinities for the compounds used in substrate binding difference spectra determinations. This observation is based on results from experiments that measured the effects of hypophysectomy on adrenal and testicular microsomal proteins. A decrease in the amounts of specific P-450 molecules was observed in hypophysectomized rats with corresponding decreases in specific P-450 enzyme activities (24,33). Hypophysectomized rats show noticeable changes in gel electrophoretic patterns of adrenal microsomal proteins relative to patterns from control animals. ACTH stimulation

of hypophysectomized animals restores the lost bands to their original intensity (33). These bands correspond to proteins of MW 53,000 Da and 57,000 Da (15). The restoration of these protein bands indicates that ACTH may induce synthesis of specific proteins therefore, increases in specific activity of P-450 proteins may not be detectable by substrate binding spectrophotometry. Peterson and Fevold (1984) attempted to determine changes in gel electrophoretic patterns of adrenal microsomal protein in control and ACTH stimulated rabbits that could be correlated with previously observed increases in P-450<sub>17 $\alpha$</sub>  enzyme activity. Results of these studies indicated definite increases in the amount of a protein band of MW 53,0000 Da in microsomes from ACTH stimulated rabbits, although this pattern of increase did not correspond with increases in 17 $\alpha$ -hydroxylase activity (34).

Bovine adrenals normally secrete large amounts of cortisol. The 17 $\alpha$ -hydroxylation of pregnenolone (or progesterone), the committed step in cortisol biosynthesis, is normally high in bovine adrenals. Recent studies by Zuber et al. (1985) on bovine adrenocortical cells in culture have shown ACTH and cAMP stimulation of 17 $\alpha$ -hydroxylase activity. In attempts to distinguish the mechanism of this increase, various subcellular fractions from these cultures were isolated and electrophoresed in a protein denaturing gel system. The gel containing separated

microsomal protein bands was electrophoretically blotted onto a nitrocellulose support. The nitrocellulose sheet was incubated in a solution containing a rabbit antibody to purified pig cytochrome P-450<sub>17 $\alpha$</sub> . This was followed by incubation in solution containing radio-labeled goat anti-rabbit IgG. Autoradiograms of nitrocellulose sheets revealed intense bands in lanes containing protein from ACTH stimulated cells. Control cell protein showed little or no radioactivity, thereby indicating ACTH induced synthesis of P-450<sub>17 $\alpha$</sub>  as a possible activation mechanism.

The quantity of P-450<sub>17 $\alpha$</sub>  in cultured, control and ACTH stimulated cells was compared with levels from fresh unstimulated gland cortex. The level of P-450<sub>17 $\alpha$</sub>  in cultured cells did not exceed or even meet levels found in normal fresh cortical cells (35). This experiment clearly shows that an increase in 17 $\alpha$ -hydroxylase enzyme activity was effected by ACTH and was obtained through an increase in the specific content of P-450<sub>17 $\alpha$</sub> . Further experiments by Zuber et al. (1986) showed that this activation occurred by induction of P-450<sub>17 $\alpha$</sub>  gene through a cAMP mediated signal (36). However, a problem is encountered in using cultures of bovine adrenocortical cells as controls indicative of the bovine adrenal cortex. This may be more of a general problem encountered when using cell cultures in place of animals. Bovine adrenocortical cells representing controls exhibit about 5% of the 17 $\alpha$ -hydroxylase specific activity

found in fresh, unstimulated cortex. Furthermore, no more than 90% of the normal  $17\alpha$ -hydroxylase activity was restored in cultured cells in the presence of ACTH (Table I,35). These results indicate that bovine adrenal cells in culture, do not function as a control indicative of the normal bovine adrenal  $17\alpha$ -hydroxylase activity. The effects in bovine adrenal cell culture are similar to those seen by Guenther and Nebert with hypophysectomized rats and restoration of aryl hydrocarbon hydroxylase activity as well as 53,000 D molecular weight protein band (24,33). Zuber's results may also indicate an intracellular regulation of the effects of ACTH. This regulation may prevent stimulation of  $17\alpha$ -hydroxylase levels above normal.



### Statement of the Problem

The treatment of most corticosterone producing species with ACTH does not result in cortisol formation. However, ACTH treatment of rabbit, a corticosterone producer, results in cortisol formation with a concomitant decrease in corticosterone production. The increased production of cortisol occurs through ACTH stimulation of  $17\alpha$ -hydroxylase activity. Therefore, it was of interest to determine if the mechanism of the ACTH-induced increase in  $17\alpha$ -hydroxylase activity occurs through an increase in P-450 $_{17\alpha}$  protein as in bovine adrenocortical cells in culture.

The purpose of this research was to determine if ACTH injections of rabbits caused an increase in P-450 $_{17\alpha}$  protein proportional to the previously observed ACTH-induced increase in  $17\alpha$ -hydroxylase activity. This was accomplished by Western blot analysis using a rabbit antibody to pig P-450 $_{17\alpha}$ . Western blot analysis of rabbit adrenal microsomal P-450 $_{17\alpha}$  involved the electrophoretic separation of microsomal protein in sodium dodecyl sulfate polyacrylamide gels followed by the electrophoretic transfer of microsomal protein bands onto a nitrocellulose sheet. The immunospecific P-450 $_{17\alpha}$  bands on the nitrocellulose sheet were detected using the rabbit anti-pig antibody followed by an immunological enzyme-linked or radiolabeled probe. The first objective of this research was to determine if the

rabbit P-450<sub>17 $\alpha$</sub>  protein cross-reacts with the antibody to the pig P-450<sub>17 $\alpha$</sub>  protein. The second objective was to quantify immunospecific bands by densito-metric analysis. Results of Western blot analysis were compared with 17 $\alpha$ -hydroxylase activity. The secondary purpose of this experiment was to determine if ACTH caused an increase in P-450<sub>21</sub> protein. This was also done by Western blot analysis using an antibody to bovine P-450<sub>21</sub> protein.

### Experimental Methods

Male New Zealand White rabbits were injected intramuscularly twice daily (once every 12 hours) for four days. Twelve to twenty rabbits were used per experiment. Half the number of rabbits were injected with 40 IU of porcine ACTH in 0.2 ml of an injection vehicle of 5% beeswax in peanut oil containing 0.5% phenol (18). The rabbits representing the other half of the group were injected with an equal volume of injection vehicle as a control, or in one experiment not injected. After the injection period the rabbits were anesthetized with carbon dioxide and sacrificed by exsanguination (21).

The adrenals were removed from the rabbits and microsomes were prepared by the method of Fevold (19). The glands were trimmed of fat and connective tissue on a 0.9% saline-saturated gauze pad in a Petri dish chilled on ice. The cleaned adrenals were weighed in pairs and pooled into a Petri dish containing 0.9% saline solution and labeled as ACTH or control. The adrenals were kept on ice to prevent loss of enzyme activity. Each group of pooled glands was minced into small pieces with surgical sissors in pH 6.8, 0.1 M sodium phosphate buffer containing 0.25 M sucrose and

0.001 M dithiothreitol. The 10% (w/v) mixture was homogenized with use of a ground glass tissue homogenizer until the homogenate was completely uniform in consistency (19-21).

The homogenate was centrifuged at 9000 X g at 4 c for 20 min to remove cell wall debris and heavy mitochondria. The supernatant was removed and centrifuged for 10 min at 12,000 X g to remove the mitochondria. The resulting supernatant was centrifuged at 17,500 X g for 30 min to remove any remaining mitochondrial and other heavy organelle fragments. The microsomal fraction was sedimented at 105,000 X g (60 min) from the 17,500 X g supernatant. The pellet was resuspended in a volume of pH 6.8, 0.1 M sodium phosphate buffer containing 0.154 M KCl and 0.001 M dithiothreitol. The volume used was one third of the volume used in the homogenization. The suspension was centrifuged for 60 min at 105,000 X g. This final pellet was resuspended (using a ground glass homogenizer in a volume of 0.1 M sodium phosphate buffer pH 7.0 containing 0.25 M sucrose in order to obtain eight adrenal equivalents per milliliter (19,21)). The microsomal preparation was kept chilled throughout the procedure.

A volume of the microsome suspension representing 0.4 of the total number of adrenals was removed from each of the preparations and reserved for electrophoretic immunoblot analysis (39) and for Lowry protein determination (40). The

remaining fraction, 0.6 of the total number of adrenals, was diluted with pre-chilled Krebs-Ringer phosphate buffer pH 7.0 and assayed for hydroxylase activity. The protein suspension was diluted to 0.5 adrenal equivalents per ml. One ml of the diluted suspension was added to each flask to initiate the enzyme assay.

Protein quantity of the microsome preparations was determined by the method of Lowry (40) using bovine serum albumin as a standard and a Gilford spectrophotometer. Readings of samples were taken at 500 nm and 700 nm and the assay was done in duplicate (40).

### Enzyme Assay

The hydroxylase assays were carried out in 25 ml Erlenmeyer flasks in a total volume of 2.0 ml. The flasks were incubated for 0 and 4 minutes in a shaking water bath at 37.5 c. The substrate for the reaction was [4,7-<sup>3</sup>H]-3 $\beta$ -hydroxy-5-pregnen-3-one (<sup>3</sup>H-pregnenolone), obtained from the Amersham corporation (specific activity 10 Ci/mmol). The 3 $\beta$ -hydroxysteroid dehydrogenase inhibitor 2 $\alpha$ -cyano-4,4,trimethylandroster-5-en-ol-3-one (cyanoketone) was present to prevent conversion of the substrate to progesterone derivatives. The cofactor NADPH was also present along with a NADPH-generating system to prevent a loss of enzyme activity due to lack of the cofactor. Final concentrations of these additions were 1.0 mM NADPH, 0.1 mM cyanoketone,

4.0 mg glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase (3).

Substrate purity was checked by thin layer chromatography before addition to the assay flasks. The original [ $^3\text{H}$ ]-pregnenolone solution was diluted to 25 ml in benzene-methanol (9:1). The amount of this solution used was sufficient to allow for 5  $\mu\text{Ci}$  (0.5 nmol) per assay flask. The benzene:methanol mixture was evaporated under a stream of nitrogen and the  $^3\text{H}$ -pregnenolone was redissolved in ethanol:propylene glycol (1:1, v/v) to produce a concentration of 2.5 nmol/ml, (25  $\mu\text{Ci}/\text{ml}$ ). The addition of 0.2 ml of substrate solution to each assay flask was followed by the addition of 50  $\mu\text{l}$  of a 40 mM cyanoketone inhibitor in an identical solvent mixture. The ethanol component of these solutions was evaporated under nitrogen before other assay ingredients were added (3).

Antibodies graciously provided by Drs. Peter Hall (P-450 $_{17\alpha}$ : antibody 1), Anita Payne (P-450 $_{17\alpha}$ : antibody 2) and Michael Waterman (P-450 $_{21}$ : antibody 3) were included in some assays in attempts to show specific inhibition of the cytochrome P-450 17 $\alpha$ -hydroxylase enzyme. Samples containing 50  $\mu\text{g}$ , 100  $\mu\text{g}$  and 200  $\mu\text{g}$  of rabbit anti-pig P-450 $_{17\alpha}$ , anti-bovine P-450 $_{21}$  or normal rabbit IgG were assayed. Experiments were performed with either fresh or frozen microsomes. There was a one hour pre-incubation between

microsomal protein and antibody prior to the start of the assays in one of the experiments. The hydroxylase assays were initiated by the addition of 1.0 ml of diluted microsomal suspension equivalent to 160 mg to 300 mg of protein. The flasks were incubated in a shaker-bath at 37.5 °C for 0-4 min. The reactions were terminated by the addition of 5 ml of cold dichloromethane, mixing, and placing the stoppered flasks in a -20 °C freezer until they were analyzed for product formation (34).

#### Product Isolation and Quantitation

The contents of each assay flask were thawed and diluted with 3.0 ml of glass redistilled (GRD) water and extracted twice with double volumes of cold dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), by the method of Fevold (34). The two volumes of  $\text{CH}_2\text{Cl}_2$  were combined and evaporated under nitrogen. The residue was concentrated in the tip of a citric acid tube using ethanol washes followed by evaporation under  $\text{N}_2$ . The extract was redissolved in 1.0 ml of ethanol and two 50  $\mu\text{l}$  aliquots were removed for the determination of the percent of total radioactivity recovered in the extraction process. Ten  $\mu\text{g}$  each of  $17\alpha$ -hydroxyprogesterone, progesterone, deoxycorticosterone (DOC), and 11-deoxycortisol were added to the remaining extract as internal chromatography standards, and the dried sample plus the standards were redissolved in 100  $\mu\text{l}$  of ethanol.

A 50  $\mu$ l sample of each extract was applied to odd-numbered lanes of Whatman LK5DF silica gel TLC plates (5 X 20 cm X 250  $\mu$ ). Even numbered lanes were used for non-labeled chromatography standards, those added to the samples plus pregnenolone and hydroxypregnenolone. The plates were developed in a solvent system of benzene-methanol (98:2, v/v) for one hour followed by an additional 90 min, with a 15 min drying period between developments (19,21). The developed plates were scanned on a radiochromatogram scanner and the areas corresponding to 17 $\alpha$ -hydroxy-[<sup>3</sup>H]-pregnenolone were scraped into vials and quantified by liquid scintillation spectrophotometry using Beckman Ready-Solv as the counting fluid. The regions corresponding to <sup>3</sup>H-DOC and <sup>3</sup>H-pregnenolone were also scraped and quantified. The percent of substrate converted into 17 $\alpha$ -hydroxypregnenolone was calculated for each of the assay flasks.

The peaks corresponding to DOC and 17 $\alpha$ -hydroxypregnenolone were further identified by paper chromatography of the remaining 50  $\mu$ l of extract.\* The chromatograms were developed in a heptane-formamide solvent system for one hour after the solvent front reached the end of the strip. This was followed by a drying period and development in benzene-formamide until the solvent front was

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\* This work was done by Tonya Holonko and Rich Cronn.

2 cm from the bottom of the strip. The paper strips were allowed to dry and the ultraviolet absorbing areas corresponding to standards were marked. The chromatograms were scanned for [ $^3\text{H}$ ]-labeled steroid (20,21).

The areas corresponding to  $^3\text{H}$ -DOC and  $17\alpha$ -hydroxy- $^3\text{H}$ -progesterone were cut from the strips and eluted with 15ml of ethanol. The solvent was evaporated and the extract was concentrated as before. The residue was acetylated and analyzed by thin layer chromatography (21).

#### SDS-PAGE and Western Blotting of Microsomal Protein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (41). A separating gel with the dimensions 130 mm X 150 mm X 1.5 mm was cast using 30 ml of a solution containing 10% acrylamide-bis monomer, 0.375 M trihydroxymethylaminomethane hydrochloride (tris-HCl), pH 8.8, 2 mM ethylene-diaminetetraacetic acid (EDTA), 0.025% (v/v) N,N,N',N'-tetramethylenediamine (TEMED), and 0.1% ammonium persulfate. A stacking gel mixture containing 5% acrylamide-bis monomer, 0.125 M tris-HCl pH 6.8, 2 mM EDTA 0.5% (v/v) TEMED, and 0.1% (w/v) ammonium persulfate was mixed thoroughly and pipeted between the glass plates on top of the separating gel. A 20 well comb was set in the stacking gel to allow

for sample application. After polymerization occurred the wells were washed with electrophoresis buffer and the gel sandwich was placed in the electrophoresis apparatus.

Electrophoresis buffer pH 8.3, 25 mM tris, 192 mM glycine, 0.1% SDS was added to the upper and lower buffer chambers.

Microsomal resuspension was mixed with sample buffer pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue tracking dye, to obtain a 1 mg/ml sample solution. Sample amounts that were based on volume were mixed with sample buffer in a ratio of 1:3, boiled for 10 min and vortexed for 2 min to denature the protein. Twenty microliters of each sample was applied to a sample well using 20  $\mu$ l disposable pipets. Duplicate and sometimes triplicate samples of ACTH and control microsomes were run on a single gel. Samples were applied to individual wells underneath the level of electrophoresis buffer. The samples were electrophoresed at 30 mA until bromophenol blue tracking dye had left the gel (34).

### Western Blotting

Following electrophoresis, the gel plates were separated and the gel was placed in degassed blotting buffer pH 8.3, 20 mM tris, 150 mM glycine in 20% (v/v) methanol (4). The Genie blotting apparatus (Idea Scientific) was prepared by filling the blotting tray with buffer, placing the plate-cathode in the bottom of the tray followed by a scotch-brite

pad, three pieces of Whatman 3 MM chromatography paper and the gel. A pre-wetted nitrocellulose membrane, cut to the dimensions of the gel, was placed on the gel taking care not to trap air bubbles between the gel and the membrane. Three pieces of 3 MM chromatography paper were placed on top of the nitrocellulose followed by a scotch-brite pad and the graphite plate-anode. The blotting tray was fitted into the blotting chamber and the gel was electrophoresed at 12 volts for 6 hours (42).

### ImmunoAssay

After the blotting was completed, the blotting sandwich was disassembled from the cathode (gel) side, the gel kept in contact with the nitrocellulose so that lanes could be located. This allowed the membrane to be cut into sections so that multiple immuno-assays could be performed. Sections of the membrane were immersed in 20 mM tris buffered saline (1 M NaCl), pH 7.5, 0.05% Tween-20 containing 0.1% sodium azide as a bacteriostat (TBS). The immersed membranes were put on a shaker for 30 min to insure that reactive sites on the nitrocellulose were blocked by the Tween-20 detergent (43). The membrane was washed in TBS six times for 5 min following the blocking step. The membrane was then incubated in a solution containing 1-2  $\mu\text{g/ml}$  of rabbit anti-pig P-450<sub>17 $\alpha$</sub>  or anti-bovine P-450<sub>21</sub> IgG in TBS for at least 16 hours with constant shaking. Antibody 2 was used for all

P-450<sub>17α</sub> assays unless otherwise stated. Antibody 3 was used for all P-450<sub>21</sub> assays. This first antibody incubation was followed by six wash steps and a second antibody incubation in a solution of goat anti-rabbit IgG coupled to the alkaline phosphatase enzyme. The concentration of goat anti-rabbit IgG was unknown but represented a 1:3000 dilution of the commercially produced BioRad titer. The 1 hour second antibody incubation period was followed by three 5 min washes in TBS containing Tween-20 and one wash in TBS without Tween-20. The membrane was immediately placed in a color development solution pH 9.8, 0.1 M NaHCO<sub>3</sub>, 1.0 mM MgCl<sub>2</sub>, 150 μg/ml 5-bromo-4-chloroindoxyl phosphate, and 300 μg/ml of nitro blue tetrazolium until bands developed to a suitable intensity. The nitrocellulose sections were immersed in GRD-water to stop the color development (10). Marker proteins were prepared in sample solubilizing buffer in 5 μg/μl solutions. The marker proteins bovine serum albumin, catalase, glutamate dehydrogenase, and ovalbumin were electrophoresed on lanes outside of microsomal sample lanes. The markers were revealed by total protein stain or by conjugation with fluorescein isothiocyanate (2 μg/μg of protein (45)).

Blots were prepared in a similar way with [<sup>125</sup>I]-protein A (IPA) as the detecting probe. The second antibody solution contained 2-5 X 10<sup>5</sup> CPM/ml of IPA (44). The incubation period was 2 hours with constant shaking.

Autoradiograms of the blots were prepared by placing the dried blots, wrapped in plastic wrap, on Kodak XAR-5 X-ray film. The film was exposed for three days in a X-ray cassette (which contained par speed intensifying screens) at -70 c. The X-ray film was developed with Picker International chemicals and photographs of autoradiograms and blots were taken using Kodak Panatomic-X 35 mm black and white print film.

Scanning of autoradiograms and blots was done using a Beckman CDS-100 Computing Densitometer. Areas under the peaks were obtained from the densitometer integrator.

Blots were also stained for total protein with Janssen Aurodye or Pelikan brand India ink. The staining procedure was initiated by blocking unoccupied binding sites of the nitocellulose immersing the blot in TBS with 0.3% Tween-20 at 37 c for 30 min with constant shaking. Following the blocking step the blots were washed 3 times for 15 min in TBS containing Tween-20. The blots were removed from the last wash solution and placed in undiluted Aurodye or in TBS Tween-20 containing 0.1% India ink and stained for 14-16 hours. Staining of the blots was followed by two 5 min washes in TBS with 0.1% tween. The blots were air-dried and stored between sheets of Whatman 3 MM chromatography paper (43).

## Results

### Microsomal Protein Recovery

The effects of intramuscular ACTH injections on rabbit adrenal size and microsomal protein yield are shown in Table 1. Adrenals obtained from ACTH-stimulated rabbits were an average 15% heavier than glands obtained from control rabbits. The total microsomal protein yields from ACTH-stimulated rabbits, in each microsome preparation, were greater than yields from controls (Table 1, column 5). The data in Table 1 for experiments 2 and 3, shows that protein yields were 23 and 27% greater respectively, relative to control rabbit protein yields. In experiment 1, only a 4% increase in protein yield was obtained, despite a significant difference in average animal weight favoring control rabbits. Experiments 2 and 3 also showed an increase in microsomal protein yield from ACTH-stimulated rabbits per gram of adrenal weight with 13 and 22% increases respectively (Table 1, column 6). The weight percent of microsomal protein to total adrenal weight remained relatively unchanged between ACTH-stimulated animals and controls. The microsomal protein fraction represented an average 0.53% of the total adrenal weight.

### Immunoblot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

**Table 1**  
**ACTH-Stimulated and Control Rabbit Adrenal Weights**  
**and Microsomal Protein Yields**

Exp. No.	Number of Adrenals	Ave. Adrenal Weight (mg)		Total Microsomal Protein (mg)		Microsomal Protein (mg/gram of gland weight)		Ave. Weight of Rabbit (g)	
		ACTH	Control	ACTH	Control	ACTH	Control	ACTH	Control
1	12	88.6	73.6	5.8	5.6	5.4	6.3	2419	2581
2	20	98.1	84.0	10.9	8.4	6.1	5.3	2368	2286
3	20	80.9	69.8	8.5	6.2	5.7	4.4	2533	2481

(SDS-PAGE) followed by Western blotting of 5  $\mu$ g of adrenal microsomal protein from ACTH-stimulated and control rabbits revealed approximately 40 electrophoretic bands as detected by Aurodye stain shown in Figure 5. The major band migrating as a 53 kDa protein appeared to be present in equal concentrations in both ACTH-stimulated rabbit and control rabbit microsome preparations on a per milligram basis. Figure 6 shows nitrocellulose sections containing 0.04 adrenal equivalents (18 to 24  $\mu$ g) of electrophoretically separated microsomal protein from ACTH-stimulated rabbits and control rabbits which were treated with rabbit anti-pig P-450<sub>17 $\alpha$</sub>  IgG followed by goat anti-rabbit IgG, conjugated to alkaline phosphatase. These immunoblots revealed monospecific bands, the most intense of which amounted from ACTH-stimulated rabbit microsomes. Figure 6 also shows a total protein pattern as detected by India ink stain of blotted protein from respective sources. The immunospecific bands appear to migrate at the leading (lower) edge of the major microsomal protein band, approximately 51 kDa. Immunoblot analysis of 20  $\mu$ g of microsomal protein from each tissue is shown in Figure 7. Quantitation of band intensity from these blots is shown in Table 2. Densitometric scanning of alkaline phosphatase immunoblots showed that microsome fractions from ACTH-stimulated rabbits contained 6 to 9 times the amount of immunoreactive P-450<sub>17 $\alpha$</sub>  per adrenal equivalent (Table 2 A.). An



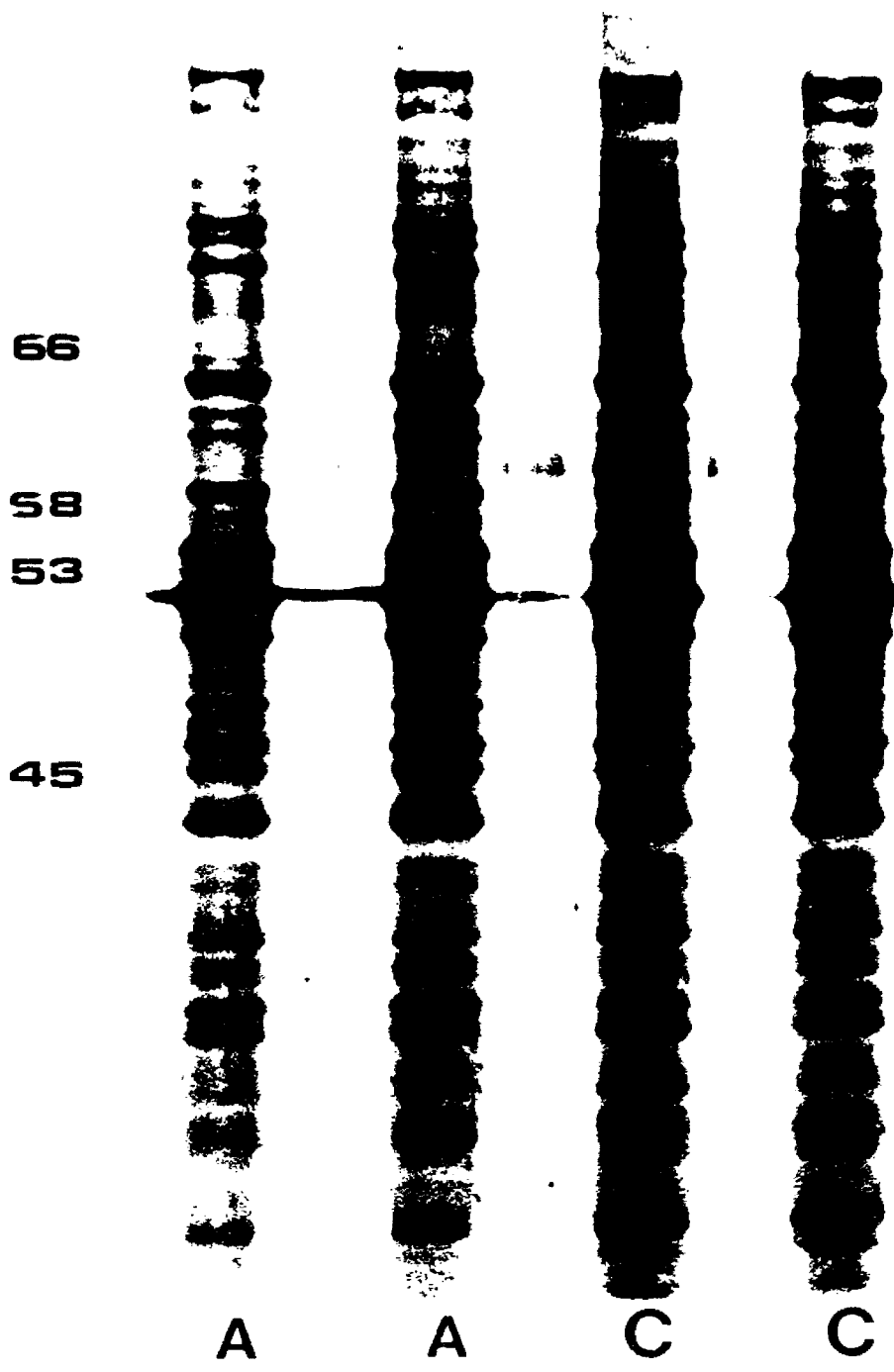


Figure 5. Aurodye-Stained Western Blot. Lanes contain 5  $\mu$ g of adrenal microsomal protein from ACTH-stimulated rabbits (A) or control rabbits (C). The migration of molecular weight markers is indicated on the left.

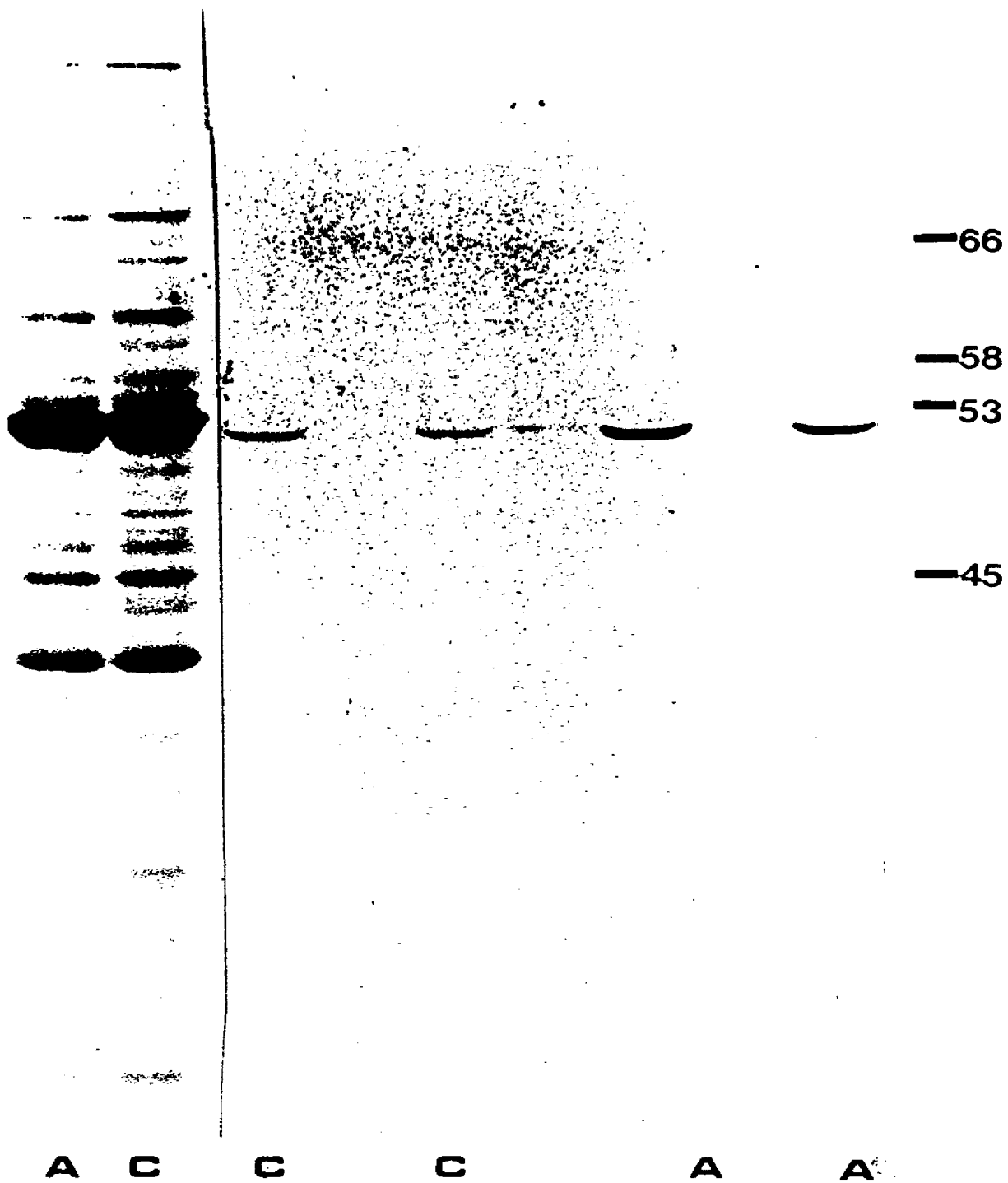


Figure 6. Enzyme Linked Immunoblot Assay of P-450<sub>17α</sub>. Lanes contain 0.04 adrenal equivalents (18-24 μg of protein) from ACTH-stimulated, (A) or control rabbits (C). The migration of total protein (TP) was detected by India ink stain. Markers are shown on the right.

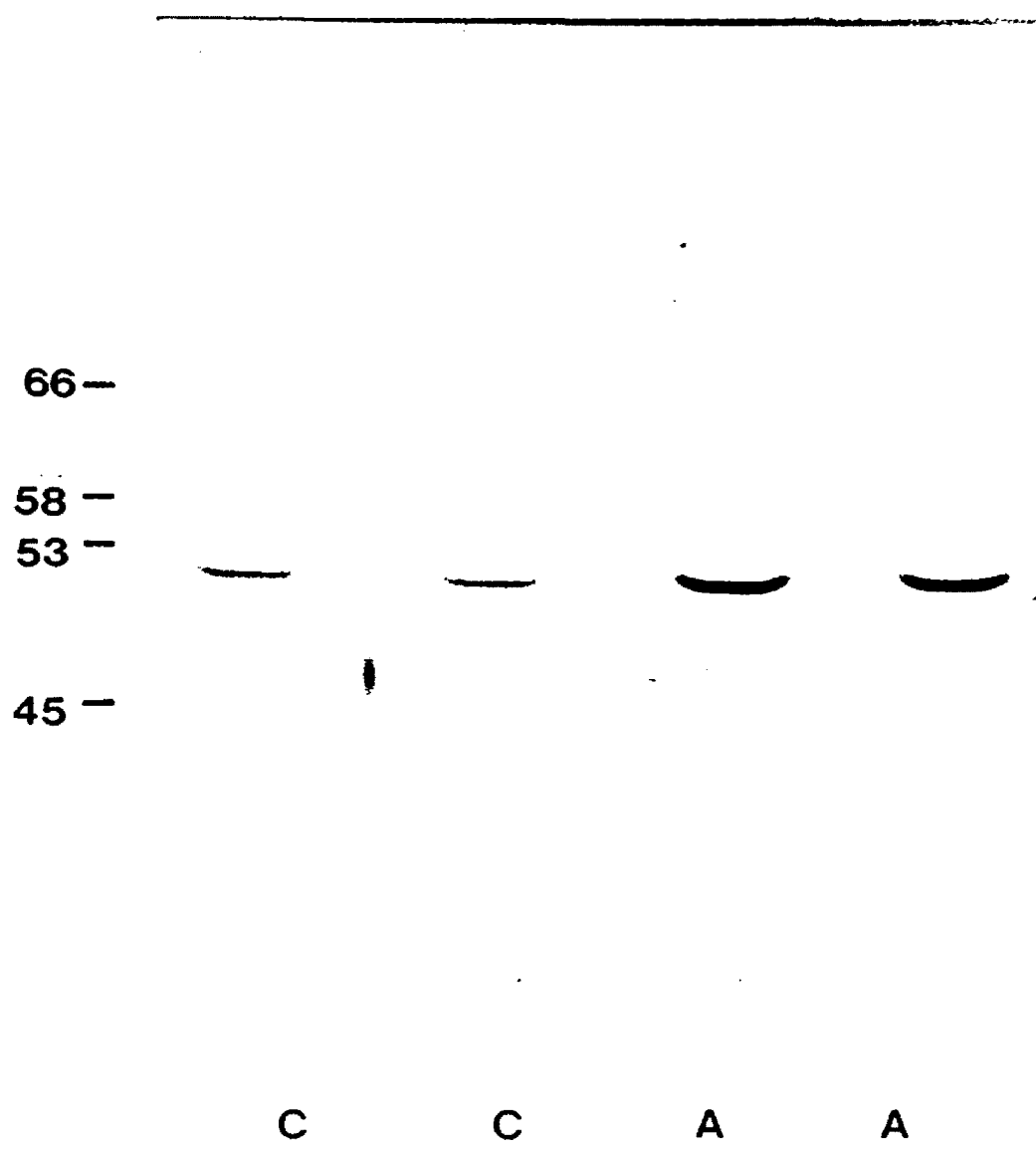


Figure 7. Enzyme Linked Immunoblot Assay of P-450<sub>17α</sub>. Lanes contain 20 μg of adrenal microsomal protein from ACTH-stimulated, (A) and control, (C) rabbits. Molecular weight markers are shown to the left (MW).

Table 2

Densitometric Quantitation of P-450<sub>17 $\alpha$</sub>  Electrophoretic Bands  
from Enzyme Linked Immunoblot Assay

A. Per Adrenal Equivalent

Microsome Suspension #	Intensity/Units ACTH	of Area Control	Ratio of ACTH/Control
I.	370	40	9.2
II.	382	62	6.2
II.	532	64	8.3
Average ratio			8.2 $\pm$ 1.4

B. Per Milligram of Protein

I.	522	122	4.3
I.	248	54	4.6
I.	134	35	3.8
II.	238	84	2.8
II.	580	195	3.0
Average ratio			3.6 $\pm$ 0.9

average 8.2 fold increase in immunospecific P-450<sub>17 $\alpha$</sub>  was observed in microsomes from ACTH-stimulated rabbits. Alkaline phosphatase immunoblot analysis on a per milligram basis showed an 3 to 4 fold increase in P-450<sub>17 $\alpha$</sub>  protein content from ACTH-stimulated rabbits. The average ACTH-stimulated increase in P-450<sub>17 $\alpha$</sub>  was found to be 3.6 fold over controls. The values in Table 2 appear to be indicating a doubling of total adrenal microsomal protein synthesis but actually reflect a large deviation involved in the measurements of enzyme linked immunoblot stains by transmittance densitometry.

In another type of immunoblot analysis <sup>125</sup>I-Protein A was used as the detecting probe. Figure 8 shows an autoradiogram from a <sup>125</sup>I-Protein A immunoblot using anti-P-450<sub>17 $\alpha$</sub>  IgG as the specific antibody. This figure shows relative band intensity on an adrenal equivalent and per milligram of protein basis and offers a side by side comparison of ACTH-stimulated specific and total increases in protein. Table 3 shows densitometric quantitation of band intensity from autoradiograms of radio-immunoblots. Densitometric analysis of autoradiograms led to more consistently reproducible results as opposed to scanning of enzyme linked immunoblots (see Discussion).

Quantitation of P-450<sub>17 $\alpha$</sub>  on a per adrenal equivalent basis showed an average ACTH-effected increase of 6.6 fold over control microsome content. A 4.1 fold ACTH-effected total

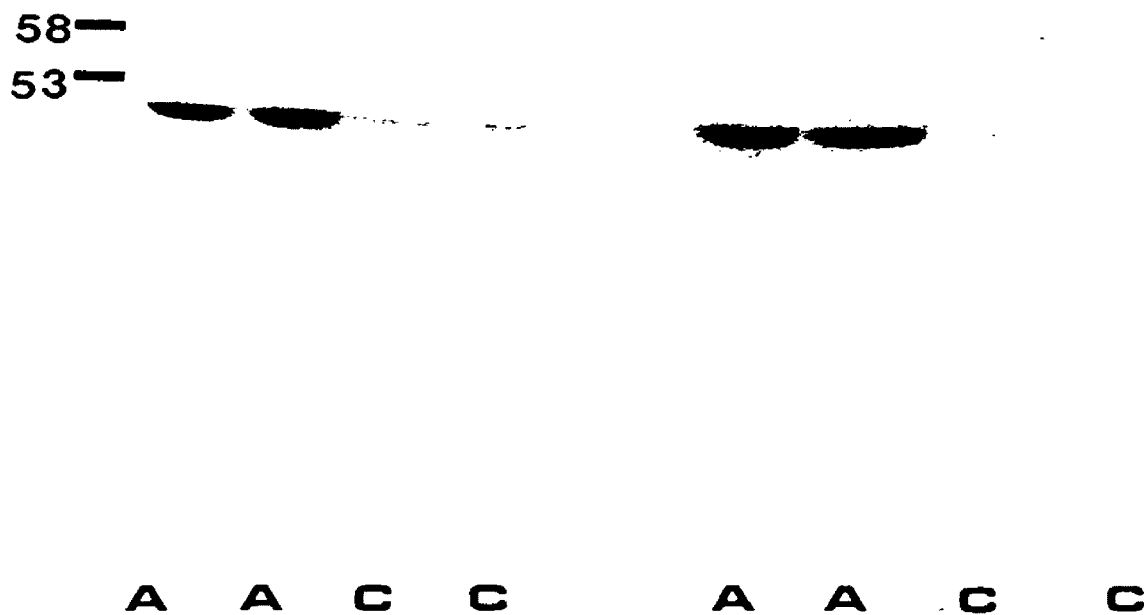


Figure 8. Autoradiogram from a Radio-Immunoblot Assay of P-450<sub>17 $\alpha$</sub> . Lanes on the left contain 20  $\mu$ g of protein from ACTH-stimulated (A) or control (C) rabbits. The four lanes on the right contain 0.04 adrenal equivalents (18-24  $\mu$ g of protein) from each source.

Table 3

Densitometric Quantitation of P-450<sub>17 $\alpha$</sub>  Electrophoretic Bands from Radio-Immunoblot Assay

A. Per Adrenal Equivalent

Microsome Suspension #	Intensity/Units ACTH	of Area Control	Ratio of ACTH/Control
I.	334	50	6.7
I.	330	56	5.9
Average ratio			6.3 $\pm$ 0.6
II.	327	45	7.3
II.	333	50	6.7
Average ratio			7.0 $\pm$ 0.4
Total average ratio			6.6 $\pm$ 0.5

B. Per Milligram of Protein

I.	316	96	3.3
I.	358	83	4.3
I.	373	92	4.1
Average ratio			3.9 $\pm$ 0.6
II.	313	70	4.5
II.	358	80	4.5
II.	373	74	5.0
Average ratio			4.7 $\pm$ 0.2
Total average ratio			4.3 $\pm$ 0.4

increase of  $17\alpha$ -hydroxylase activity (see Table 6) can be correlated to a 6.6 fold increase in P-450 $_{17\alpha}$  protein, with differences in these values attributed to losses in enzyme activity. Results of  $^{125}\text{I}$ -Protein A immunoblot analysis of P-450 $_{17\alpha}$  on a per milligram basis showed an ACTH-stimulated 4.1 fold increase. This increase in protein correlates with the specific increase in enzyme activity shown in Table 6.

Immunospecific detection and quantitation of microsomal P-450 $_{21}$  was accomplished with rabbit anti-bovine antibody. Analysis of P-450 $_{21}$  was performed in order to measure the effects of ACTH on a prominent microsomal P-450 species relative to P-450 $_{17\alpha}$ . Figure 9 shows the large ACTH-effected increase in P-450 $_{17\alpha}$  (Fig. 9, left) adjacent to the slight increase P-450 $_{21}$  (Fig. 9, right). Table 4 shows densitometric quantitation of P-450 $_{21}$  from ACTH-injected rabbits and control rabbits. An average 1.3 fold increase in ACTH/Control ratio of P-450 $_{21}$  per unit weight of protein, is effected by ACTH.

A modification of enzyme linked immunoblot analysis is shown in Figure 10. Blots of adrenal microsomal protein were assayed for P-450 $_{17\alpha}$  and photographed immediately after alkaline phosphatase color development. The P-450 $_{17\alpha}$ -stained blots were then assayed for P-450 $_{21}$  and photographed. This method, referred to as a enzyme linked immunoblot (dual) assay, allows a more precise evaluation of the relative positions of the two P-450 proteins. Figure 10



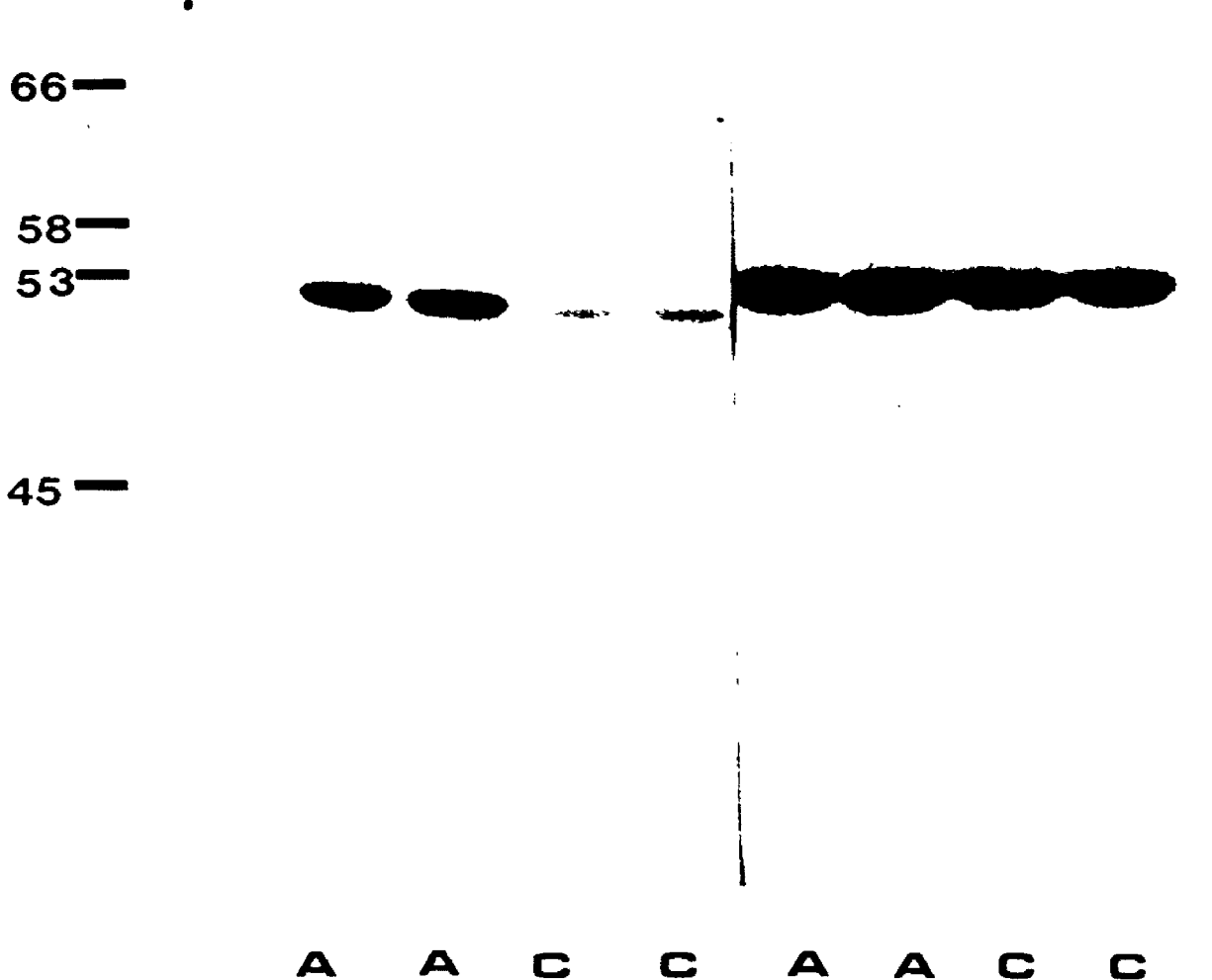


Figure 9. Autoradiogram from a Radio-Immunoblot Assay of P-450<sub>17α</sub> and P-450<sub>21</sub>. The four lanes on the left show immunoreactive P-450<sub>17α</sub> from 25 μg of microsomal protein from stimulated (A) and control (C) rabbits. Lanes on the right show immunoreactive P-450<sub>21</sub> from each source. Molecular weight markers are shown to the far left.

**Table 4**

**Densitometric Quantitation of P-450<sub>21</sub> Electrophoretic Bands  
from Radio-Immunoblot Assay**

**A. Per Adrenal Equivalent**

<b>Blot Number</b>	<b>Intensity/Units ACTH</b>	<b>of Area Control</b>	<b>Ratio of ACTH/Control</b>
1.	316	218	1.4
2.	441	355	1.2
3.	287	240	1.2
4.	435	365	1.2
<b>Average ratio</b>			<b>1.3 ± 0.1</b>

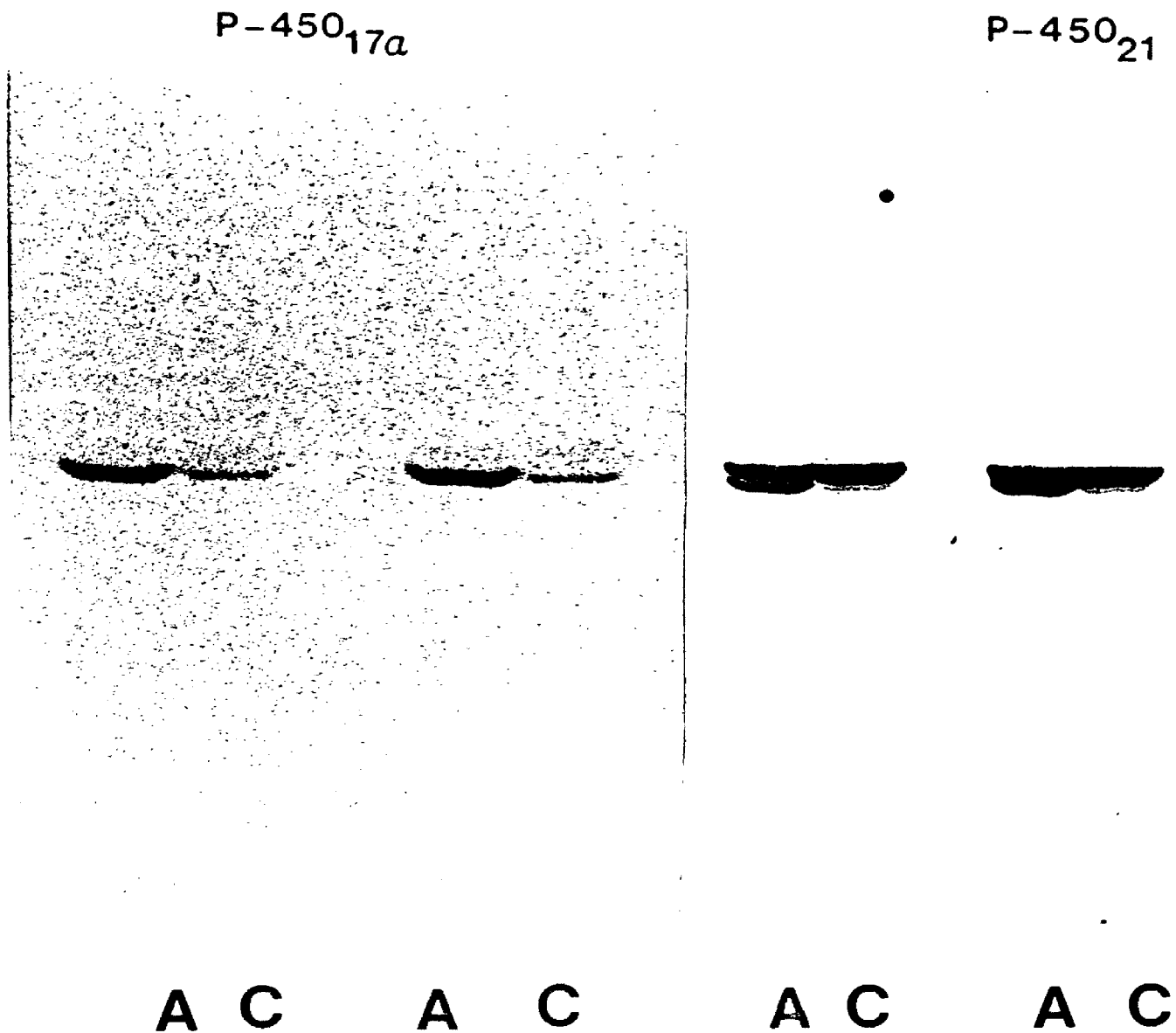


Figure 10. Enzyme Linked Immunoblot (Dual) Assay of P-450<sub>17α</sub> and P-450<sub>21</sub>. This figure shows two photographs of the same blot. The blot was first treated with anti-P-450<sub>17α</sub>, assayed with probe and photographed (left). Following the photography of P-450<sub>17α</sub> immunospecific bands, the blot was assayed for P-450<sub>21</sub> and photographed (right). Lanes contain 20 μg of microsomal protein from stimulated (A), or Control (C) rabbits.

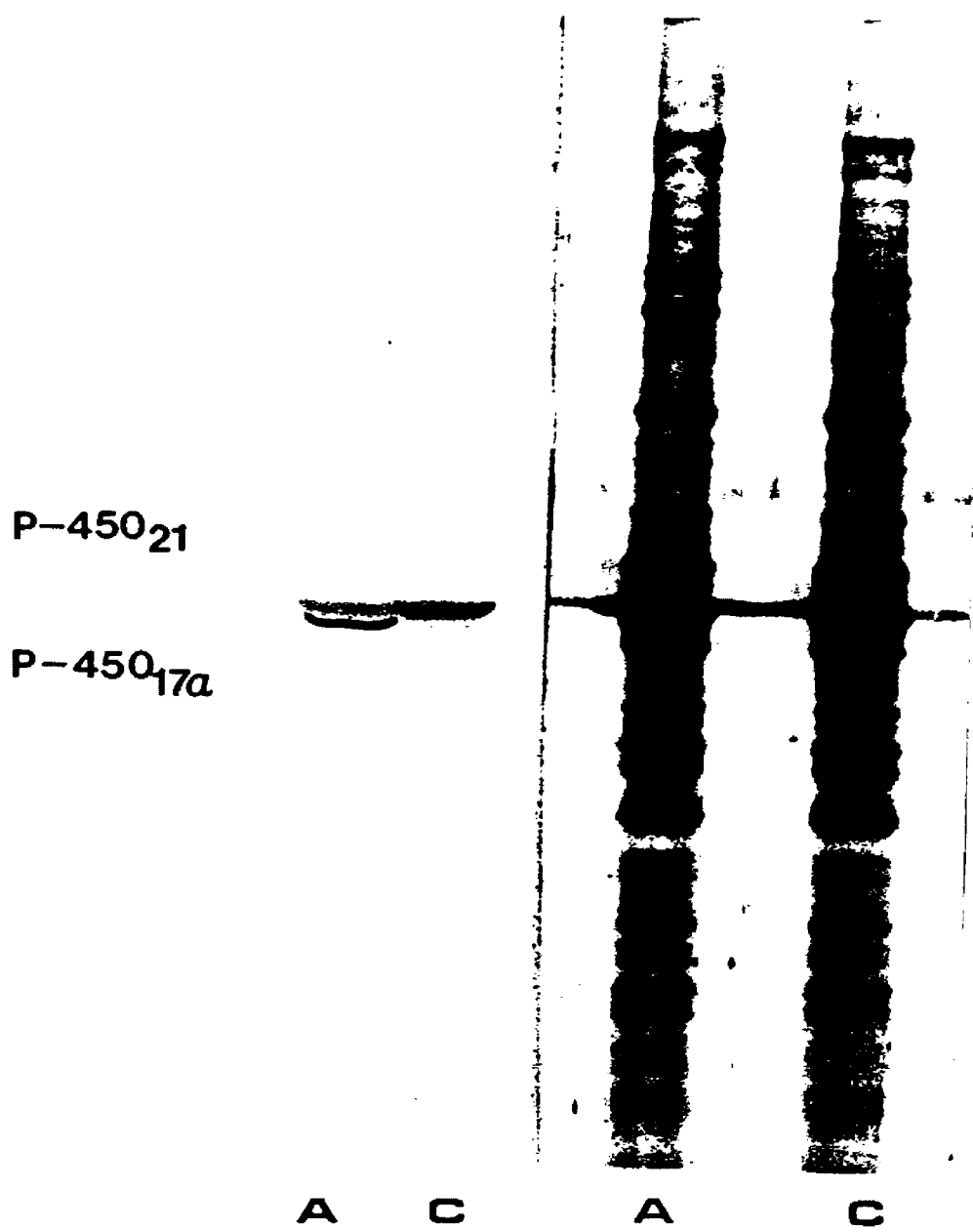


Figure 11. Enzyme Linked Immunoblot (Dual) Assay Adjacent to Total Protein. The dual assay procedure shows the two immunospecific bands obtained by the procedure shown in Figure 10 relative to the migration of other microsomal proteins. The P-450<sub>21</sub> protein migrates parallel to the major microsomal band of 53 kDa. Lanes contain 20  $\mu$ g of protein from stimulated (A) and control (C) rabbits. The difference in staining intensity of the two bands is due to the time period and probe (alkaline phosphatase) activity between assays (2 months).

also shows that P-450<sub>21</sub> concentration appears to be only slightly affected by ACTH (see Discussion). Figure 11 shows the final staining of a enzyme linked immunoblot dual assay of immunoreactive P-450<sub>21</sub> relative to P-450<sub>17 $\alpha$</sub> ; total microsomal protein (TP) detected by Aurodye stain is also shown. The immunospecific P-450<sub>21</sub> band appears to comigrate with the most prominent microsomal band, in the 53 kDa region. The lower intensity of staining of P-450<sub>21</sub> was due to an extended time period between the two assays (3 months). The more sensitive ACTH induced response of P-450<sub>17 $\alpha$</sub>  relative to P-450<sub>21</sub> can be seen in ratios of the two protein levels from normal and stimulated rabbits. Densitometric quantitation of P-450<sub>17 $\alpha$</sub>  band intensity relative to P-450<sub>21</sub>, indicates that normal rabbits have a P-450<sub>21</sub>/P-450<sub>17 $\alpha$</sub>  ratio of 5.3 per milligram of adrenal microsomal protein. In ACTH-stimulated rabbits this ratio is approximately 3.

#### Conditions Affecting Immunoblot Assay

The difference in the amount of immunoreactive P-450<sub>17 $\alpha$</sub>  relative to P-450<sub>21</sub> appears to be the cause of some of the multispecific binding of antibody observed in initial immunoblots. Nitrocellulose blots were first assayed using goat anti-rabbit IgG conjugated to horse raddish peroxidase with little or no significant band formation. This was followed by alkaline phosphatase immunoblot assay.

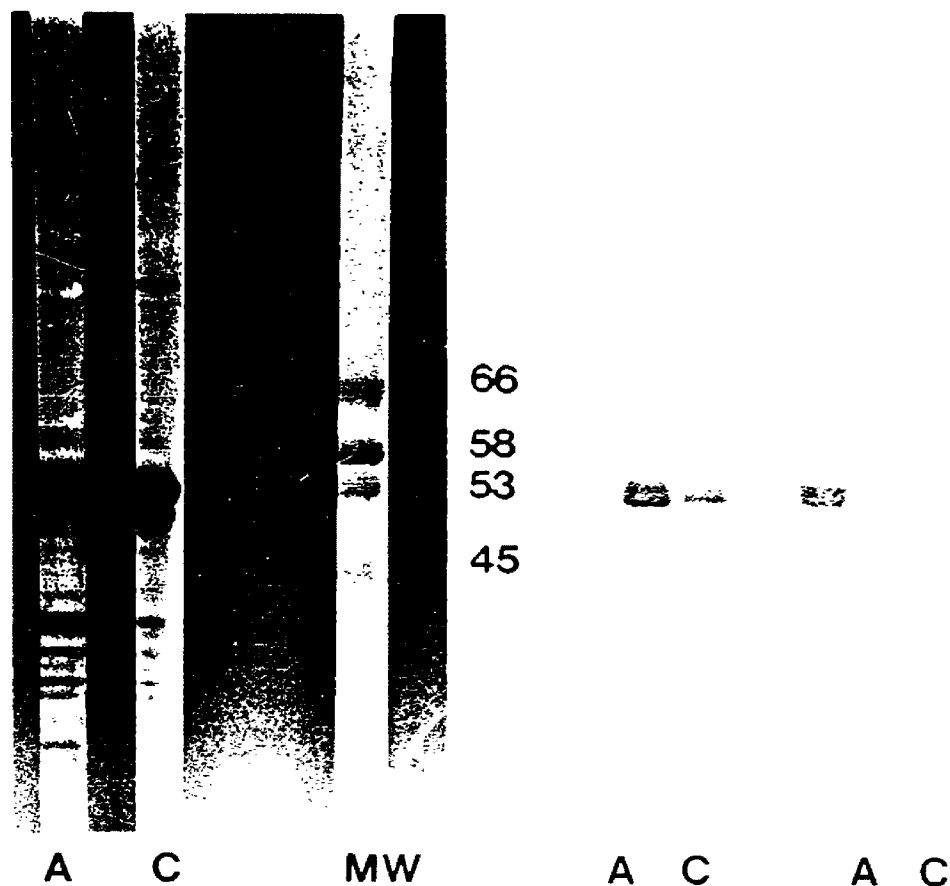


Figure 12. Enzyme linked Immunoblot Assay: Effects of Different Blotting Conditions. Lanes contain 30  $\mu\text{g}$  of microsomal protein from stimulated (A) or control (C) rabbits. Amido black stained molecular weight markers are shown in the center lane (MW). Lanes on the left show nonspecific binding to various microsomal proteins, using gelatin as blocker, 0.5 M NaCl and 0.3  $\mu\text{g}/\text{ml}$  of specific antibody. Lanes on the right show relatively specific binding in the absence of gelatin and presence of 1.5 M NaCl and with Tween-20 as the only blocker of nonspecific binding. Immunospecific bands were revealed using antibody 1.

Figure 12 shows initial results from alkaline phosphatase enzyme linked immunoblots using 0.3  $\mu\text{g}$  of anti-P-450<sub>17 $\alpha$</sub>  IgG (antibody 1) and 0.5 M NaCl. Most of the nonspecific binding shown in Figure 12 was eliminated by increasing the salt concentration to 1.5 M and using only Tween-20 as a blocker of random protein attachment to nitrocellulose. This led to typical results shown on the right in Figure 12. Immunoblots on the right of Figure 12 appear to indicate a competition between P-450<sub>17 $\alpha$</sub>  and P-450<sub>21</sub> for anti-P-450<sub>17 $\alpha$</sub>  IgG. Amido Black-stained molecular weight markers are shown in the center lane (MW). Further modifications in antibody concentration and antibody incubation time led to complete monospecific cross-reactivity. The final assay conditions consisted of an antibody solution containing 2  $\mu\text{g}/\text{ml}$  of anti-P-450<sub>17 $\alpha$</sub>  IgG and 1.5 M NaCl with typical incubation times of 18 hr.

### Interspecies Cross-Reactivity

Immunoblot analysis was also performed with guinea pig adrenal microsomes which were prepared in the same way as those isolated from rabbits in attempts to show cross-reactivity between guinea pig P-450 proteins and antibody to purified pig P-450<sub>17 $\alpha$</sub> , or purified bovine P-450<sub>21</sub>. Figures 13 and 14 show monospecific cross-reactivity between species for both P-450<sub>17 $\alpha$</sub>  and P-450<sub>21</sub>. A significant difference exists in the amount of P-450<sub>17 $\alpha$</sub>  and P-450<sub>21</sub> between guinea

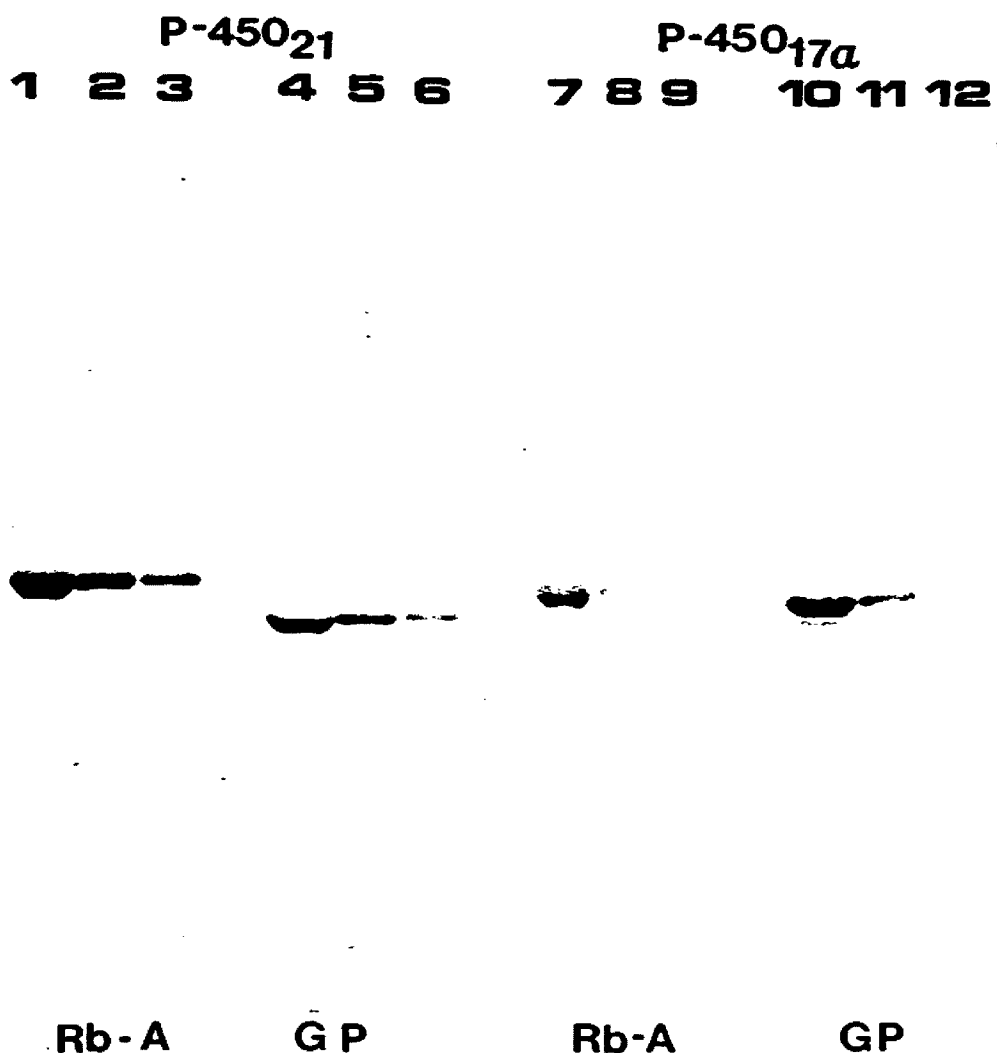


Figure 13. Enzyme Linked Immunoblot Assay of Guinea Pig P-450<sub>17α</sub> and P-450<sub>21</sub> Relative to Rabbit Proteins. Lanes are in groups of three, containing (l-r) 40, 10, and 4 μg of adrenal microsomal protein from guinea pig, GP, stimulated rabbit, Rb-A. The left portion of the blot shows P-450<sub>21</sub> the right side shows P-450<sub>17α</sub> from respective sources. Immuno-specific bands on this blot were revealed using antibodies 1 and 3.



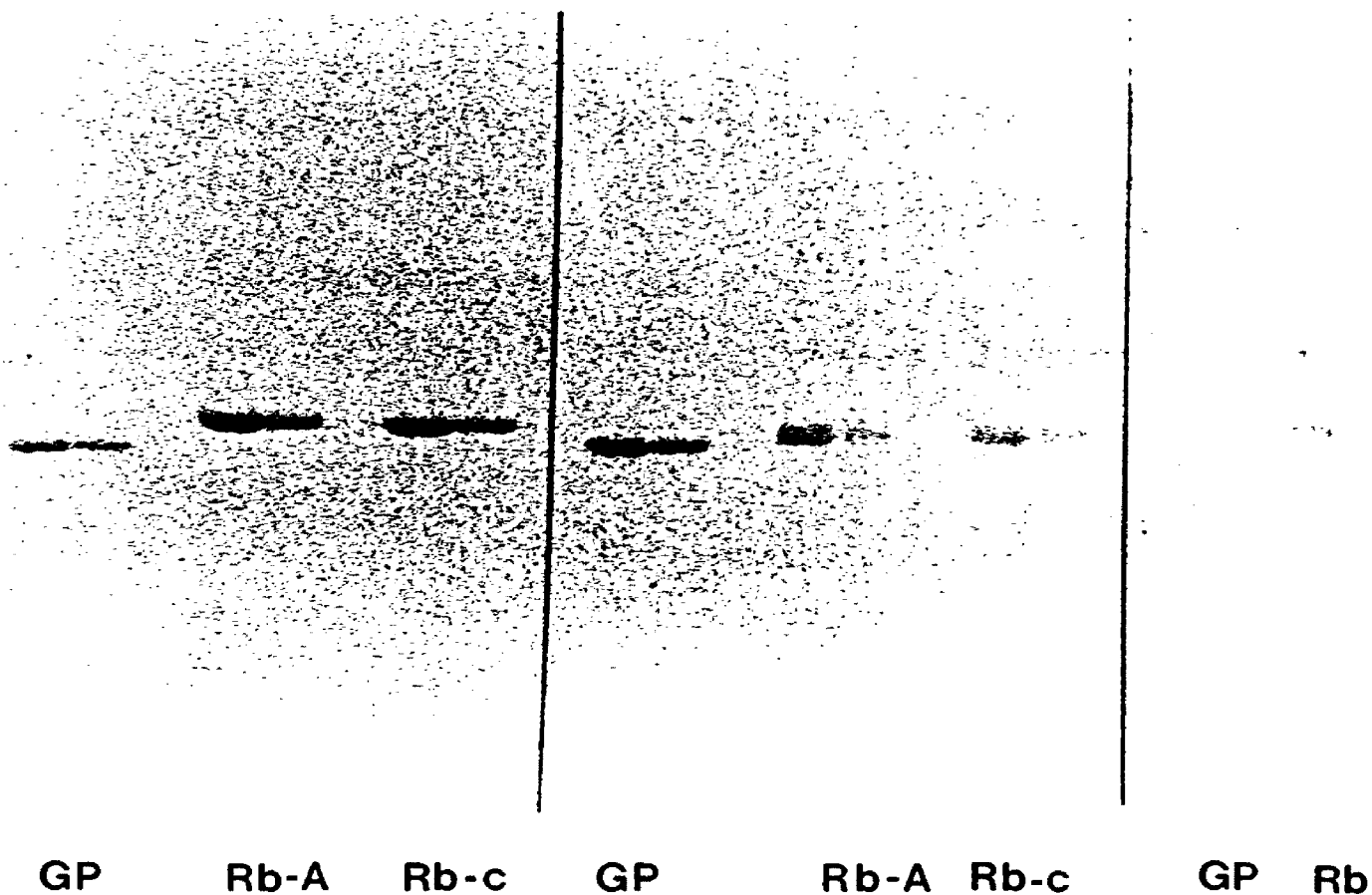


Figure 14. Enzyme Linked Immunoblot Assay of Rabbit and Guinea Pig Proteins. Lanes are arranged in pairs containing 20 and 10  $\mu\text{g}$ , respectively, of microsomal protein from guinea pig, GP, stimulated rabbit, Rb-A, and control rabbit Rb-C. This blot shows P-450<sub>21</sub> (left) and P-450<sub>17 $\alpha$</sub>  (middle). Lanes at left were assayed with preimmune IgG as a control for revealing nonspecific binding. Immuno-specific bands were revealed using antibodies 1 and 3.

pig and rabbit, favoring guinea pig. This P-450<sub>17 $\alpha$</sub>  guinea pig/rabbit ratio was quantified as a 20 fold difference between species, but, due to background on nitrocellulose membranes a high, average deviation of band intensities was obtained. Differences in P-450<sub>21</sub> concentrations appear to favor normal rabbit, although this difference was only a 2-3 fold ratio of rabbit/guinea pig. The rabbit and guinea pig P-450<sub>17 $\alpha$</sub>  proteins appear to comigrate in the 51 to 52 kDa region whereas rabbit P-450<sub>21</sub> appears to have a 53 kDa molecular weight relative to 49 kDa for the guinea pig protein.

### Enzyme Activity

The enzymatic activity of adrenal microsomal 17 $\alpha$ -hydroxylase was measured in terms of percent of substrate, <sup>3</sup>H-pregnenolone, converted to product, <sup>3</sup>H-17 $\alpha$ -hydroxypregnenolone. The detector tracings produced from the scanning of thin layer chromatograms allowed the detection of labeled substrate, labeled product and other labeled substrate metabolites. These tracings also showed separation between radiolabeled substrate metabolites. The migration of internal and external steroid standards allowed the peaks on the TLC radiochromatogram tracings to be identified. The internal standards migrated the same distances as external standards. Identification of individual peaks was accomplished as described in Materials and Methods. Percent

conversion of substrate was expressed on a per adrenal equivalent basis (Table 5) and also on a per milligram basis (Table 6). The percent conversion per milligram of protein shows possible ACTH-stimulated increases in specific activity whereas results expressed in terms of equal adrenal microsomal fractions (adrenal equivalents), shows possible ACTH-stimulated increases in total activity. Enzyme activity was measured in the presence and absence of anti-P-450<sub>17 $\alpha$</sub>  IgG, anti-P-450<sub>21</sub> IgG, and normal rabbit IgG at varying concentrations of antibody in attempts to show specific inhibition of enzyme activity. Values in Tables 5 and 6 are averages of two measurements with corresponding average deviation between measurements. Results expressed in adrenal equivalents in Table 5 show that in the absence of antibody, enzyme activity was stimulated 3.2 fold over controls. Results from a similar experiment showed an average 5.5 fold ACTH-stimulated increase in total activity. The average ACTH effected total increase 17 $\alpha$ -hydroxylase activity was 4.3. The enzyme activity from ACTH-injected rabbits, in the presence of 50  $\mu$ g of anti-P-450<sub>17 $\alpha$</sub>  IgG, shows a 3.5 fold stimulation of activity. This ratio of rates. ACTH/Control, dropped to approximately 2.5 fold in the presence of 100 and 200  $\mu$ g of antibody. Enzyme activity was inhibited 60% by 50  $\mu$ g of anti-P-450<sub>17 $\alpha$</sub>  IgG. The presence of 100 and 200  $\mu$ g of anti-P-450<sub>17 $\alpha$</sub>  antibody appeared to cause further inhibition in some cases, but the

Table 5

The Effect of Cytochrome P-450 Antibodies on ACTH-Stimulated and Control Rabbit Adrenal  $17\alpha$ -Hydroxylase Activity: Per Adrenal Equivalent.

<u>Antibody Addition</u>	<u>Activity</u>	
	$^3\text{H}$ - $17\alpha$ -Hydroxypregnenolone Percent of Substrate/ 4 Min	
	<u>ACTH</u>	<u>Control</u>
None	22.9 $\pm$ 1.1	7.7 $\pm$ 1.5
<u>Anti-P-450<sub>17<math>\alpha</math></sub> IgG</u>		
50 $\mu\text{g}$	9.4 $\pm$ 0.4	2.7 $\pm$ 0.4
100 $\mu\text{g}$	9.6 $\pm$ 1.5	3.8 $\pm$ 1.0
200 $\mu\text{g}$	6.3 $\pm$ 2.7	2.3 $\pm$ 0.2
<u>Anti-P-450<sub>21</sub> IgG</u>		
50 $\mu\text{g}$	13.8 $\pm$ 2.1	5.0 $\pm$ 0.0
100 $\mu\text{g}$	12.3 $\pm$ 0.8	2.9 $\pm$ 1.5
200 $\mu\text{g}$	11.9 $\pm$ 0.6	1.7 $\pm$ 0.4
<u>Pre-immune IgG</u>		
50 $\mu\text{g}$	23.5 $\pm$ 2.9	4.0 $\pm$ 0.1
100 $\mu\text{g}$	19.6 $\pm$ 4.8	4.8 $\pm$ 0.4
200 $\mu\text{g}$	11.9 $\pm$ 0.6	7.1 $\pm$ 0.4

Table 6

The Effect of Cytochrome P-450 Antibodies on ACTH-Stimulated and Control Rabbit Adrenal 17 $\alpha$ -Hydroxylase Activity: Per Milligram of Protein.

<u>Antibody Addition</u>	<u>Activity</u>	
	<sup>3</sup> H-17 $\alpha$ -Hydroxypregnenolone Percent of Substrate/ 4 Min	
	<u>ACTH</u>	<u>Control</u>
None	53.2 $\pm$ 2.7	24.5 $\pm$ 4.9
<u>Anti-P-450<sub>17</sub><math>\alpha</math> IgG</u>		
50 $\mu$ g	22.1 $\pm$ 0.9	8.6 $\pm$ 1.2
100 $\mu$ g	22.5 $\pm$ 3.6	12.3 $\pm$ 3.1
200 $\mu$ g	14.9 $\pm$ 6.0	7.4 $\pm$ 0.6
<u>Anti-P-450<sub>21</sub> IgG</u>		
50 $\mu$ g	32.6 $\pm$ 5.0	15.9 $\pm$ 0.0
100 $\mu$ g	28.9 $\pm$ 1.8	9.2 $\pm$ 4.9
200 $\mu$ g	28.1 $\pm$ 1.3	5.5 $\pm$ 1.2
<u>Pre-immune IgG</u>		
50 $\mu$ g	55.2 $\pm$ 6.8	12.9 $\pm$ 0.6
100 $\mu$ g	46.2 $\pm$ 0.0	15.3 $\pm$ 1.2
200 $\mu$ g	48.0 $\pm$ 6.7	22.7 $\pm$ 1.2

large deviation between duplicate measurements (shown in Tables 5 and 6) indicate the possibility of a lack of inhibition with increasing antibody concentration.

Results obtained from assays with 300  $\mu\text{g}$  of prefrozen microsomal protein and a one hour preincubation with 200  $\mu\text{g}$  of anti-P-450<sub>17 $\alpha$</sub>  IgG showed a 87% inhibition of 17 $\alpha$ -hydroxylase, indicating that increased antibody concentrations do in fact show further inhibition of enzyme activity. The activity of 17 $\alpha$ -hydroxylase from frozen microsomes preincubated with 200  $\mu\text{g}$  of preimmune IgG was 9.1 + 0.1% conversion of substrate per milligram of protein, per 4 minutes. This activity was inhibited to 1.2 + 0.1% conversion in the presence of 200  $\mu\text{g}$  of anti-P-450<sub>17 $\alpha$</sub>  antibody. The low average deviations obtained with the preincubation period indicate that the inhibition pattern seen in Tables 5 and 6 is real and is not as ambiguous as the respective average deviations may imply. Control rabbit enzyme activity showed the same pattern of inhibition and this pattern adds to the evidence that approximately 80% inhibition is obtained with 200  $\mu\text{g}$  of anti-P-450<sub>17 $\alpha$</sub> .

Inhibition studies with anti-P-450<sub>21</sub> IgG showed a significant increase in the ratio of enzyme activities with increasing concentrations of anti-P-450<sub>21</sub> IgG. The 17 $\alpha$ -hydroxylase activity of ACTH-stimulated microsomes appeared to be inhibited 40% by anti-P-450<sub>21</sub> IgG. Increasing concentrations of antibody did not cause further inhibition

of ACTH-stimulated rabbit  $17\alpha$ -hydroxylase activity. Inhibition of control  $17\alpha$ -hydroxylase activity however, caused the increase in the ratio of ACTH-stimulated enzyme activity over control. Ratio values ranged from 2.8 to 4.3 to 7 fold in the presence of 50, 100, and 200  $\mu\text{g}$  respectively, of anti-P-450<sub>21</sub> IgG (see Discussion). Enzyme activity from ACTH-stimulated rabbits did not appear to be significantly inhibited by normal rabbit IgG, although the ratio of  $17\alpha$ -hydroxylase activity dropped in the presence of increasing amounts of hyperimmune IgG. The ratios of ACTH/Control activities were used in the calculation of ACTH effected total and specific increases in  $17\alpha$ -hydroxylase rates. Enzyme activity from ACTH-injected rabbits showed a 6 fold increase over control in the presence of 50  $\mu\text{g}$  of normal rabbit IgG. Activity dropped to a 4 fold ratio in the presence of 100  $\mu\text{g}$  of normal rabbit IgG antibody, as shown in Tables 5 and 6.

Enzyme activity expressed on a per milligram basis shows that microsomal  $17\alpha$ -hydroxylase activity was stimulated 3.2 fold in ACTH-injected rabbits relative to controls, per milligram of protein (Table 6). The pattern of antibody inhibition was identical to values expressed per adrenal equivalent. An average value of 55.2% conversion of substrate per adrenal equivalent was obtained from ACTH-stimulated rabbit microsomes in the presence of 200  $\mu\text{g}$  of preimmune IgG (Table 6). In the presence of 200  $\mu\text{g}$  of anti-

P-450<sub>17α</sub> IgG, average conversion was inhibited to 14.9%. It appears that the preincubation of microsomes with 200 μg of normal rabbit IgG showed a more accurate inhibition of enzyme activity than that observed in the absence of the preincubation period and there was a slightly greater degree of inhibition obtained with the preincubation period.



### Discussion

The increase in adrenal microsomal protein yields and adrenal weights obtained from ACTH-injected rabbits indicates an overall increase in adrenocortical protein synthesis which has previously been correlated to ACTH stimulation of rabbits (Table 1) (18-21).

Immunoblot analysis of rabbit adrenal microsomal protein revealed that the rabbit antibody to purified porcine microsomal P-450<sub>17 $\alpha$</sub>  protein reacts in a monospecific manner with the rabbit enzyme. This implies that rabbit and pig share adequate sequence homology at their antigenic sites and appear to be similar in monomeric (14) size as well (Figures 6-14) (35). This interspecies cross-reactivity was used in the detection and quantitation of P-450<sub>17 $\alpha$</sub>  protein from rabbit and guinea pig adrenal, with alkaline phosphatase enzyme linked immunoassay (Blake et al. 1984; Figures 6,7,10,11,13 and 14) and <sup>125</sup>I-Protein A (Burnette et al. 1981; Figures 8,9), but bands were not detectable using horse raddish peroxidase enzyme linked immunoblot assay.

The results presented here have shown that the 4 fold ACTH-stimulated total increase in microsomal 17 $\alpha$ -hydroxylase activity, is attributed to the greater than 6 fold increase in the content of immunoreactive P-450<sub>17 $\alpha$</sub>  (Tables 2 and 4).

The increase in P-450<sub>17 $\alpha$</sub>  protein content and 17 $\alpha$ -

hydroxylase total activity appears to occur through induction of enzyme expression (35,39). Furthermore, this increase in activity of  $17\alpha$ -hydroxylase exceeds the ACTH-stimulated increase in total adrenal protein synthesis, showing a 3 fold specific increase in activity (Tables 6). This activity corresponds to a 4 fold increase in the specific content of P-450<sub>17 $\alpha$</sub>  protein. The inhibition experiments shown in Tables 5 and 6 indicate the possibility of multispecific binding between  $17\alpha$ -hydroxylase and anti-P-450<sub>21</sub> IgG. The 40% inhibition of ACTH-stimulated enzyme activity, by anti-P-450<sub>21</sub> relative to the greater than 70% inhibition by P-450<sub>17 $\alpha$</sub> , indicates the possibility of sequence homology between antigenic sites of the two enzymes. This is also indicated by the multispecific binding of anti-P-450<sub>17 $\alpha$</sub>  IgG at low concentrations of antibody, as shown in Figure 12. It appears that lack of complete inhibition by anti-P-450<sub>17 $\alpha$</sub>  is not simply a factor of antibody concentration but also shows that access to the antigenic site may be limited by the internalization of that region of P-450<sub>17 $\alpha$</sub>  in microsomal membrane. The limitation of access of antibodies to the antigenic site may also explain the partial inhibition of  $17\alpha$ -hydroxylase by anti-P-450<sub>21</sub> antibody.

The ratio of enzyme activity between ACTH-stimulated and Control tissue is lower in each experiment than is the corresponding ratio of the amounts of protein. The increase

in rabbit adrenal  $17\alpha$ -hydroxylase specific activity per milligram of protein, which was obtained in experiments presented here, was not as high as values obtained previously (Table 6) (18-21). Based on the assumption that control rabbits were not under any type of stress, the lack of reproducible results may be due to human error involved in isolating adrenal microsomes and assaying microsomal activity. A loss in activity of both microsomal preparations, ACTH and control, may have resulted in a decrease in the magnitude of stimulation. The ratio of the amount of enzyme "may appear" slightly higher than the ratio of enzyme activity possibly due to conditions less stringent than those previously used in the analysis of enzyme assay extracts (21) and also due possibly to the competition between  $3\beta$ -hydroxy steroid dehydrogenase and  $17\alpha$ -hydroxylase for substrate pregnenolone. Normally this  $3\beta$ -ol-dehydrogenase activity is inhibited by cyanoketone but, the presence of radioactive peaks comigrating with both progesterone standards and 11-deoxycorticosterone indicates that this activity was at best, partially inhibited. Denaturation of active multimeric forms of the enzyme which would not affect the immunospecific quantitation of  $P-450_{17\alpha}$  but would certainly inhibit  $17\alpha$ -hydroxylase.

The difference could also be explained by the detection of newly synthesized immunoreactive monomeric  $P-450_{17\alpha}$ , not yet associated in active multimeric form (14). An alter-native

explanation is also proposed, which is supported by data from previous experiments and theoretical treatment of the data shown in Table 6.

Studies involving the ACTH-stimulation of live animals must take into account all possible factors associated with stress. This stress may be attributed to, among other causes, animal handling, disease, a change in environment, such as a change in altitude or a change in the quality of food. The death of two experimental animals immediately following air transportation from Seattle, Washington to Missoula, Montana, implied stress due to the conditions encountered in air travel. Subjects that appeared healthy were allowed to adjust to their new surroundings for a period of at least 10 days before the injection period. Unhealthy subjects were replaced with healthy animals, and allowed to adjust to their new environment. In previous studies rabbits were not shipped by air, but were obtained locally. Therefore a comparison between enzyme activity of control rabbits from previous experiments was compared to activity from control rabbits shown in Tables 5 and 6.

Previous studies of the kinetic properties of rabbit  $17\alpha$ -hydroxylase by (Fevold et al. 1978) showed that a microsome preparation containing 0.4 mg of protein from ACTH-stimulated rabbits, converted 7% of the substrate to product, per minute, in the presence of  $0.5 \mu\text{M}$  substrate. The activity of control rabbit  $17\alpha$ -hydroxylase was cited as

being less than 5% of the ACTH-stimulated activity or 3% conversion, per 4 minutes, per mg of protein (21). This value compares directly with the value of control rabbit enzyme activity of 24% per 4 minutes per mg of protein in Table 6. This was done by assuming linear catalytic rates at low substrate concentration (0.25-0.5  $\mu$ M) (21). The control rabbit 17 $\alpha$ -hydroxylase activity cited in Table 6 is 8 fold over the conversion obtained previously, lending credence to the suspicion of a response to stress present in control animals used in this experiment. Assuming that this was infact, the case, ACTH-stimulated animals may have reached a point where increases in enzyme activity and P-450<sub>17 $\alpha$</sub>  protein content were no longer inducible by ACTH. This chronic stimulation by native ACTH would thereby lower the ratio of activities between control and ACTH-stimulated rabbits, an effect seen previously in both rabbit and bovine adrenocortical cells in culture (18-21,35). Despite this possible stress factor, the results presently discussed indicate an ACTH-stimulated increase in P-450<sub>17 $\alpha$</sub>  protein and also in 17 $\alpha$ -hydroxylase above levels found in normal rabbits.

Injection of rabbits appears to effect an increase in 17 $\alpha$ -hydroxylase in the same way that ACTH and cAMP effect an increase in cultured bovine adrenocortical. This conclusion would have to be verified by Northern blot quantitation of mRNA using puromycin D and cycloheximide to specifically

show stimulation of transcription. This induction of enzyme expression recently reported for bovine adrenocortical cells in monolayer culture, shows a ACTH-cAMP dependent stimulation of transcription (17,35,39). However, rabbit  $17\alpha$ -hydroxylase differs from the bovine system by the fact that ACTH-injected rabbits exceed at least by 3 fold, their natural activity of  $17\alpha$ -hydroxylase and P-450 $_{17\alpha}$  content. This has not been shown in other tissues and in bovine adrenal cell cultures evidence suggests that this may not be possible in cortisol secreting species (35) due to the fact that ACTH treatment of cultured bovine adrenocortical cells for greater than 72 hours leads to a decrease in newly transcribed P-450 $_{17\alpha}$  gene, although the stability of mRNA is greatly enhanced in the presence of ACTH (39). ACTH stimulation of  $17\alpha$ -hydroxylase and P-450 $_{17\alpha}$  content of bovine cells in culture, appears to taper as normal levels found in fresh cortex are approached (35). The rabbit, thus far, is the only species to show an ACTH-stimulated increase in the specific activity of  $17\alpha$ -hydroxylase which significantly exceeds levels found in fresh cortex of normal animals. This may imply that in vivo, bovine levels of ACTH keep  $17\alpha$ -hydroxylase activity at a level, high enough to result in cortisol production in preference to corticosterone. This may be the case for guinea pig, as indicated by the relative staining of P-450 proteins shown in Figures 13 and 14.

Increases in P-450<sub>21</sub> protein induced by ACTH, shown in the quantification results in Table 6 have been observed in bovine adrenocortical cells in culture as well. This ACTH-stimulated increase in P-450<sub>21</sub> protein has been found to occur in the absence of 21-hydroxylase activity in bovine cells and a lack of response in 21-hydroxylase activity in ACTH stimulated rabbits has also been observed (32,40). The reason for the absence of activity in the presence of newly synthesized protein remains to be found.

Three major conclusions can be formulated by the research presented here, the first of which is that ACTH treatment of rabbits induces an increase in the content of adrenal P-450<sub>17 $\alpha$</sub> . Secondly, the increase in content of this specific P-450 protein appears to be responsible for the corresponding increases in 17 $\alpha$ -hydroxylase activity, lowering the V<sub>max</sub> of the enzyme by increasing the amount of protein. Last of all, this increase in adrenal microsomal P-450 protein appears to be more specific for P-450<sub>17 $\alpha$</sub>  relative to other microsomal P-450's such as P-450<sub>21</sub>.

### The Role of the SHIP Protein

The synthesis of a labile protein prior to transcription of bovine adrenal steroid hydroxylases has been found to be a requirement of the ACTH regulated initiation of induction of gene expression. This protein factor has been termed as a steroid hydroxylase inducing protein or SHIP. It is not

known at this time, whether a separate SHIP protein is responsible for regulating an individual steroid hydroxylase gene or if a general protein activates initiation of transcription of all steroid hydroxylase genes. Furthermore it is not known if the SHIP protein binds a second messenger ligand to activate transcription of its own gene or of steroid hydroxylase genes. In any case, function of this protein has been shown to come under regulation of cAMP and therefore ACTH. Future experiments will be directed toward characterizing the mechanism of ACTH/cAMP/SHIP induction of P-450 enzyme expression and toward the regulatory differences between P-450<sub>17 $\alpha$</sub>  and other steroid metabolizing P-450 proteins (43).

#### Discussion of Techniques

Densitometric scanning of autoradiograms showed reproducible results relative to the scanning of nitrocellulose membranes. This is probably due to unstained protein on nitrocellulose membranes that blocks the transmittance of light, interfering with the background of the blots and lowering quantitative differences between lanes. Therefore reflectance densitometry is the method of choice when quantifying alkaline phosphatase chromogen on nitrocellulose (Blake et al. 1984). Band broadening produced in autoradiography is probably not an issue of concern here since broadening occurs in proportion to the amount of antibody bound and is not a factor in relative measurements.



This broadening is a factor in the determination of molecular weights of proteins, especially when analyzing intra- and interspecies forms of P-450 proteins. Immunoblot experiments not shown in the results section showed that fluorescein labeled adrenal microsomal protein was easily detectable on nitrocellulose. The proteins remained antigenically stable, allowing detection with  $^{125}\text{I}$ -Protein A probe or alkaline phosphatase ELISA. This is a useful technique in isolating and identifying molecular weight markers throughout the electrophoresis step and after blotting to nitrocellulose.

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