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The in vivo effect of ACTH on CYP17 mRNA in the rabbit adrenal

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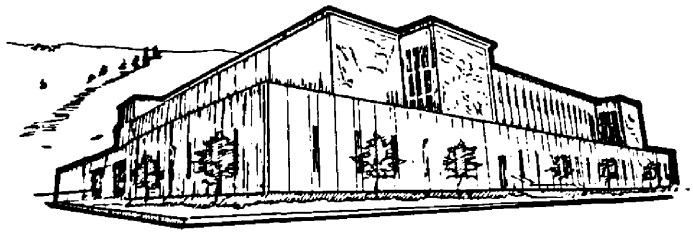

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The *In vivo* Effect of ACTH on CYP17 mRNA in the Rabbit Adrenal

by

Kathleen E. Mach

Presented in partial fulfillment of the requirements

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The in vivo effect of ACTH on CYP17 mRNA in the rabbit adrenal.

Director: H. Richard Fevold

Cytochrome P450 17α/17,20 lyase ($P450_{17\alpha}$) is encoded by the CYP17 gene. Upon ACTH stimulation the amount of this protein and its enzymatic activity have been shown to increase in the rabbit adrenal. The present study was designed to determine whether these increases could be correlated to an increase in CYP17 mRNA. Adrenal RNA isolated from ACTH-stimulated, control rabbits injected with the injection vehicle, and uninjected control rabbits were compared for the level of CYP17 mRNA. After four days of ACTH injections little change (1.07 fold increase) in CYP17 mRNA could be detected. With six days of ACTH injection 1.34 fold increase in CYP17 mRNA was found compared to the injected controls and a 1.7 fold increase compared to the uninjected controls. Western analysis confirmed ACTH stimulation with a 21 fold increase in $P450_{17\alpha}$ protein in ACTH-stimulated rabbits compared to the injected control and a greater than 50 fold increase compared to the uninjected control rabbits. The difference between injected control rabbits and uninjected control rabbits is presumably caused secretion of endogenous ACTH. The large elevation in $P450_{17\alpha}$ protein levels with a minimal increase in CYP17 mRNA indicates the increase in $P450_{17\alpha}$ protein with ACTH stimulation is due to translation of mRNA already present in the adrenals.

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INTRODUCTION

$P450_{17\alpha}$

Cytochrome P450 17α hydroxylase/17,20 lyase ($P450_{17\alpha}$) is a key enzyme in the synthesis of glucocorticosteroids, estrogens and androgens from cholesterol. In all species investigated it is encoded by a single copy gene named CYP17 (Nelson *et al.* 1993). This gene or its cDNA has been isolated from several species, including bovine (Zuber *et al.* 1986a, Bhasker *et al.* 1989), human (Chung *et al.* 1987, Picado-Leonard and Miller 1987, Bradshaw *et al.* 1987, Kagimoto *et al.* 1988, Brentano *et al.* 1990), rat (Namiki *et al.* 1988, Nishihara *et al.* 1988, Fevold *et al.* 1989, Zhang *et al.* 1992), mouse (Youngblood and Payne 1992), and porcine (Conley *et al.* 1992 and Zhang *et al.* 1992). The degree of homology among these genes is high enough to allow hybridization of CYP17 cDNAs from different species. In humans this gene is located on chromosome 10 at q24.3 (Fan *et al.* 1992). Expression of CYP17 is tissue specific. While it is expressed in the testes and ovaries of all mammals, expression in the adrenal glands varies among species.

$P450_{17\alpha}$ is an integral membrane hemoprotein located in the smooth endoplasmic reticulum. This enzyme can be expressed in COS 1 cells; for active expression in this system membrane attachment is required. The amino terminal sequence functions as a signal anchor sequence for membrane attachment and may be important for proper folding of the protein (Clark and Waterman 1991 and Clark and Waterman 1992).

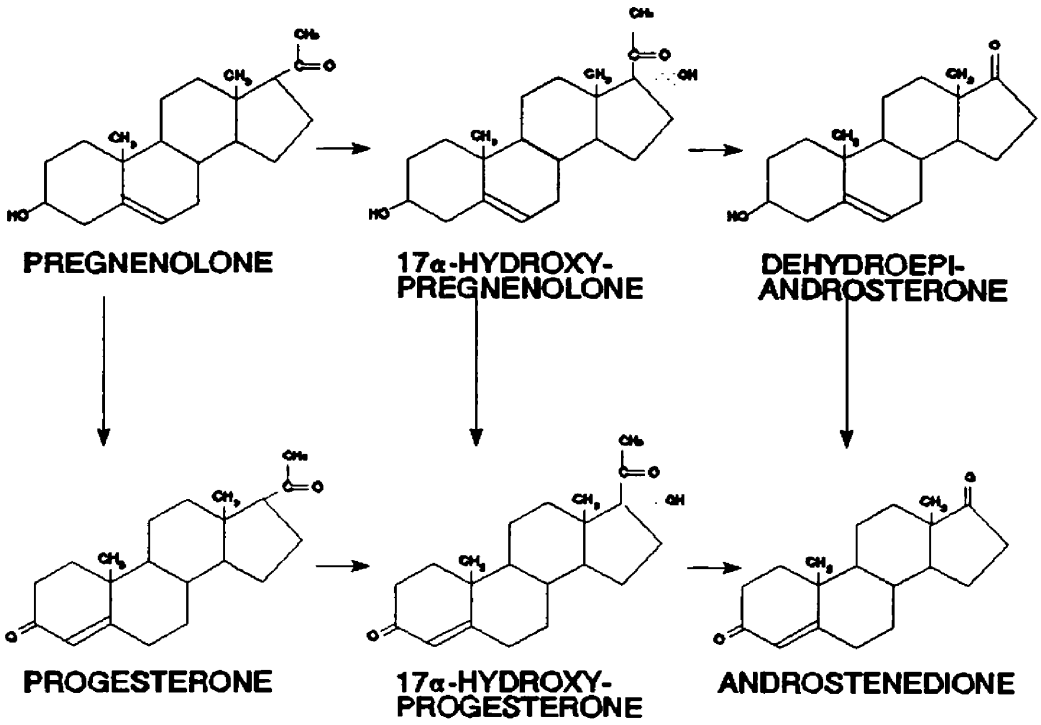

Figure 1: Reactions catalyzed by $P450_{17\alpha}$ represented by horizontal arrows.

$P450_{17\alpha}$ catalyzes two separate types of reactions (Figure 1). The first reaction is the addition of a hydroxyl group in the alpha position of carbon 17 of pregnenolone or progesterone (list of abbreviations and common names used are found at end of thesis). In this reaction the heme prosthetic group plays a key role; Figure 2 depicts this mechanism (Guengerich 1991). The second reaction is the cleavage between carbons 17 and 20 of 17α-hydroxypregnenolone or 17α-hydroxyprogesterone. The 17-hydroxylase and 17,20-lyase catalytic activities are located in the same polypeptide chain. This has been shown by isolation and characterization of the protein (Nakajin and Hall 1981 and Nakajin *et al.* 1983)

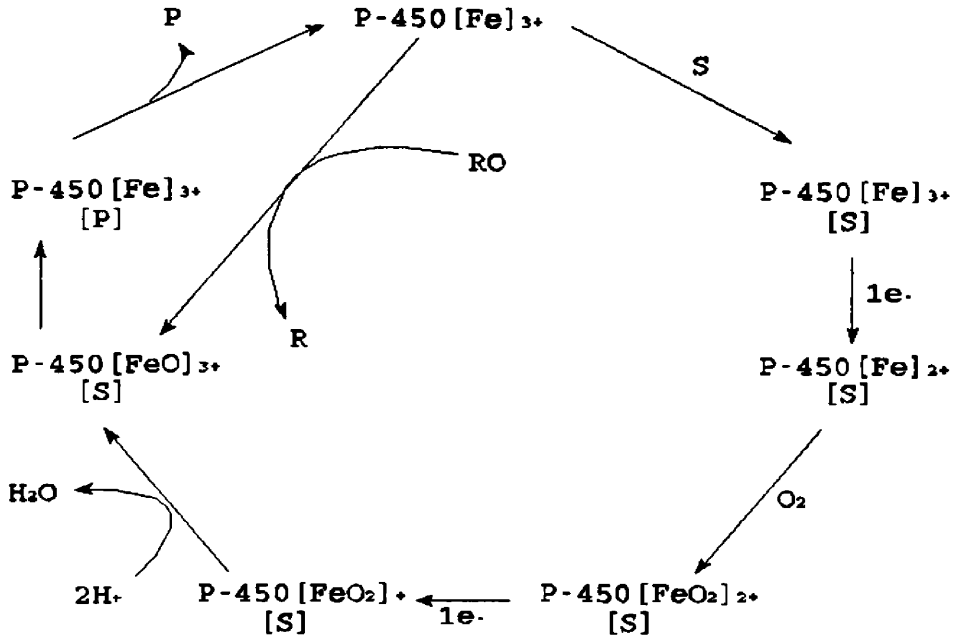

Figure 2: Scheme for P-450 oxygen activation and oxygenation. S,substrate; P, product; and RO, artificial active oxygen donor. (Guengerich 1990).

and expression of the CYP17 cDNA in nonsteroidogenic cells (Zuber *et al.* 1986b).

$P450_{17a}$, and the other microsomal P450 proteins receive their reducing equivalents from nicotinamide adenine dinucleotide phosphate (NADPH) via NADPH cytochrome P-450 reductase. Two electrons from NADPH are transferred to NADPH cytochrome P-450 reductase and then to the P450 protein (Omura *et al.* 1966 and Hiwatashi and Ichikawa 1979).

THE ADRENAL GLAND

The adrenal glands are part of the mammalian endocrine system. They are composed of two sections, the medulla in the center and the outer cortex. The medulla is responsible for synthesizing catecholamines and the cortex produces aldosterone, glucocorticoids, and adrenal androgens. The cortex can be further divided into three layers. The outer layer of the cortex, the zona glomerulosa, produces primarily aldosterone and does not express $P450_{17\alpha}$. The inner two layers, zona fasciculata and zona reticularis are responsible for the production of the glucocorticoids, cortisol or corticosterone, and adrenal androgens. In cortisol producing species $P450_{17\alpha}$ is expressed in the inner two layers of the adrenal cortex (as reviewed by Norman and Litwack 1987).

STEROID HORMONE PRODUCTION

The production of steroid hormones begins in the mitochondria where cholesterol is converted to pregnenolone by $P450_{scc}$ (Figure 3). Pregnenolone then moves to the endoplasmic reticulum. Steroidogenic enzymes $P450_{17\alpha}$, 3ß-hydroxysteroid dehydrogenase (3ßHSD), and 21-hydroxylase ($P450_{C21}$) are all located in the endoplasmic reticulum. Products of the sequence of reactions catalyzed by these enzymes are transported back to the mitochondria where they are converted to cortisol or corticosterone by 11ß-hydroxylation. Since $P450_{C21}$ and 11ß-hydroxylase ($P450_{11\beta}$) are expressed only in the adrenal glands, the adrenal is the only organ to produce glucocorticosteroids.

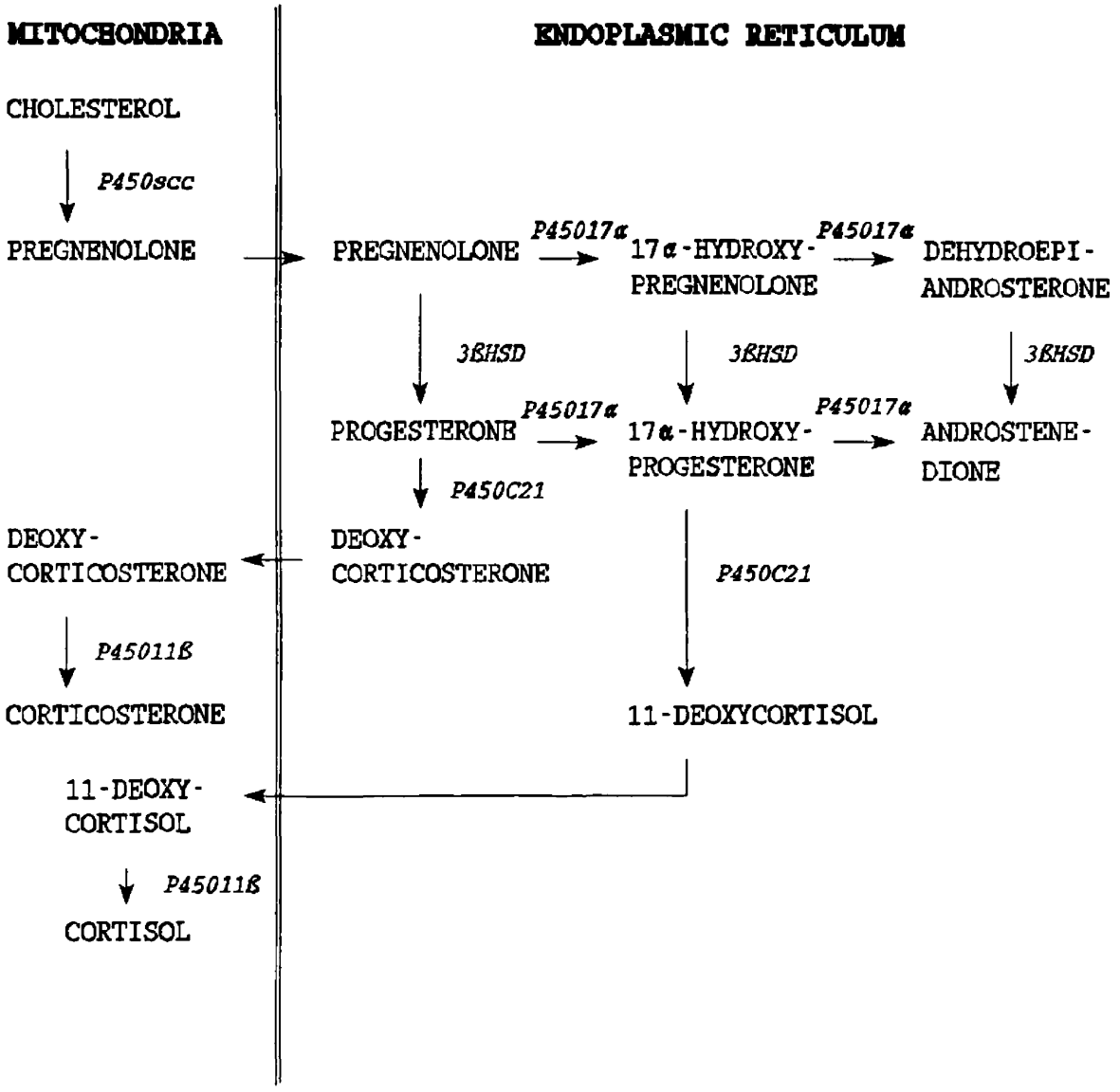

Figure 3: Metabolic pathway for production of glucocorticosteroids in the adrenal glands (Waterman and Simpson 1989).

ACTH AND STEROIDOGENESIS

Glucocorticoid production is under the control of the peptide hormone, ACTH (Figure 4). ACTH is synthesized in the anterior pituitary as part of a polyprotein, POMC. POMC produces three melanocyte-stimulating hormones, three endorphins, and ACTH; there is some overlap among these sequences. The level of expression of ACTH is controlled by posttranslational processing of POMC (as reviewed by Bolander 1989).

ACTH elicits an acute effect, the mobilization of cholesterol for conversion to pregnenolone, in a matter of minutes. The cellular response to ACTH begins with binding of ACTH to specific cell surface receptors in the zona reticularis and zona fasciculata. This activates adenylate cyclase causing an increase in the production of cAMP. The resulting increased concentration in of cAMP activates the catalytic subunit of protein kinase A (Haynes 1958 and Haynes *et al.* 1959). Active protein kinase A phosphorylates other proteins in a cascade of events resulting in hydrolysis of the stored cholesterol esters (Jefcoate *et al.* 1987). The free cholesterol is transported to the mitochondria by sterol carrier protein 2 (Chanderbhan *et al.* 1982) where it is the substrate for $P450_{scc}$. $P450_{scc}$ catalyzes the initial and rate limiting step in the synthesis of glucocorticosteroids. The acute action of ACTH is to increase the amount of initial substrate, and thereby escalate steroid hormone production.

ACTH also has a chronic or long term effect on steroidogenesis (Kass *et al.* 1954). It maintains optimal levels of steroidogenic enzymes in the adrenal cortex

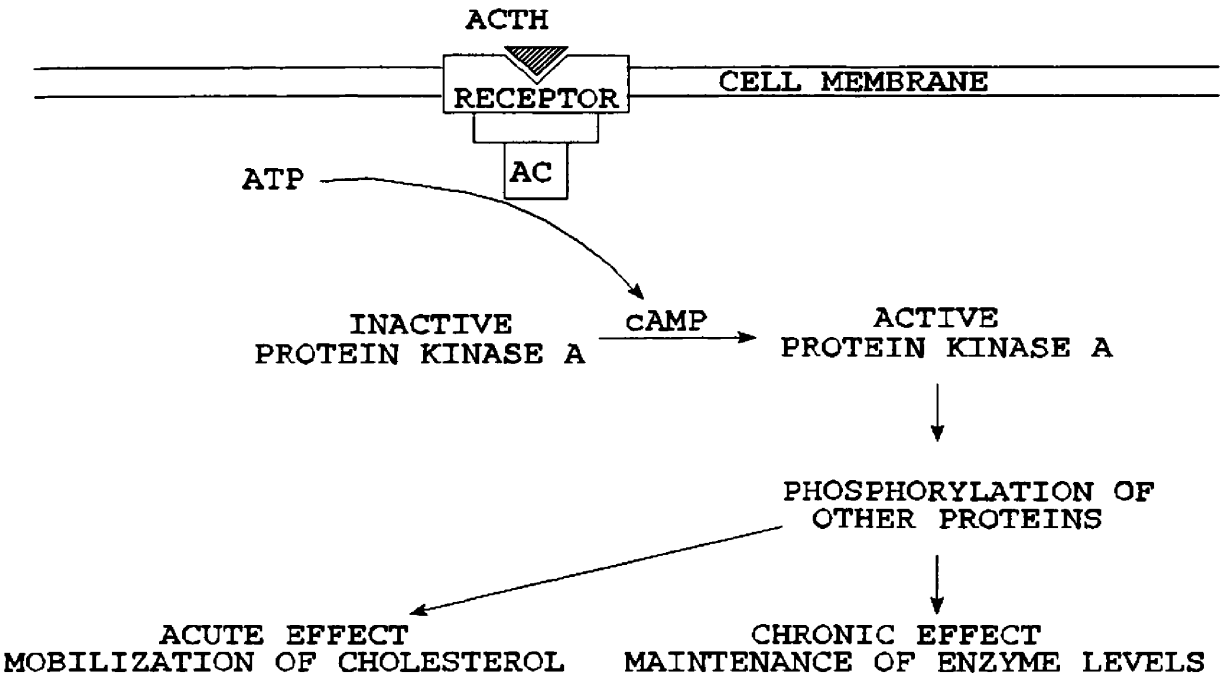

Figure 4: Through a cascade of reactions ACTH acts to increase the amount of available substrate and maintain enzyme levels for steroidogenesis.

and alters steroidogenic pathways. Adrenocortical P450 levels fall after hypophysectomy and are increased by the administration of ACTH (Purvis *et al.* 1973). Many studies have focused on elucidating the mechanism by which ACTH controls enzyme levels. Considering the pleiotropic effects of ACTH, the control mechanisms in the adrenal gland is probably variable among species.

Bovine adrenocortical cells grown in primary culture are found to produce substantially less cortisol in the absence of ACTH compared to cells grown with ACTH (Goodyer *et al.* 1976, Simonian *et al.* 1979, Kramer *et al.* 1983). The

ability of these cells to produce cortisol was linked to the activity of 17α-hydroxylase (McCarthy *et al.* 1983). In cultures lacking ACTH little to no P45017α protein or CYP17 mRNA was detected. If the same cells were grown in the presence of ACTH or cAMP, levels of CYP17 mRNA (John *et al.* 1986) and $P450_{17\alpha}$ protein (Zuber *et al.* 1985) returned to nearly normal levels. This demonstrates the requirement of ACTH for maintenance of $P450_{17\alpha}$ levels and indicates that in cattle ACTH control is at the transcriptional level. Studies of bovine fetuses showed adrenal expression of $P450_{17\alpha}$ in the first 100 days of gestation; between days 100 and 230 $P450_{17\alpha}$ and CYP17 mRNA were not detectable; and after day 230 P45017α and CYP17 mRNA were again expressed. Blood levels of ACTH and cortisol also follow the same pattern as $P450_{17\alpha}$, present early and late in gestation but absent between days 100 and 230 (Lund *et al.* 1989). During ovine fetus development the same presence, absence, reappearance pattern of expression of P45017α, CYP17 mRNA, ACTH, and cortisol was found (Tangalakis *et al.* 1990). These results provide further evidence that ACTH can function *in vivo* as well as in cell culture to induce transcription of the CYP17 gene . The factors affecting ACTH inducibility of CYP17 may vary throughout the cattle's life as seen by the observance of lower levels of CYP17 mRNA in the adrenals of older (10-12 years old) cow and primary adrenal cell cultures from old cattle produce less CYP17 mRNA than that from young (1 year old) cattle (Ogo *et al.* 1991).

Hamsters, a cortisol producing rodent, express $P450_{17\alpha}$ in response to

ACTH stimulation (LeHoux *et al.* 1987). When the effect of ACTH was followed over time LeHoux *et al.* (1992) found that CYP17 mRNA is maximally increased at 2.5 hours after injection, while the levels of P45017α protein increase gradually between 5 and 10 hours after ACTH injection and reached a constant level after 15 hours. The pronounced lag between mRNA synthesis and protein production indicated a control point between transcription and translation.

Guinea pigs, another cortisol producing rodent, express some of the highest levels of adrenal $P450_{17\alpha}$ of any species. The level of adrenal $P450_{17\alpha}$ expression can be increased slightly with ACTH stimulation. Upon ACTH stimulation of guinea pig adrenal cells in culture, both CYP17 mRNA and P45017α protein increased (Provencher *et al.* 1992a). However, *in vivo* an apparent increase in 17α-hydroxylase activity was observed with no corresponding increase in CYP17 mRNA. In northern blot analyses the level of CYP17 mRNA appeared to drop (Provencher *et al.* 1992b). *In vivo* observations of the CYP17 mRNA levels in these studies did not take into account the 35% increase in adrenal weight observed in the ACTH stimulated animals, however along with the increase in adrenal size is a larger pool of RNAs. This could mask increases in the level of CYP17 mRNA.

Rats and mice produce no 17α-hydroxylated steroids in their adrenal cortex, and no 17α-hydroxylase activity can be detected in the adrenals of these rodents (LaPlante *et al.* 1964). When injected with ACTH an increase in the blood levels of corticosterone is seen. This is due, at least in part, to the acute

action of ACTH, increasing the availability of initial substrate (Koritz and Kumar 1970). In these species ACTH does not induce adrenal 17α-hydroxylase activity and does not stimulate production of cortisol (Slaga and Krum 1976). Although cAMP does not induce CYP17 expression in the adrenal, in testicular leydig cells luteinizing hormone, which also acts via a cAMP second messenger, can induces transcription of CYP17 (Nishihara *et al.* 1988). This indicates that other factors that control transcription of CYP17 are tissue specific.

RABBITS, ACTH, AND $P450_{17\alpha}$

The link between ACTH stimulation and an increase in 17α-hydroxylated steroid hormone production was first described in rabbits (Kass *et al* 1954). Rabbits are mainly a corticosterone producing mammal; little or no cortisol can be detected in their blood (Bush 1953). In 1954 Kass *et al.* reported that when rabbits were injected with porcine ACTH the size of the adrenal glands increased, and increasing amounts of cortisol could be found in the adrenal vein blood. The ratio of cortisol to corticosterone in untreated animals was <0.05, after 7 days of ACTH injections the ratio rose to 0.5, and after 21-28 days of ACTH stimulation the cortisol level was four time higher than that of corticosterone. When Krum and Glenn (1965) repeated this work they also reported a cortisol to corticosterone ratio of 0.05 in control animals and ratios of 0.36 and 0.81 after 15 and 30 days of stimulation with porcine ACTH. It has been repeatedly confirmed that there is an increase in cortisol production in ACTH stimulated rabbits.

The increase in cortisol levels of rabbits is due to an increase in 17α-hydroxylase activity (Fevold 1969; Fevold *et al.* 1978). The increase in hydroxylase activity with ACTH stimulation can be shown *in vitro* (Fevold 1984) and *in vivo* (Fevold *et al.* 1978) When comparing adrenal levels of $P450_{17\alpha}$ protein, ACTH stimulated rabbits showed a 4 fold increase over unstimulated animals (Chouinard and Fevold, 1990). The effect of ACTH on adrenal steroidogenic enzymes has been shown to be specific to $P450_{17\alpha}$, the levels of 21-hydroxylase and 3ßHSD showed little to no change upon ACTH stimulation (Slanina and Fevold 1982).

STATEMENT OF THE PROBLEM

Rabbits are unusual in that they normally produce mainly corticosterone yet through ACTH stimulation begin producing more cortisol and less corticosterone (Kass *et al.* 1954 and Krum and Glenn 1965). The increase in cortisol production has been linked to an increase in 17α-hydroxylase activity (Fevold *et al.* 1978) with a corresponding increase in the amount of $P450_{17\alpha}$ protein (Chouinard and Fevold 1990).

Upon ACTH stimulation an increase in adrenal CYP17 mRNA resulting in an increase in adrenal $P450_{17\alpha}$ has been demonstrated in cattle (John *et al.* 1986) and humans (Brentano *et al.* 1990). The purpose of this research was to determine whether the increase in $P450_{17\alpha}$ in rabbits is due to an increase in CYP17 mRNA as is seen in other species.

EXPERIMENTAL METHODS

Male New Zealand White rabbits from R&R Rabbitry (Stanwood, WA) were housed at the University of Montana animal laboratory and given food and water *ad libitum*. The rabbits were injected intramuscularly twice daily, in the morning and evening. Rabbits were injected for either a four or six day period. Experimental rabbits were injected with 28 to 40 IU ACTH (Armour Pharmaceutical Co., Kankakee, IL). ACTH was injected in 5% beeswax in peanut oil containing 0.5% phenol (ICN, Irving, CA) as a preservative (Fevold 1967) or from the commercially prepared Acthar gel. All ACTH was a gift from Dr. R. Schlueter, Armour Pharmaceutical Co. Control rabbits were either injected with the control vehicle or left uninjected.

Following completion of the injection period, rabbits were anesthetized with sodium pentobarbital then sacrificed by exsanguination. The adrenal glands, testes, and a portion of the liver were removed from the animals. The excised tissues were immediately frozen in liquid nitrogen and used for isolation of either total RNA or microsomal protein.

RNA ISOLATION AND QUANTITATION

RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (1987). The tissue was

weighed and homogenized in 4M guanidinium thiocyanate (Fisher, Fair Lawn NJ), 25mM sodium citrate (Sigma, St. Louis, MO), 0.5% sodium sarcosyl (Sigma), and 0.1 M ß-mercaptoethanol (Sigma) using a Tissuemizer homogenizer (Tekmar, Cincinnati, OH). One tenth volume 3M sodium acetate (Fisher), pH 4.0; an equal volume water saturated phenol (ICN); and two tenths volume chloroform (Fisher) were added to the homogenized tissue and mixed by inversion. The mixture was centrifuged to separate the aqueous phase from the phenol/chloroform phase. The aqueous phase was collected and the RNA precipitated by adding two volumes isopropanol and storing at -20°C for at least one hour. The precipitate was collected by centrifugation and the supernatant discarded. The pellet was redissolved in homogenate buffer and reprecipitated with isopropanol as above. The RNA pellet was air dried then redissolved in 10mM Tris (USB, Cleveland, OH) and 1mM EDTA (Sigma), pH 7.2. The quantity and quality of RNA isolated was determined spectrophotometrically by measuring the optical density of a 1:300 dilution of the RNA in 10 mM Tris and 1 mM EDTA at 260 and 280 nm using a Gilford model 2400 spectrophotometer. For all manipulations of RNA DEPC treated solutions and RNase free glass wear was used.

Northern blot analysis was preformed on the isolated RNA samples. RNAs were separated by electrophoresis through agarose gels containing formaldehyde (protocol from Sambrook *et al.* 1989). Gels contained 1.0% agarose GTG (FMC, Rockland ME), 2.0 mM MOPS (Boehringer Mannheim GbmH, Mannheim W. Germany), 5.0 mM sodium acetate, 1.0 mM EDTA, 0.25 $\mu g\ ml^{-1}$ ethidium

bromide (Sigma), and 1.0 M formaldehyde (Fisher). To prepare samples for electrophoresis RNA was precipitated by adding sodium acetate to 0.3M and 2 volumes ethanol and storing at -20°C for at least one hour, collected by centrifugation, washed with an equal volume of 70% ethanol, and dried. The RNA was redissolved in a buffer containing 2.0 mM MOPS, 5.0 mM sodium acetate, 1.0 mM EDTA, 1.0 M formaldehyde, and 50% deionized formamide (Fisher). The solution was heated to 65°C for 15 minutes to denature the RNA, then cooled on ice. To each RNA sample 2μl bromophenol blue/xylene cyanol tracking dye mixture was added. Samples were loaded onto a gel submerged in running buffer, 2.0 mM MOPS, 5.0 mM sodium acetate, 1.0 mM EDTA, and 1.0 M formaldehyde. Samples were electrophoresed at 80 volts until the bromophenol blue ran out of the gel. After electrophoresis RNA was visualized under ultraviolet light and photographed with a Polaroid MP-4 Land camera and Polaroid type 55 film. The locations of 28S and 18S ribosomal RNA bands were noted.

After electrophoresis the RNA was transferred to a Zeta Probe (BioRad, Richmond, CA) nylon membrane by wicking from below with a solution of 1.5 M sodium chloride (Fisher) and 1.5 M sodium citrate, 10X SSC (Thomas 1983). After transfer was complete the locations of the 28S and 18S ribosomal RNA bands were marked and the membrane baked in a 80°C vacuum oven for one hour to irreversibly bind the RNA. Membranes with bound RNA were prehybridized at 45°C with gentle shaking over-night in Seal-A-Meal bags in a buffer of 50%

formamide; 25 mM potassium phosphate (EM Science, Cherry Hill, NJ), pH 7.4; 5X Denhardt's solution, and 0.5 mg ml^{-1} sheared denatured salmon sperm DNA (Sigma). The membranes were hybridized in a buffer containing 50% fromamide; 25 mM potassium phosphate, pH 7.4; 5X Denhardt's solution, 0.5 mg/ml sheared denatured salmon sperm DNA, 10% dextran sulfate (Sigma), and the denatured ^{32}P labeled cDNA probe at 45°C in a shaking waterbath (Thomas 1983).

For analysis of RNA spotted onto nylon membranes, RNA samples were dissolved in a mixture of 2X SSC, 2.0 M formaldehyde, and 50% formamide. A Zeta Probe nylon membrane (BioRad) was wetted in 20X SSC and placed in a hybridot dot blot manifold (BRL, Gaithersburg, MD). RNA samples were spotted onto the membrane using suction. Slots containing RNA samples were washed twice with 10X SSC, and the membrane was baked in an 80° C vacuum oven to bind the RNA to the membrane. Membranes were prehybridized and hybridized in the same manner as northern blots.

Complementary DNA probes were used for northern blot and dot blot hybridizations. These probes were labeled with (α-^{32}P)dCTP 3000Ci $mmol^{-1}$ (NEN Research products, Boston, MA) using a Boehringer Mannheim random primed DNA labeling kit or by PCR (Perkin Elmer, Norwalk, CT) incorporation of (α-^{32}P)dCTP 3000Ci/mmol (NEN Research products). Probes were purified to remove unincorporated nucleotides by centrifugation through a G-50 Sephadex column as described by Sambrook *et al* 1989.

CYP17 mRNA was detected on each membrane with a rat CYP17 cDNA

probe (Fevold *et al.* 1989). CYP17 probes were either a 1.1 kb KpnI (Promega, Madison, WI) - SacI (Promega) restriction fragment representing bases 102 to 1352, a 0.4 kb BamHI (Boehringer Mannheim) - PstI (Promega) restriction fragment representing bases 936 to 1376, or 0.5 kb PCR amplified fragment of the rat CYP17 cDNA. The sequences of the PCR primers used were TTCAATGACCGGTCT and CTAGAGCCACGCGGTCC, which amplified the sequence from base 1026 to 1489.

Hybridized membranses were washed twice in 2X SSC and 0.1% SDS at room temperature for 5 minutes per wash, in 1X SSC and 0.1% SDS at room temperature for 30 minutes, and in 0.5X SSC and 0.1% SDS at 50°C for 30 minutes. Binding of the probe was detected by autoradiography using Kodak XAR-5 film. Blots were exposed to film in a cassette with intensifying screens for two to ten days at -80° C. Film was developed using Kodak GBX (Eastman Kodak, Rochester NY) developer and fixer. Density of the signal on the developed film representing the hybridized bands was determined by scanning densitometry using an EC densitometer (St. Petersburg FL).

Membranse with RNA were then stripped of probe by submerging in 0.1X SSC and 0.1% SDS for 30 minutes at 80°C, prehybridizing, and rehybridized as above, but using a ^{32}P labeled 0.9 kb EcoRI/BamHI restriction fragment representing bases 490 to 1389 of the rat CYP21 cDNA. Efficiency of stripping was checked by overnight exposure to film prior to reprobing. Hybridization was detected by autoradiography and intensity of signal determined as above.

PROTEIN ISOLATION AND DETECTION

Microsomal proteins were isolated by the procedure described by Fevold and Drummond (1972). The tissue was weighed, minced on a gauze pad saturated in 0.9% NaCl, rinsed with 0.154 M KCl (Fisher) in 0.1 M $NaPO_4$, 1 mM DTT (Sigma), pH 6.8, and homogenized in 0.25 M sucrose (Sigma) in 0.1 M $NaPO_4$,1 mM DTT, pH 6.8, using a ground glass homogenizer. Homogenate was centrifuged at 9000 xg for 20 minutes to remove plasma membranes, mitochondria, unbroken cells, and cell debris. The supernatant was recentrifuged at 17,500 xg for 30 minutes to remove light mitochondria, and the resulting supernatant centrifuged at 105,000 xg for one hour to pellet the microsomes. The pellet from the last centrifugation was washed by resuspending in 0.154 M KCl in 0.1 M $NaPO_4$, 1.0 mM DTT and recentrifuging at 105,000 xg for one hour. The final pellet was resuspended in 0.25 M sucrose in 0.1 M $NaPO_4$, 1 mM DTT, and 20% glycerol and stored at -20° C. Quantity of protein isolated was determined using the Lowry method (Lowry *et al.* 1951).

Proteins from the microsomal faction were separated by SDS-PAGE (Laemmli 1970) for western blotting as described by Chouinard and Fevold (1990). The gel consisted of a separating gel of 10% acrylamide (Sigma), 0.27% N,N-methylene bis acrylamide (Sigma), 0.375 M Tris-HCl, pH 8.8, 2 mM EDTA, 0.025% (v/v) TEMED (Sigma), and 0.1% ammonium persulfate (Sigma), with a stacking gel of 5% acrylamide, 0.13% N,N-methylene bis acrylamide, 0.125M Tris-HCl, pH 6.8, 2 mM EDTA, 0.5% (v/v) TEMED, and 0.1% ammonium persulfate.

Gels were run with a buffer of 25 mM Tris, 192 mM glycine (Sigma), and 0.1% SDS at pH8.3.

Protein samples of 20 to 40 µg were mixed with an equal volume of sample solubilizing buffer to a final concentration of 2% SDS, 1 M ß-mercaptoethanol, 20% glycerol, 0.125 M tris-HCl, pH 6.8, and 0.01% bromophenol blue as a tracking dye. Molecular weight markers (BioRad low range) were treated the same as the microsomal protein samples. Samples in solubilizing solution were vortexed to thoroughly mix then boiled for 10 minutes in a water bath to denature the proteins. Immediately after heating, the samples were loaded onto the gel and electrophoresed until the dye front reached the end of the gel.

After electrophoresis the molecular weight marker lanes were removed, fixed in 45% methanol, 1% acetic acid for one hour and stained with 0.1% (w/v) Coomassie brilliant blue in 50% methanol for one hour. The marker lanes were cleared of excess dye by soaking in 45% methanol, 1% acetic acid for two hours. Microsomal proteins were transfered to a nitrocellulose membrane (BioRad) by electroblotting in a Genie blotting apparatus (Idea Scientific, Corvallis, OR) filled with 20 mM sodium phosphate buffer, pH 8.0. The nitrocellulose membrane with the transferred microsomal protein was removed from the blotting apparatus and soaked in a blocking solution of 10 mM Tris in 150 mM NaCl, pH 7.4, with 0.3% Tween-20 (BioRad), TTBS, and 2% nonfat milk for one hour. Blots were soaked overnight in TTBS containing 1 µg ml^{-1} primary antibody. After allowing the primary antibody to complex with the protein, the blots were washed six times, 15

minutes per wash, in TTBS, followed by an incubation for one hour in TTBS containing 2-5 X 10^5 cpm/ml ^{125}I labeled protein A, specific activity > 30 $\mu Ci\ \mu g^{-1}$ (ICN). The blots were washed again six times in TTBS, wrapped in plastic wrap, and exposed to Kodak XAR-5 film in a cassette with intensifying screens at -70° C for 2 days. After exposure the film was developed with Kodak GBX chemicals. Intensity of bands on autoradiograms was determined by scanning densitometry with an EC densitometer.

$P450_{17\alpha}$ protein was detected on the western blots using a rabbit anti-pig $P450_{17\alpha}$ antibody provided by Dr. Anita Payne. Some blots were then stripped of antibody by incubation at 70° C for 30 minutes in a solution of 2% (w/v) SDS, containing 100 mM ß-mercaptoethanol and 62.5 mM Tris-HCl, pH 6.8, (Kaufmann *et al.* 1987), both stripped or unstripped blots were reprobed with a rabbit anti-bovine $P450_{c21}$ antibody provided by Dr. Michael Waterman.

RESULTS

EFFECTS OF ACTH STIMULATION

The effects of ACTH stimulation on adrenal size and adrenal RNA yield are shown in Table 1. Adrenal glands from rabbits injected with ACTH were substantially larger then those from unstimulated animals. In the first experiment in which the rabbits were injected for four days, there was a 36% increase in adrenal weight of the ACTH-stimulated animals over the control animals. In this experiment the ACTH-injected rabbits were an average of 12% larger than those in the control group, which might account for some of the difference in adrenal size. In the first six day injection experiment a 15% increase in adrenal size was

EXP NO.			AVERAGE BODY WEIGHT, kg	AVERAGE ADRENAL WEIGHT, g	TOTAL RNA/ ADRENAL, µg	TOTAL RNA/ g TISSUE, µg
1	4 DAY	ACTH	3.05 ± 0.18	0.1375	177	1288
		CONTROL	2.68 ± 0.29	0.0875	148	1697
2	6 DAY	ACTH	*	0.0875	118	1347
		CONTROL	*	0.075	27**	360**
3	6 DAY	ACTH	2.07 ± 0.19	0.117	293	2515
		INJECTED CONTROL	2.06 ± 0.11	0.080	127	1589
		UN-INJECTED CONTROL	2.25 ± 0.12	0.082	154	1889

Table 1: ACTH-stimulated and control rabbit adrenal weights and RNA yield. All data an average of pool of adrenals from 4 rabbits

* Not recorded.

** Apparent loss of RNA during preparation.

observed in the ACTH-stimulated rabbits, the possible contribution of rabbit size to this increase in adrenal weight cannot be determined as the weights of the rabbits were not recorded. Adrenal size was also increased in the third experiment (Table 1). The ACTH-stimulated rabbit adrenal gland were 32% larger than those of both the injected control and uninjected control. The amount of RNA and protein isolated from each adrenal was greater in the ACTH-stimulated as compared to that of the control group(s) in all experiments. These results confirm effective stimulation by ACTH.

Figure 5 shows northern blot analysis of RNA isolated from rabbits after four days of stimulation with ACTH and from control rabbits injected with vehicle. This blot was probed with a 1.1 kb restriction fragment of the rat CYP17 cDNA which was labeled with (α-^{32}P) dCTP by random primed labeling. Lanes 5 and 6 contain 40 and 20 µg RNA isolated from the adrenals of ACTH-stimulated rabbits and lanes 9 and 10 contain 40 and 20 µg RNA isolated from control rabbit adrenals. Lanes 1 and 3 contain 20 µg and lanes 2 and 4 contain 10 µg of rat testis RNA, and lanes 7 and 8 contain 40 and 20 µg rabbit testis RNA, all as positive controls. Lanes 11 and 12 contain 40 and 20 µg rabbit liver RNA as negative controls. Scanning densitometry of the autoradiogram showed no significant difference in the intensity of bands of equal concentrations of adrenal RNA from ACTH-stimulated and control rabbits (Table 2). This result indicated that ACTH caused no change in the amount of CYP17 mRNA per microgram of

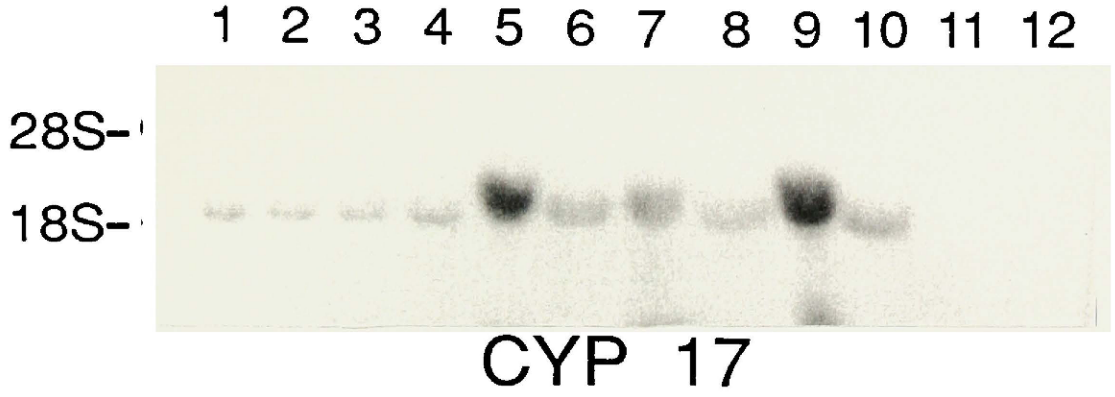

Figure 5: Northern blot analysis of RNA from rabbits after 4 days of injection with ACTH. RNA was probed with a ^{32}P labeled 1.1 kb rat CYP17 cDNA. Lanes 1-4: rat testis RNA; 5 and 6: 40 and 20 µg ACTH stimulated rabbit adrenal RNA; 7 and 8: 40 and 20 µg rabbit testis RNA; 9 and 10: 40 and 20 µg control rabbit adrenal RNA; 11 and 12: 40 and 20 µg rabbit liver RNA.

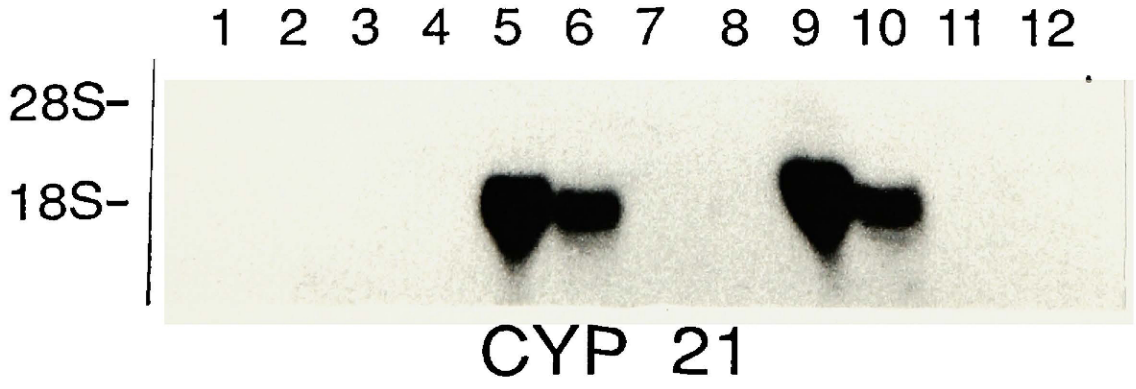

Figure 6: The filter from the experiment in Figure 5 was stripped and reprobed with the 0.9 kb rat CYP21 cDNA.

EXP. NO.	INJECTION DURATION	AMOUNT AND SOURCE OF ADRENAL RNA	INTENSITY OF CYP17 BANDS[3]	INTENSITY OF CYP21 BANDS[3,4]
1	4 DAY	20 µg ACTH- STIMULATED	2.21	21.4
		20 µg INJECTED CONTROL	2.09	22.8
		40 µg ACTH STIMULATED	9.86	56.1
		40 µg INJECTED CONTROL	12.6	57.8
2	6 DAY[1]	10 µg ACTH- STIMULATED	2.41	33.7
		10 µg INJECTED CONTROL	2.46	45.5
		20 µg ACTH- STIMULATED	9.06 *	82.5
		20 µg INJECTED CONTROL	4.45	87.4
3	6 DAY[2]	40 µg ACTH- STIMULATED	8.96	5.22
		40 µg INJECTED CONTROL	15.9	22.6
		40 µg UNINJECTED CONTROL	10.6	29.9
		80 µg ACTH- STIMULATED	19.3	17.9
		80 µg INJECTED CONTROL	29.0	52.2
		80 µg UNINJECTED CONTROL	21.4	57.3

Table 2: Intensities of CYP17 bands and CYP21 on northern blots as determined by scanning densitometry.

* Nonspecific probe binding apparent in this band.
[1] Injected twice daily with 40 IU porcine ACTH in beeswax in peanut oil.
[2] Injected twice daily: In the morning with 40 IU porcine ACTH in beeswax in peanut oil; in the evening with 40 IU ACTHar gel.
[3] Arbitrary units.
[4] Comparisons should not be drawn between CYP17 and CYP21 blots.

total RNA. The blot was stripped of CYP17 cDNA probe and reprobed with the 0.9 kb restriction fragment of CYP21 cDNA. This was done as a negative control, since previous results (Chouinard and Fevold, 1990) had demonstrated that ACTH stimulation has a negligible effect on $P450_{c21}$ protein expression. The intensity of the signal from the CYP21 probe was also the same from ACTH-stimulated and control rabbits at equal RNA concentrations (Table 2). Since microsomal protein was not isolated from these animals, a western blot analysis could not be done. From this experiment it appeared that ACTH stimulation had no effect on the amount of either CYP17 or CYP21 mRNA per microgram of total RNA.

Since a 4 day stimulation resulted in no increase in CYP17 mRNA, a longer stimulation period was used in the next experiment. Analysis of RNA isolated from rabbits injected for six days with 40 IU ACTH in 1% beeswax in peanut oil gave results similar to the 4 day injection. There appeared to be equal amounts of CYP17 mRNA per microgram of total RNA in the adrenals of both ACTH-stimulated and control rabbits by northern blot (Figure 7) as confirmed by densitometry (Table 2). The level CYP21 mRNA also appeared equal, by densitometry, in both experimental and control groups (Table 2 and Figure 8).

ACTH stimulation in Experiment 2 (Table 2) was confirmed by western blot analysis of the adrenal microsomal proteins from the ACTH-stimulated and control rabbits. Figure 9 is a western blot probed with the $P450_{17\alpha}$ antibody. Lanes 4 and 5 contain 40 and 80 μg adrenal microsomal protein isolated from ACTH stimulated animals and lanes 6 and 7 contain 40 and 80 μg adrenal

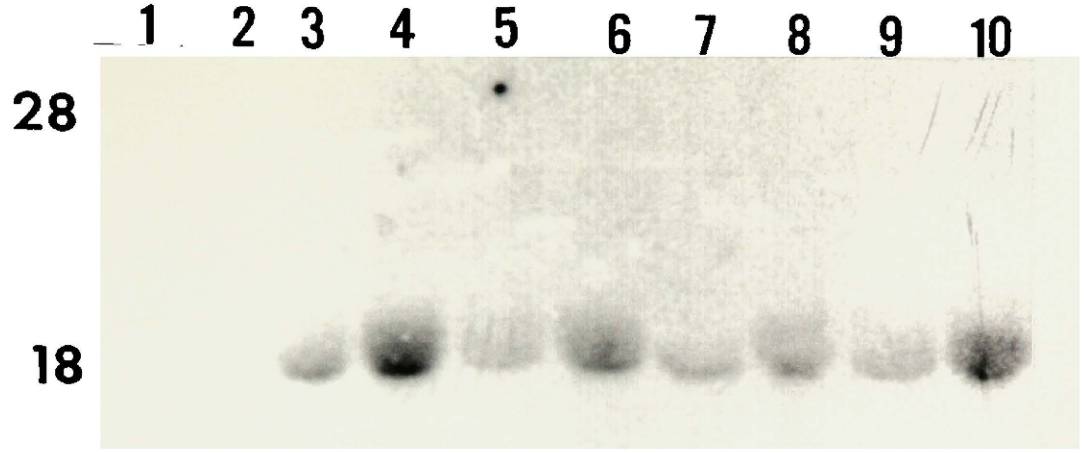

Figure 7: Northern blot analysis of RNA from rabbits after 6 days of ACTH injection using a PCR generated rat CYP17 cDNA probe. Lanes 1 and 2: 20 and 40 µg rabbit liver RNA; 3 and 5: 20 µg rabbit testis RNA; 4 and 6: 40 µg rabbit testis RNA; 7 and 8: 20 and 40 µg control rabbit adrenal RNA; 9 and 10: 20 and 40 µg ACTH stimulated rabbit adrenal RNA.

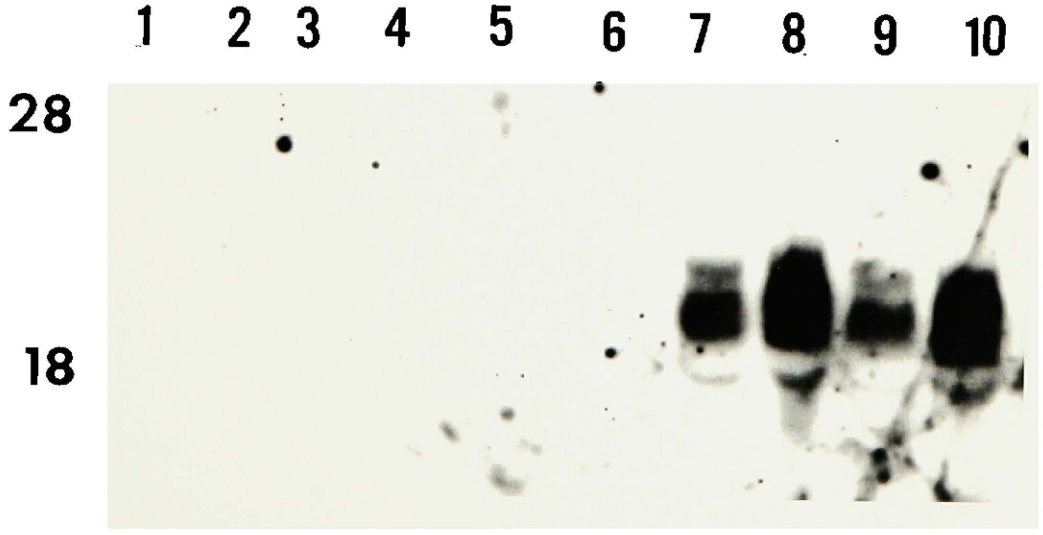

Figure 8: The filter from the experimetn in Figure 7 was stripped and reprobed with a 0.9 kb rat CYP21 cDNA.

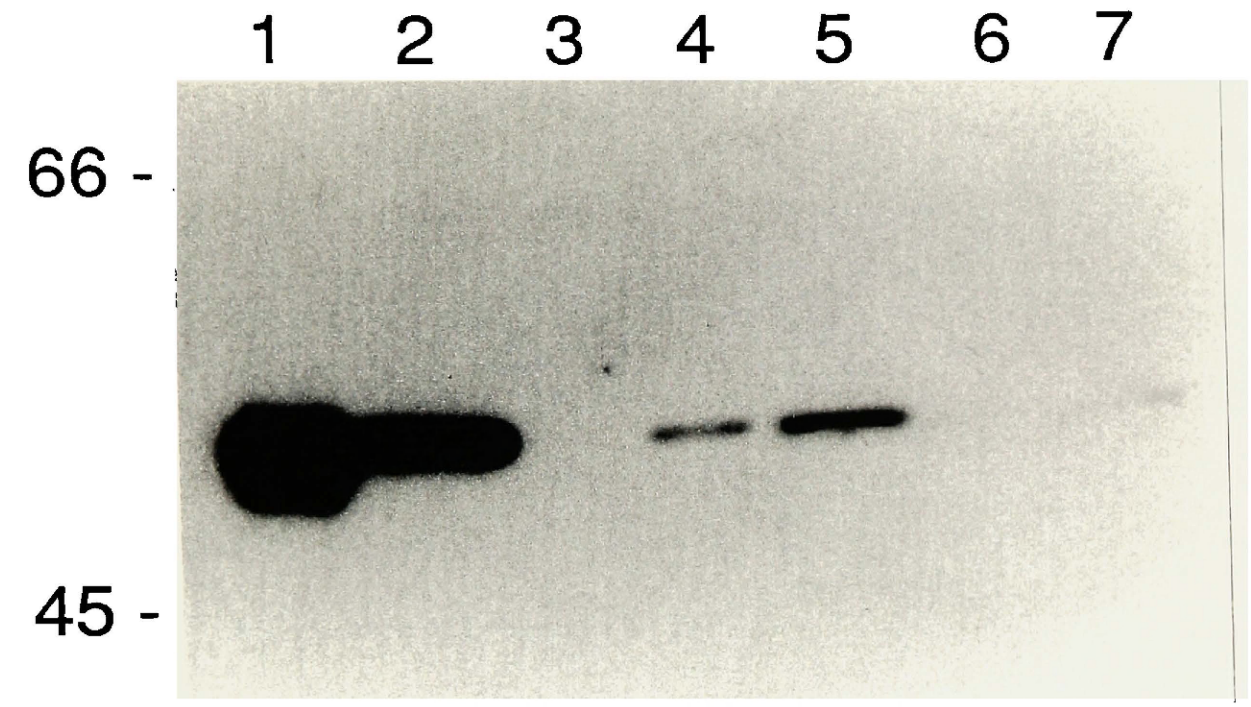

Figure 9: Western blot analysis of $P450_{17\alpha}$ protein in microsomal proteins. Lane 1: 40 µg guinea pig adrenal; 2: 40 µg rabbit testis; 3: 40 µg rabbit liver; 4 and 5: 40 and 80 µg ACTH stimulated rabbit adrenal; 6 and 7: 40 and 80 µg control rabbit adrenal.

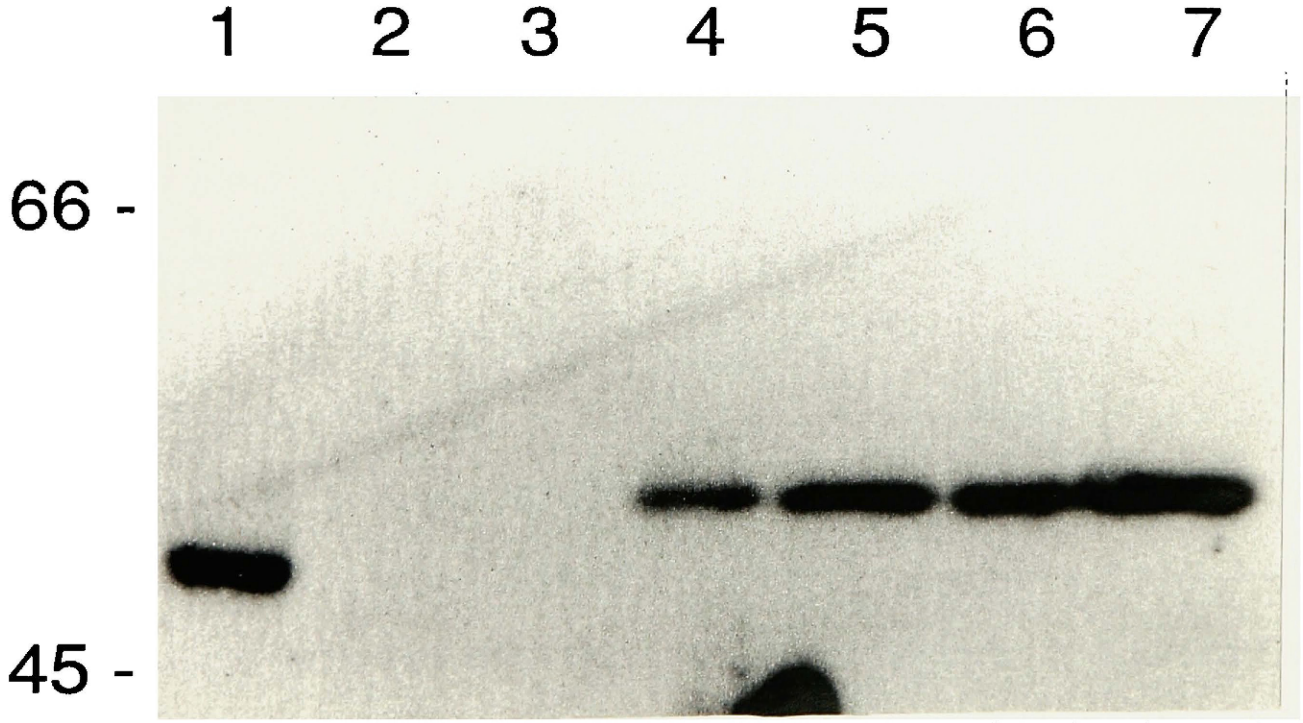

Figure 10: The filter from the experiment in Figure 5 was stripped and reprobed with the $P450_{c21}$ antibody.

microsomal protein isolated from control rabbits. The blot shown in Figure 10 is the same blot as in Figure 9 stripped of the $P450_{17\alpha}$ antibody and reprobed with the $P450_{c21}$ antibody detected with ^{125}I labeled protein A. Scanning densitometry of theses blots showed an unmeasurable amount $P450_{17\alpha}$ protein present in the unstimulated animals and a densitometry readings of 1.43 and 4.18 for 40 and 80 μg microsomal protein in the ACTH-stimulated rabbits. The $P450_{c21}$ autoradiogram had densitometry readings of 4.86 and 9.08 for 40 and 80 μg microsomal protein from ACTH stimulated rabbits, and 10.24 and 12.00 for 40 and 80 μg from the control group, an actual decrease of $P450_{c21}$ protein per microgram of adrenal microsomal protein.

The unexpected amounts of CYP17 mRNA in the injected control adrenals,and no evidence of an increase resulting from the injection of ACTH, suggested that the stimulation of endogenous ACTH secretion resulting from the act of injecting the animals might have been responsible for the CYP17 mRNA in the control animals. The six day injection experiments were repeated adding an uninjected control group and using ACTHar gel, a more potent ACTH preparation, for the evening injection of ACTH in an attempt to obtain a higher degree of stimulation. Judging by the increase in adrenal weight and total RNA, stimulation was greater in this experiment than in the previous two (Table 1). In northern blot (Figure 11) and dot blot (Figure 13) analyses of the RNA isolated from this experiment there is 15-44% less CYP17 mRNA per μg total RNA in ACTH stimulated animals compared to both control groups (Table 2). Also, when

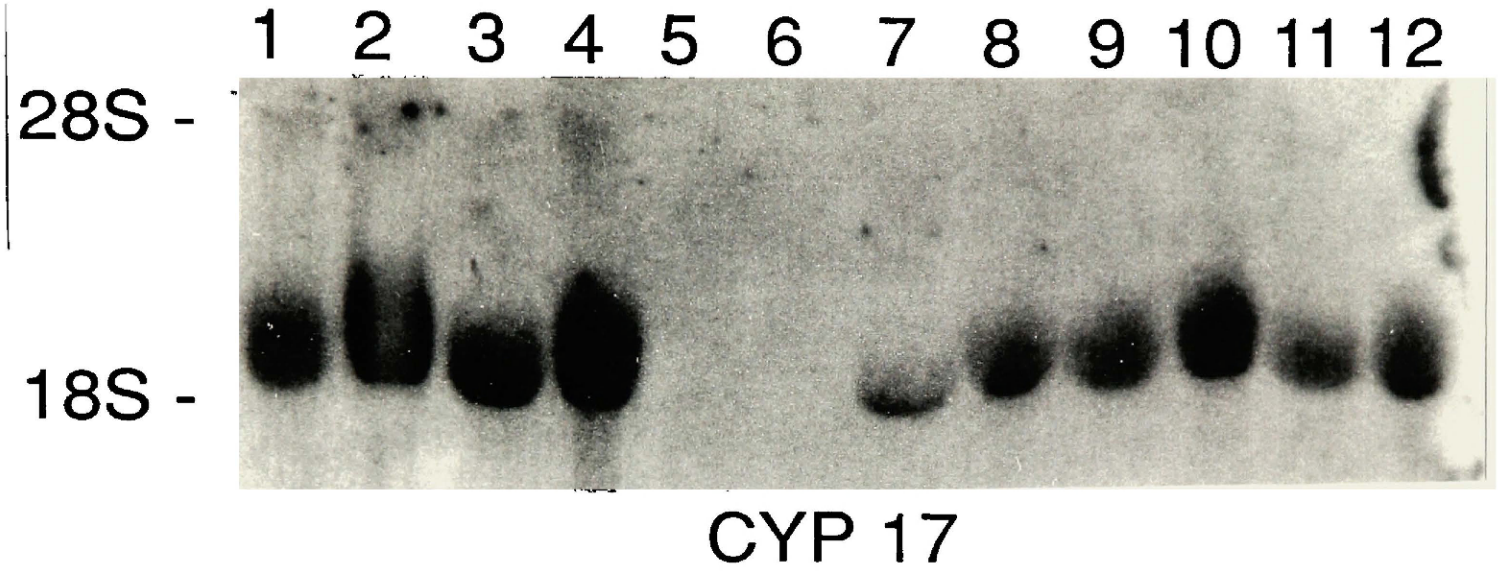

Figure 11: Northern blot analysis of RNA from rabbits after 6 days of ACTH and Acthar gel injection using a 0.4 kb rat CYP17 cDNA probe. Lanes 1 and 2, 40 and 80 µg ACTH-stimulated rabbit testis RNA; 3 and 4, 40 and 80 µg uninjected control rabbit testis RNA; 5 and 6, 40 and 80 µg liver RNA; 7 and 8, 40 and 80 µg ACTH-stimulated adrenal RNA; 9 and 10 injected control adrenal RNA; 11 and 12 uninjected control adrenal RNA.

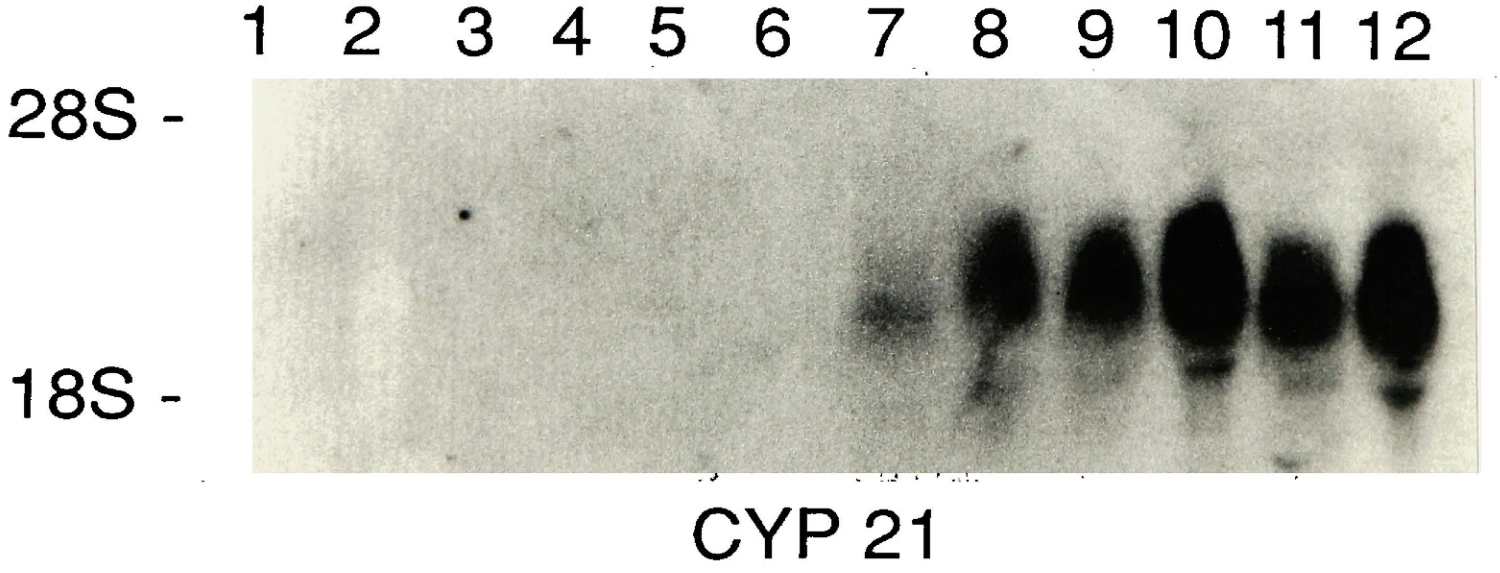

Figure 12: The filter from the experiment in Figure 11 was stripped and reprobed with a 0.9 kb rat CYP21 cDNA.

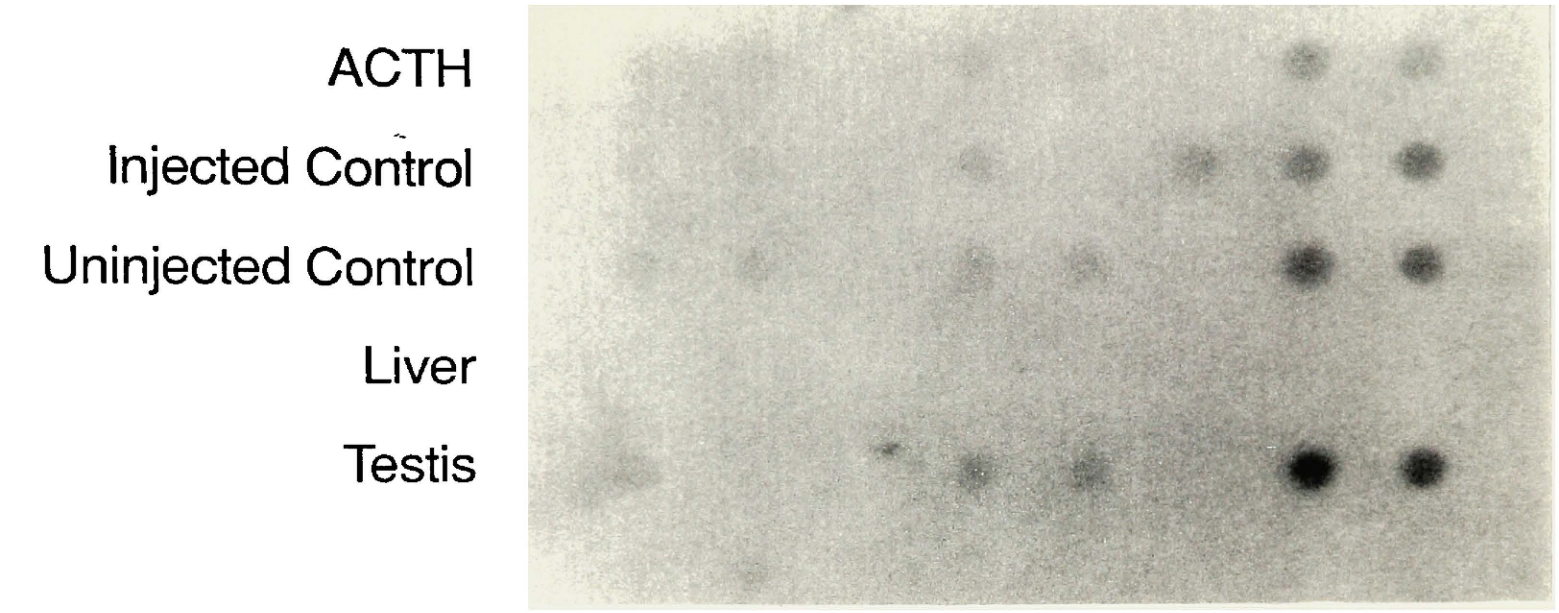

Figure 13: RNA dot blot probed with 0.4 kb rat CYP17 cDNA. All samples were applied in duplicate. The first three lines are adrenal RNA. The liver and testis were from uninjected control rabbits. The second 10 µg sample of the injected control and the second 5 µg sample of the testis RNA were inadvertently displaced one position to the right and below, respectively.

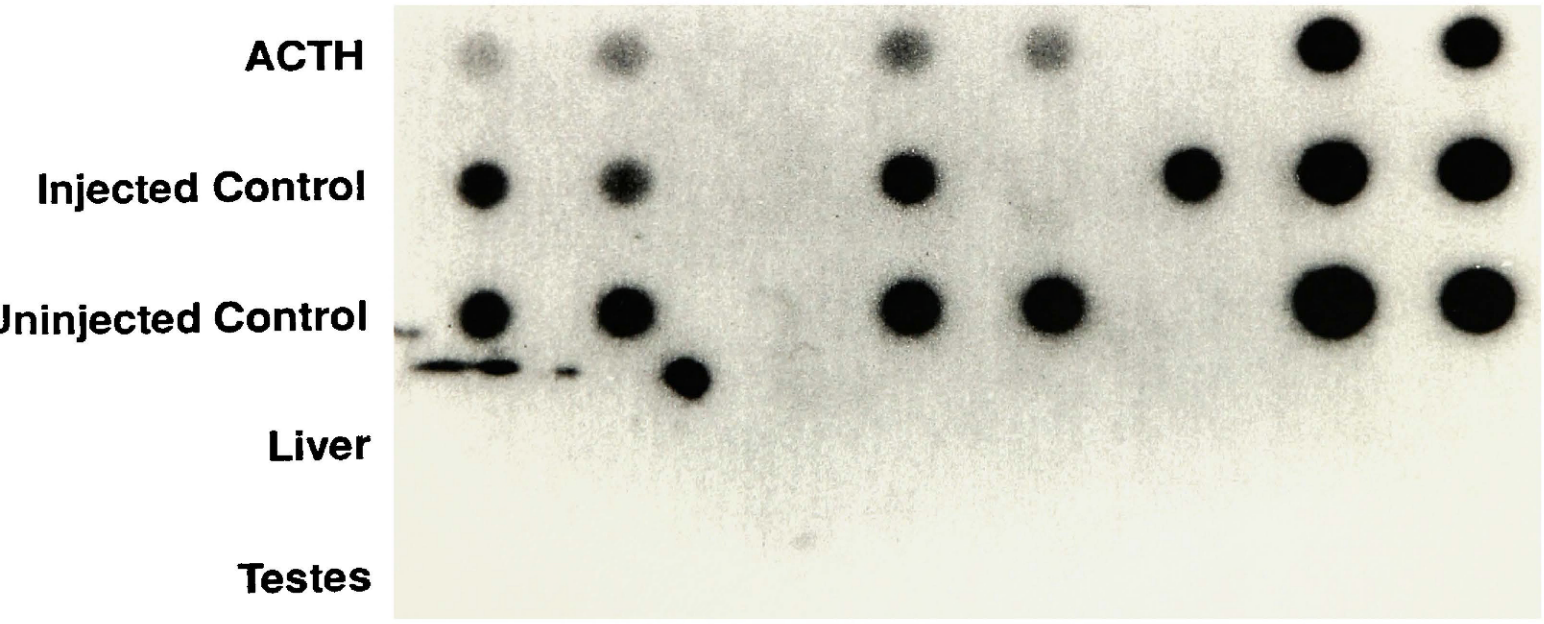

Figure 14: The filter from the experiment in Figure 13 was stripped and reprobed with a 0.9 kb rat CYP21 cDNA.

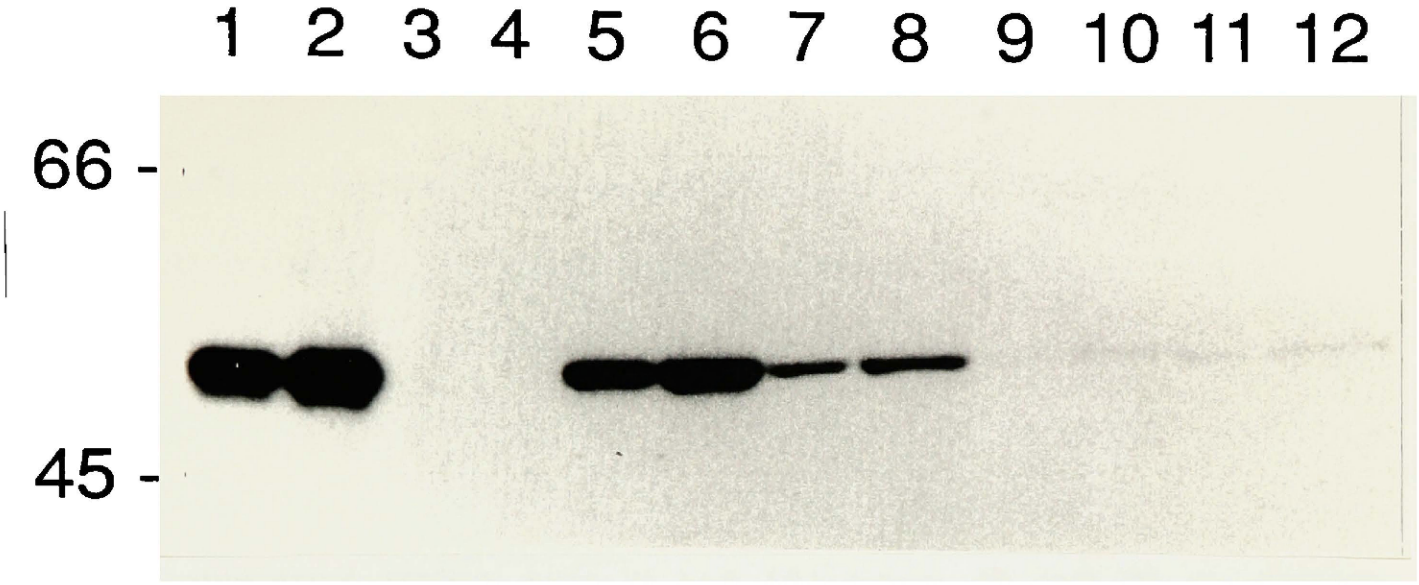

Figure 15: Western blot analysis of $P450_{17\alpha}$ protein in microsomal proteins. Lanes 1 and 2, 40 and 80 µg guinea pig adrenal; 3 and 4, 40 and 80 µg liver; 5 and 6, 40 and 80 µg rabbit testis; 7 and 8, 40 and 80 µg ACTH-stimulated adrenal; 9 and 10, 40 and 80 µg injected control adrenal; 11 and 12, 40 and 80 µg uninjected control adrenal.

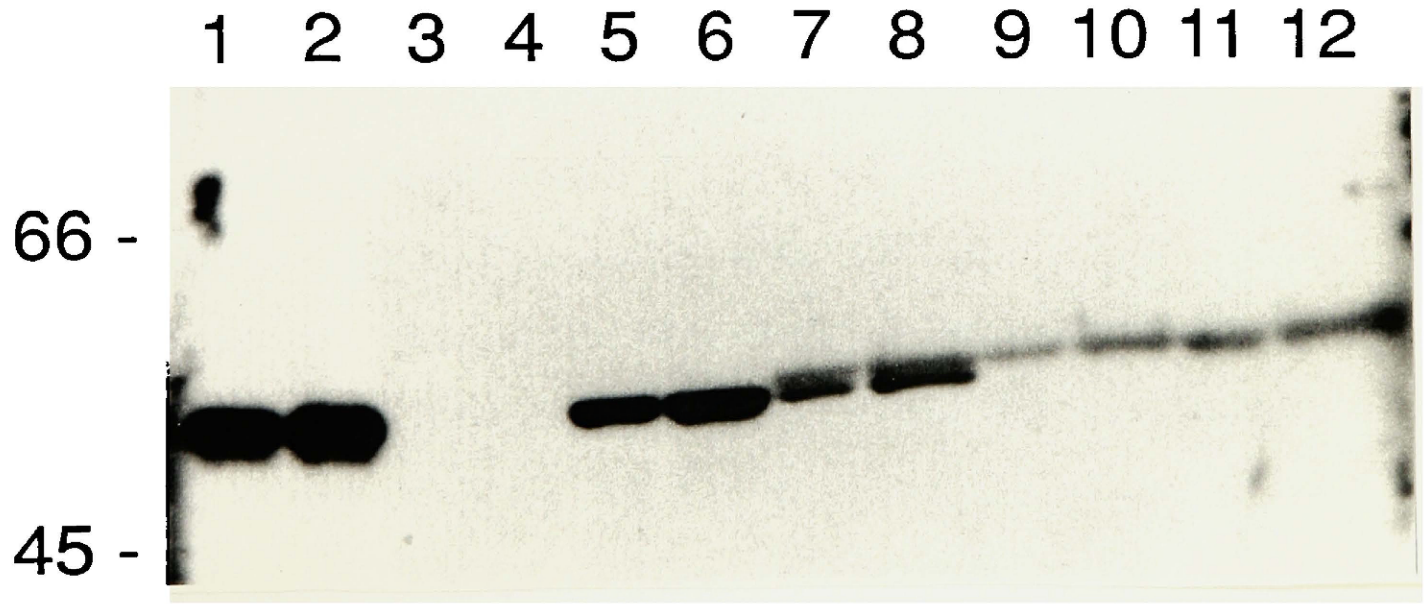

Figure 16: The filter from the experiment in Figure 15 was probed with a second

antibody, $P450_{c21}$

the blots were stripped and reprobed with the CYP21 cDNA (Figures 12 and 14) there was 18 to 39% less CYP21 mRNA per µg total RNA, presumably from the 46% stimulation in total RNA production (Table 2).

ACTH stimulation was confirmed by western blot. Figure 15 shows the $P450_{17\alpha}$ detection in microsomal protein SDS-polyacrylamide electrophoretic patterns. Lanes 1 and 2 are guinea pig adrenal, 3 and 4 are rabbit liver, 5 and 6 are rabbit testis, 7 and 8 contain 40 and 80 µg adrenal microsomal protein from ACTH stimulated rabbits, lanes 9 and 10 contain 40 and 80 µg adrenal microsomal protein from injected control rabbits, and lanes 11 and 12 contain 40 and 80 µg adrenal microsomal protein from uninjected control rabbits. Figure 16 is the same blot as 15 probed with the $P450_{c21}$ antibody without prior stripping of the $P450_{17\alpha}$ antibody. The data from this experiment confirm those of the previous experiment and show no apparent stimulation of an increase in CYP17 mRNA per microgram of total RNA but a dramatic increase in $P450_{17\alpha}$ protein per microgram of microsomal protein.

The data obtained from analysis of the CYP17 RNA and $P450_{17\alpha}$ protein were analyzed as a fraction of the pool of RNA or microsomal protein isolated per adrenal to determine whether the total amount of either had been altered due to ACTH stimulation. From the CYP17 mRNA or $P450_{17\alpha}$ levels per adrenal the ratios between ACTH stimulated rabbits and control rabbits were determined (Table 3).

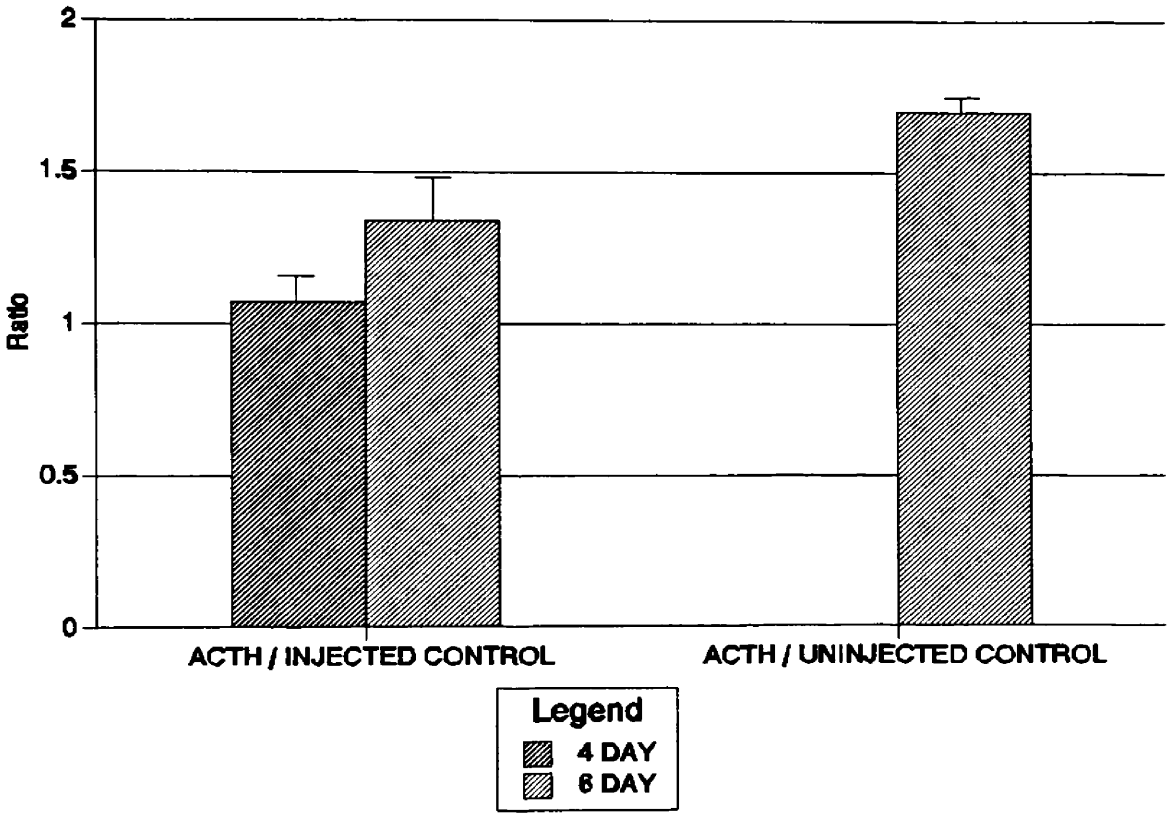

Figure 17: Ratio of CYP17 mRNA per adrenal in ACTH stimulated rabbits verses control rabbits. Error bars are the average deviation of two ratios.

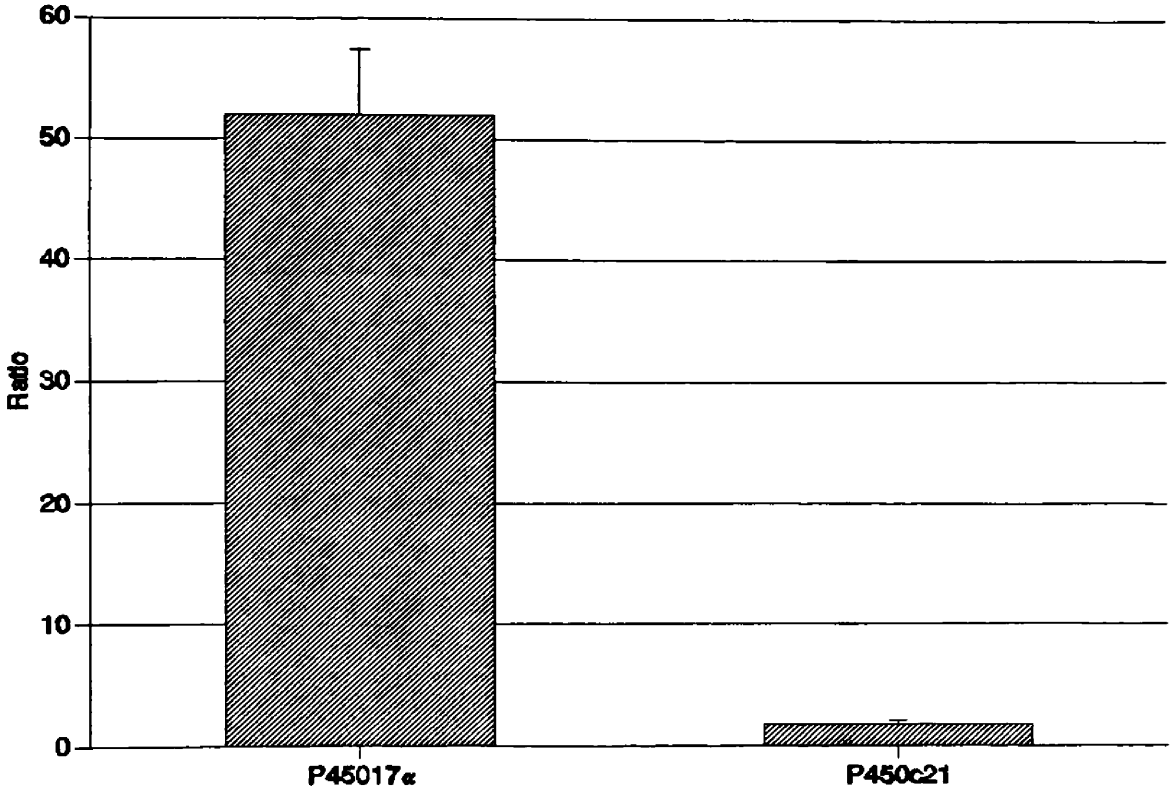

Figure 18: Ratio of $P450_{17\alpha}$ and $P450_{c21}$ protein per adrenal in ACTH stimulated verses uninjected control rabbits. Error bars are the average deviation of two ratios.

In the 4-day ACTH injection experiment the ratio of the amount of adrenal CYP17 mRNA from ACTH stimulated to control rabbits was 1.07 (Exp 1, Table 3). In the second 6-day experiment (Exp 3, Table 2) the ratio for CYP17 mRNA was 1.34 between ACTH-stimulated and injected control rabbits and 1.70 between ACTH-stimulated and uninjected controls (Figure 17). The larger amount in injected controls compared to uninjected controls (ratio 1.18) was presumably due to endogenous ACTH secretion. While the expression of CYP17 mRNA was minimally increased, the $P450_{17\alpha}$ protein per adrenal was increased over 50 fold compared to the uninjected controls (Figure 18) and 21 fold compared to the injected controls.

The level of CYP21 mRNA increased by a ratio of 1.23 for ACTH-stimulated to either injected control or uninjected control. The increase in CYP21 mRNA was accompanied by a slight increase in $P450_{c21}$ protein, a ratio of 1.8 for ACTH-stimulated to injected control and 1.12 for ACTH-stimulated to uninjected control (Figure 18). The increase in $P450_{c21}$ protein is consistent with previously reported data (Chouinard and Fevold 1990).

EXP. NO.	INJECTION DURATION		RATIO
1	4 DAYS	ACTH/INJECTED CONTROL	1.07 ± 0.14*
3	6 DAYS	ACTH/INJECTED CONTROL	1.34 ± 0.19
		ACTH/UNINJECTED CONTROL	1.70 ± 0.09

Table 3: Ratios of amount of CYP17 mRNA per adrenal between ACTH stimulated and control rabbits. *Average deviation of duplicate determinations.

DISCUSSION

ACTH stimulation of rabbits results in growth of the adrenal glands (Kass *et al.* 1954). An increase in the weight of the adrenals, an increase in microsomal protein, and an increase in RNA (Table 1) were all observed in the ACTH stimulated rabbits in these experiments. An increase in immunoreactive $P450_{17\alpha}$ protein with little increase in $P450_{c21}$ also indicated that the ACTH injections were effectively stimulating the rabbits (Chouinard and Fevold 1990).

Specific binding of the cDNA probe in northern analysis of rabbit RNA demonstrated that there was enough sequence homology between rabbit and rat CYP17 genes for cross hybridization. This allowed the use of a rat CYP17 cDNA as a probe to determine relative amounts of CYP17 mRNA isolated from ACTH-stimulated and control rabbits.

Previous results have shown an increase in the level of 17α-hydroxylase activity (Fevold 1969 and Fevold *et al.* 1978). The results presented here show a >50 fold increase in the level of adrenal $P450_{17\alpha}$ protein in ACTH stimulated rabbits verses uninjected control rabbits; this contrasted with a mere 1.7 fold increase in the amount of adrenal CYP17 mRNA (Figures 17 and 18, Table 3). Chouniard and Fevold (1990) found a 6 to 8 fold increase in immunoreactive $P450_{17\alpha}$ protein in ACTH-stimulated rabbits compared to the injected controls; in this experiment the control animals were injected with an oil injection vehicle and some stimulation of endogenous ACTH secretion may have occurred. This results

in higher $P450_{17\alpha}$ levels in controls and a smaller apparent increase in the ACTH-injected animals. This is consistent with our finding that injected controls had slightly higher CYP17 mRNA levels than did non-injected controls (Table 3). Also in this study the rabbits were injected with ACTH for 6 days, whereas in the study by Chouinard and Fevold the injection period was 3 days. The longer injection period accounts for the larger increase (21 fold) in protein in the injected controls seen in this experiment. The large increase in $P450_{17\alpha}$ protein production coupled with a significantly lower increase in CYP17 mRNA production per adrenal indicates that ACTH is mainly inducing translation of message which is already present in the adrenal. In the bovine adrenals a 2 to 4 fold increase in the ammount of CYP17 mRNA corresponds with a 4 to 10 fold increase in the ammount of $P450_{17\alpha}$ protein (John *et al.* 1986).

The effect of ACTH is not the same for all of the enzymes involved in the production of cortisol. Little to no change in 21-hydroxylase activity has been detected (Fevold and Brown 1978). At the protein level a 1.3 fold increase in $P450_{c21}$ was reported in ACTH-stimulated rabbits compared to injected control rabbits (Chouniard and Fevold 1990). In the present study the level of $P450_{c21}$ protein after 6 days of ACTH-stimulation is increased by only 1.23 fold with a 1.8 fold increase in the level of CYP21 mRNA per adrenal compared to uninjected controls. The correlation between CYP21 mRNA and $P450_{c21}$ levels here indicates that ACTH has only a slight effect at the transcriptional level of CYP21, which is consistent with pervious findings (Chouinard and Fevold 1990).

Since $P450_{17\alpha}$ is at the branch point between production of cortisol and corticosterone, it is logical that production of this enzyme is targeted as an important control point. However, it is unusual that the point of control is predominately at the translational rather than transcriptional level. This translational control seen in rabbits is unique compared to other mammals where ACTH has been shown to have a transcriptional effect on the CYP17 gene. The human (Brentano *et al.* 1990) and bovine (John *et al.* 1986) CYP17 appear to respond solely at the transcriptional level to ACTH. In hamsters ACTH-stimulated increases in CYP17 mRNA and in $P450_{17\alpha}$ have been demonstrated; however, there is a temporal lag between the peak level of CYP17 mRNA and the $P450_{17\alpha}$ protein increase. This lag suggest that there is a control point between transcription and translation. Whether this control point involves ACTH has not yet been determined (LeHoux *et al.* 1992). In guinea pigs the level of $P450_{17\alpha}$ protein is also increased upon ACTH-stimulation. *In vitro* experiments in which ACTH is added to cell cultures demonstrate a corresponding increase in the CYP17 mRNA (Provencher *et al.* 1992a); however, after *in vivo* injection of ACTH no increase in CYP17 mRNA was detected upon ACTH-stimulation (Provencher *et al.* 1992b). The discrepancy between *in vitro* and *in vivo* experiments may be due to the artificial system *in vitro*, or the high level of CYP17 mRNA already present *in vivo* in guinea pig adrenals may impair efforts to detect increases in CYP17 mRNA due to ACTH-stimulation.

TRANSLATIONAL CONTROL

There are few eukaryotic systems for which a specific translational regulatory mechanism has been defined. However, two mechanisms for translational regulation have been found: modulation of translation by initiation factor-2, which effects overall protein translation (as reviewed by Hershey 1991); and inhibition of ferritin translation by an iron-responsive mRNA-binding protein (IRE-BP). Ferritin is a protein that sequesters excess iron. IRE-BP binds to the 5' end of the ferritin mRNA when iron levels are low. When there is and excess of iron the protein dissociates from the mRNA and the message is translated (Klausner and Harford 1989; Haile *et al.* 1989).

Messenger RNAs without poly(A) tails, without an accessible 5'-m^7G cap, or with secondary structure that hinders translation have been found and are translated less efficiently. Yet no mechanism for changing the mRNA structure to allow efficient translation of these RNAs has been found. Translational activator proteins, the context of the AUG initiator codon, and compartmentalization of mRNA have also been proposed as mechanisms for translational control; however, there is no evidence of these mechanisms in eukaryotic systems (as reviewed by Kozak 1992).

FUTURE DIRECTIONS

Cloning and sequencing of the rabbit CYP17 gene, especially its 5' upstream sequences will be an important step in further understanding the control

mechanisms of this gene. Comparisons between rabbit CYP17 upstream control sequences with those of CYP17 genes from other species may be important in deciphering why there are differences in the effects of ACTH among species. Also comparison of the 5'-noncoding region of rabbit CYP17 mRNA and those of other genes under translation control may provide insight on the mechanism of regulation. Analysis of the CYP17 mRNA to determine the length of the poly(A) tail and if the mRNA is capped, may also be useful.

To determine if the barrier to translation is due to the physical subcellular location of the CYP17 mRNA, nuclear RNA could be isolated separately from cytoplasmic RNA. Localization of the CYP17 mRNA in the nucleus would indicate that ACTH promotes transport of the mRNA to the cytoplasm were it can be translated. However, if the CYP17 mRNA was found mainly in the cytoplasm this would suggest that ACTH acts to remove a repressor protein, induce an activator protein, or change the secondary structure of the message so that it can be translated.

The up to 50 fold increase in the level of P45017α with a small increase in CYP17 mRNA upon ACTH-stimulation provides a clear demonstration of translational control. This system may prove to be an excellent system in which to study translational regulation in an eukaryotic system.

List of abbreviations and commons names used. Pregnenolone, 3ß-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; 17α-hydroxypregnenolone, 3ß,17α-dihydroxy-5-pregnen-20-one; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; corticosterone, 11ß,21-dihydroxy-4-pregnene-3,20-dione; 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 11-deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11ß,17α,21-trihydroxy-4-pregnene-3,20-dione; $P450_{scc}$, cytochrome P450 side chain cleavage; 3ßHSD, 3ß-hydroxysteroid dehydrogenase/isomerase; $P450_{c21}$, cytochrome P450 21-hydroxylase; ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin; MOPS, 3-(N-morpholino) propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; tris-HCl, trihydroxymethylaminomethane hydrochloride; TEMED, N,N,N',N'-tetramethylenediamine; PCR, polymerase chain reaction.

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