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THE ROLE OF α-TOCOPHEROL ON THE REGULATION OF RAT LIVER MICROSOMAL HYDROXYLATIONS: THE EFFECTS OF CASTRATION AND ADRENALECTOMY

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

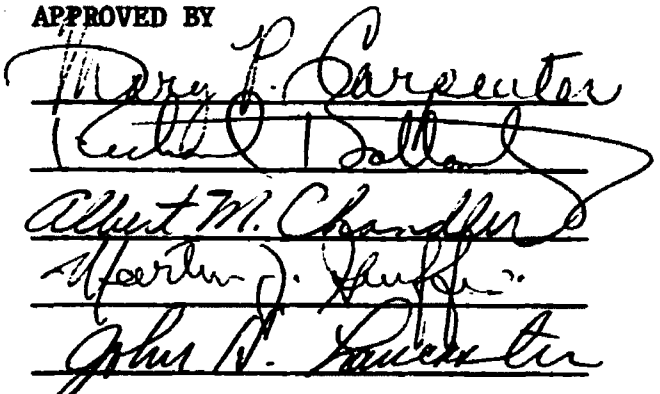
CHARLES NEILL HOWARD, JR.

Oklahoma City, Oklahoma

1972

THE ROLE OF α-TOCOPHEROL ON THE REGULATION OF RAT LIVER MICROSOMAL HYDROXYLATIONS: THE EFFECTS OF CASTRATION AND ADRENALECTOMY

APPROVED BY



DISSERTATION COMMITTEE

To

Charlotte

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THE ROLE OF α-TOCOPHEROL ON THE REGULATION OF RAT LIVER MICROSOMAL HYDROXYLATIONS: THE EFFECTS OF CASTRATION AND ADRENALECTOMY

CHAPTER I

INTRODUCTION

Liver microsomes carry out hydroxylations of a wide variety of substrates including certain drugs, carcinogenic hydrocarbons and insecticides (1-3), as well as steroids, fatty acids and other endogenous substrates (31,75). The enzyme system involved in these transformations is tightly bound to the endoplasmic reticulum, is NADPH-dependent, requires molecular oxygen and has a heme protein component, cytochrome P-450 (4-6). Electrons are transferred from NADPH to NADPH cytochrome c reductase, a flavoprotein containing FAD, then to cytochrome P-450, the active hydroxylase which binds both the substrate and oxygen (2,7,14-18). The pathway may be represented as follows:

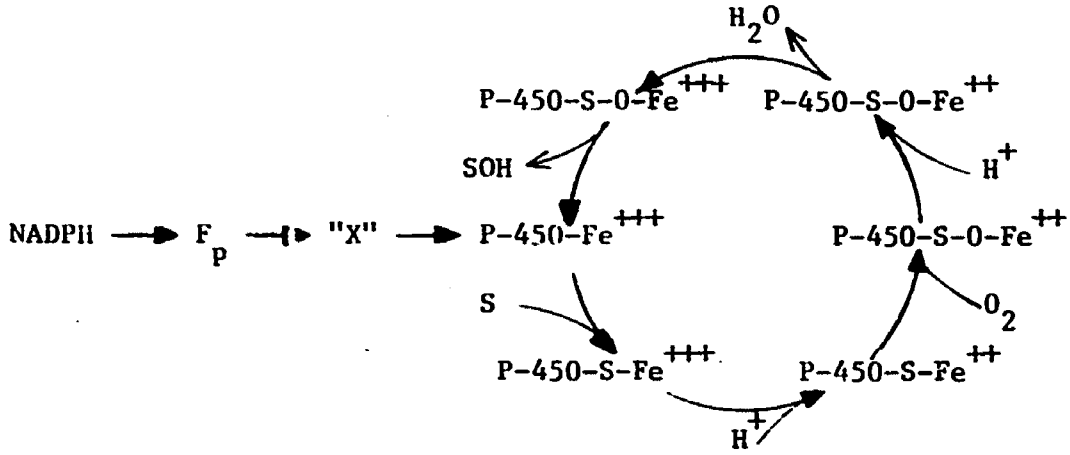
NADPH ⟶ FP ⟶ "X" ⟶ P-450

The flavoprotein shown above in the pathway for microsomal hydroxylations, which uses NADPH as an electron donor (28-30), has been solubilized and purified from liver microsomes (31-34). Purification and subsequent immunological studies, in which antibody to NADPH cytochrome c reductase was added to the system with resultant loss of drug hydroxylation,

have shown this flavoprotein-containing molecule is indeed a component of the electron transport chain (33,34,153,154).

Evidence for the "X" component in this system is very controversial. The possibility that "X" exists is supported by observations which have been made in several laboratories (158,164,139). Estabrook, et al. (13) believe that "X" may represent a crossover point from the microsomal NADH, cytochrome b_5 pathway, to the NADPH cytochrome P-450 pathway.

Cytochrome P-450 is a carbon monoxide binding pigment which, when reduced and complexed with carbon monoxide, has a difference spectrum with a maximum at 450 mμ and a minimum at 408 mμ. The cytochrome serves, not only as the oxygen activating moiety (2,15,16) of the microsomal hydroxylating system, but also as the site for substrate binding (17,18). Cytochrome P-450 apparently has two different types of binding sites for substrates, "type I" and "type II". "Type I" substrates such as codeine, aminopyrine, hexabarbital and ethylmorphine are thought to bind to the lipid moiety of the cytochrome. When bound to cytochrome P-450, they show a spectrum that has a maximum at about 385 mμ and a minimum at about 420 mμ. "Type II" substrates, such as aniline and nicotinamide, are believed to be bound to the iron moiety of the heme and they give spectrum with a maximum at about 430 mμ and a minimum at approximately 393 mμ (155,156,157). The P-450 electron transport system with addition of substrate and oxygen may be represented as follows:



Solubilization of this electron transport system has now been accomplished (158,164). When the components of this mixed function oxidase system were reconstituted by Lu, et al., they found some enzyme activity using drugs or fatty acids as substrates; however, the activities were extremely low (159,160). In regard to the two types of substrate binding, there is evidence that binding may not occur at two separate sites on the same molecule but on different molecules i.e., there may be multiple forms of cytochrome P-450 which have different physical and chemical characteristics (20,23,161).

Cytochrome b_5 is another heme protein associated with the microsomal fraction. This heme protein is reduced via NADH cytochrome c reductase, a non-heme iron FAD-containing protein, by NADH (7-12). The specific function of this system shown below is not understood.

$$\text{NADH} \longrightarrow F_p \longrightarrow b_5 \longrightarrow \text{"Y"}$$

The terminal electron acceptor for this system is still not known, lending even more ambiguity to its function.

The possibility that cytochromes b_5 and P-450 may be components of the same electron transport pathway has been considered. In general, studies that have been done to demonstrate a link between these components

have indicated they are separate, but a recent report (13) supports the possibility of a crossover from cytochrome b_5 to the "X" component of the P-450 system.

A similar NADPH-linked, oxygen-dependent electron transport system that metabolizes steroids (2,33,35-37) has been observed in mitochondria of the adrenal cortex. The enzymes in this system have physical and chemical properties different from those found in liver microsomes (2,33, 34,38). Masters, et al. (33,140) have clearly shown that the NADPH reductase which reduces cytochrome c and cytochrome P-450 in adrenal mitochrondria is immunochemically different from that found in liver microsomes. In addition, the cytochrome P-450 system of adrenal mitochondria contains a ferrodoxin-like component, adrenodoxin, an iron-sulfur containing protein. This protein has been shown to function as an intermediate between NADPH and cytochrome P-450.

Several of the compounds which are metabolized by the cytochrome P-450-dependent oxidase system, but are foreign to the cell i.e., various drugs, carcinogenic hydrocarbons and insecticides, as well as substances which are native to the cell i.e., steroids, bring about increased enzyme activity after in vivo administration (39-42). Many studies in which phenobarbital was given to animals have shown that the activity of the microsomal hydroxylating system is increased. After phenobarbital induction, NADPH cytochrome c reductase, cytochrome P-450, microsomal protein, RNA and lipid increase (43). In addition, it has been clearly shown that phenobarbital induces formation of new messenger RNA and protein. Another effect of phenobarbital which has been studied is the accumulation of the smooth endoplasmic reticulum (17,42-49). This appears to be the result

of enhanced synthesis and decreased turnover of both the protein and phospholipid moieties of this membrane.

Induction by drugs depends on the formation of new messenger RNA as indicated by its sensitivity to actinomycin D (43,54), a potent inhibitor of RNA and protein synthesis. This inhibitor, however, has limited ability to depress phospholipid synthesis and proliferation of smooth endoplasmic reticulum, suggesting that these latter processes are not controlled by syntheses of new m-RNA or enzymes (45). Since both enzyme and membrane syntheses are abolished by puromycin, the entire process, including formation of new phospholipid, involves synthesis of new protein (43,45). Other studies dealing with turnover rates (31,55-58) after phenobarbital treatment have revealed that both increased rates of protein and phospholipid synthesis and decreased rates of degradation are brought about by drug treatment. Recent investigations have suggested that there are early effects in the nucleus; inductions by phenobarbital and methylcholanthrene are accompanied by changes in nuclear protein formation (165,166).

Studies with another substrate of the mixed-function oxidase system, the carcinogen, 3-methylcholanthrene, have revealed a specific inductive effect. This molecule induces the formation of a form of cytochrome P-450 called P_1-450 (19-27,162). This cytochrome P_1-450 reacts preferentially with "type II" compounds in opposition to the P-450 induced by phenobarbital which reacts readily with either "type I" or "type II" substrates. The data with 3-methylcholanthrene suggest that there are either multiple forms of cytochrome P-450 or there are different species.

Investigations carried out in this laboratory have suggested that vitamin E has a role in regulating the activity of the microsomal hydroxylating system. Carpenter (60) has shown that NADPH-linked drug metabolism by rat liver microsomes of vitamin E-deficient animals is depressed. Microsomes from animals supplemented with vitamin E have specific activities about twice those of preparations from vitamin E-deficient animals. This effect of α-tocopherol can be seen in 48 hours after transfer of vitamin E-deficient animals to a vitamin E-supplemented diet and 12 hours after an oral dose. This apparent induction is prevented if the animals are pretreated with actinomycin D (60).

The well known antioxidant properties of vitamin E (61,62,68,71) do not appear tc account for the effect of α-tocopherol on drug metabolism. Carpenter (82) has observed and Gram and Fouts (59) have confirmed that the addition of α-tocopherol in vitro eliminates lipid peroxidation but has no effect on rates of drug metabolism. Other antioxidants were added in vitro with the same results as were seen with the in vitro addition of α-tocopherol i.e., decreased lipid peroxidation but no enhancement of drug hydroxylation (82). N,N'-diphenyl-p-phenylenediamine (DPPD), another antioxidant, was given to the vitamin E-deficient rats in the diet. DPPD is known to reverse many of the effects caused by vitamin E-deficiency and, therefore, appeared a logical antioxidant to use. It was administered in the diet for three days, the amount of time required for reversal of enzyme activity by α-tocopherol in this enzyme system. That DPPD was incorporated into the liver microsomes was shown by elimination of lipid peroxidation; however, there was no effect on drug hydroxylation (82),

Early work by Dinning, et al. (90) and later work by others (65-67,69,70) led Nair (66) to postulate that vitamin E may be important in heme biosynthesis of primates, since these animals develope anemia during vitamin E-deficiency. The studies by Murty et al. (69) have shown that the levels of rate limiting enzymes in porphyrin biosynthesis, δ-aminolevulinic acid synthetase (in bone marrow) and δ-aminolevulenic acid dehydratase (in liver), are depressed in vitamin E-deficient animals. Therefore, the suggestion has been made that α-tocopherol may be involved as an "inducer" of rate limiting enzymes in porphyrin biosynthesis.

The regulation of protein synthesis and turnover of rat liver microsomes have been studied by Tsai (163) with the following observations: the rates of synthesis and turnover of total microsomal protein in vitamin E-supplemented and vitamin E-deficient animals appear to be the same; polyacrylamide gel electrophoresis indicates no new species of protein are produced by vitamin E treatment; and the relative concentration of the major protein bands, with the exception of two, was very constant, indicating vitamin E action is specifically on a few protein species only.

Work that has been done in regard to the possible control mechanisms for microsomal hydroxylations has suggested steroids may be very important. That steroid hormones, especially androgens and estrogens, are very important in the regulation of the P-450 hydroxylase system has been documented in the work of R. Kato and colleagues (73,74,83). Their investigations and those of others (75-78,84,85,86) reveal that higher hydroxylation activities are observed in males than in females,

that administration of androgens to immature males, females, or castrated males, increases the activity of the liver microsomal hydroxylating system. In addition, it is known that either castration of males or administration of estrogens to them results in a decrease in activity. When estrogens are administered, there is a decrease in production of androgens; the effect may, therefore, be that of a lowered amount of androgen as opposed to a primary inhibition by estrogen. All these observations suggest that steroid hormones, which are themselves metabolized by the cytochrome P-450 hydroxylase system, are the regulators of this enzymic pathway. These compounds cause changes in both levels of cytochrome P-450 and in rates of microsomal hydroxylations.

Investigators in other laboratories have observed that adrenal steroids are also important in the control of the liver cytochrome P-450 system (86,88,89,92-94). Adrenalectomy was observed to cause both a decrease in content of cytochrome P-450 as well as a decrease in microsomal hydroxylase activity. Adrenalectomy seems to cause the greatest changes in male rats, as there seems to be no change in these parameters in the adrenalectomized female. In addition to the above findings, Orrenius, et al. (97) have observed a decrease in both P-450 content and microsomal hydroxylase activity in adrenectomized-castrated animals. Orrenius also observed that the enzyme activity in the castrated-adrenalectomized rat could not be induced with phenobarbital unless it was first treated with testosterone.

A new role for hormones and fat-soluble vitamins has been recently postulated. Observations which have been made suggest that the

effect is at the nuclear level and is one of nucleoprotein modification (63,148). Murthy, et al. (167) have observed that hydrocortisone induces phosphorylation of histones, which is then associated with increased m-RNA synthesis. Later work by Barker (168) has shown that estrogen may be responsible for histone synthesis as well as synthesis of a particular non-histone chromatin protein. Because there may be a difference in the content and/or species of nucleoproteins which regulate transcription, some work has been done in regard to establishing the exact role of nucleoproteins.

The purpose of the present study was to determine whether the effect of α-tocopherol on liver microsomal hydroxylation was mediated via androgens or if the effect was independent of those steroids. The experimental design used was to compare certain parameters of liver microsomal metabolism by control rats, fed a vitamin E-supplemented diet, and experimental rats, fed a vitamin E-deficient diet. Identical analyses were made of both control and experimental rats that were either castrated to remove the influence of testosterone or adrenalectomized to delete the effect of the adrenal steroids; some animals were subjected to both castration and adrenalectomy. Groups of rats, in which steroid producing organs were surgically removed, were treated either with appropriate steroids or with α-tocopherol. To determine whether the effects of α-tocopherol on microsomal hydroxylation were similar to those of phenobarbital, induction by this drug was studied in castrated, adrenalectomized, and castrated-adrenalectomized rats. Some preliminary studies have been made of the histone components in the liver nuclei of the control and experimental rats.

The results of these studies have provided information about the mechanism involved in the induction of miscrosomal hydroxylation by α-tocopherol as well as the relationship between the effects of vitamin E and phenobarbital.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Adult male albino rats, derived from the Holtzman-Sprague Dawley strain, bred and maintained in this laboratory were used for these experiments. Animals received either distilled water or saline (0.9%) and were fed ad libitum a synthetic diet described below which was prepared in our laboratory.

Materials for Diets

Casein, vitamins (except α-tocopherol acetate), cod liver oil, and Alphacel (a pure, powdered cellulose added for bulk) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Stripped lard (tocopherol and other volatile materials removed by molecular distillation) and α-tocopherol acetate were obtained from Distillation Products Industries, Rochester, New York.

Synthetic Diet

Experimental Diet. The α-tocopherol deficient diet used has been previously described (141) and is a modification of the diet of

Young and Dinning (149). The salt mixture and vitamin mixture of this diet were prepared according to the methods of Hubbell, et al. (150). The general composition of the experimental diet was:

Composition of Basal Diet

	Percent Composition
Casein, vitamin free	20.0
Sucrose	37.3
Corn starch	36.0
Lard, stripped	3.0
Cod liver oil	3.0
Salt mixture	3.0
Vitamin mixture	0.7

The basal diet was mixed with Alphacel in a ratio of 10 to 1.

Control diet. The α-tocopherol supplemented diet fed was the same as the experimental, except that α-tocopherol acetate was added, 100 mg/kg diet.

Stock animals were maintained on the Rockland diet (Rockland Laboratories, Teckland Incorporated, Manmouth, Illinois).

Reagent Chemicals

All chemicals and solvents were reagent grade and were used as obtained, except where otherwise specified.

Nicotinamide adenine diphosphonucleotide phosphate (NADP), glucose-6-phosphate (sodium salt), glucose-6-phosphate dehydrogenase (type VII), testosterone propionate, hydrocortisone, corticosterone, and aniline were purchased from Sigma Chemical Company, St. Louis, Missouri.

The following chemicals were obtained from J.T.Baker Chemical Company, Phillipsburg, New Jersey: barium hydroxide, ammonium acetate, acetic acid, urea, copper sulfate, sodium hydroxide, anhydrous sodium carbonate, sodium potassium tartrate, formaldehyde, magnesium chloride, potassium chloride, glycerol, potassium phosphate, sulphuric acid, ether, sodium phosphate, zinc sulfate and sucrose.

The following chemicals were obtained from Fisher Scientific Company, Pittsburg, Pennsylvania: sodium chloride, trichloroacetic acid, acetylacetone, sodium dodecyl sulfate, tris (hydroxy methyl) aminomethane, potassium ferrous cyanide, Folin reagent.

The following chemicals were obtained from Eastman Organic Chemicals, Rochester, New York: thiobarbituric acid, phenol, nicotinimide, β-mercaptoethanol, sodium dithionite and β-alanine.

Acrylamide, N,N' methylene-bis-acrylamide, N,N,N',N'-tetramethylene ethylene diamine (TEMED), and ammonium persulfate were obtained from Canal Industrial Corporation, Rockville, Maryland.

Codeine was obtained from S.B.Penick and Company, Chicago, Illinois.

Buffalo black NBR (amido swartz) was obtained from Allied Chemicals, Chicago Heights, Illinois.

Lubrol was obtained from I.C.I. Organics Inc., Providence, Rhode Island.

Ethanol was obtained from U.S.Industrial Chemicals Company, New York, New York.

Aminopyrine was obtained from K. & K. Laboratories, Plainview, New York.

Bovine serum albumin was obtained from Armour Pharmaceutical Company.

Penthrane was obtained from Abbott Laboratories, North Chicago, Illinois.

Carbon monoxide was obtained from Matheson Company, Chicago, Illinois.

Standard calf thymus histone was obtained from Worthington Biochemicals Corporation, Freehold, New Jersey.

Phenobarbital (sodium salt) was purchased locally.

Instruments and Equipment

Spectrophotometric measurements were performed using a Beckman DU-2 from Beckman Instruments Company, Cary Model 14 Recording Spectrophotometer, Applied Physics Corporation, and Gilford 2400 Spectrophotometer from Gilford Instrument Company. Centrifugation were performed using either a Spinco Model L ultracentrifuge and a Beckman L-265 ultracentrifuge, Beckman Instruments Company, or a LC-1, Ivan Sorval, Incorporated. Beckman centrifuge rotor heads were used for differential centrifugation and gradient centrifugation.

Polyacrylamide gel electrophoresis was carried out using a Canalco Model 300B, Canal Industrial Corporation.

Incubations and digestions were performed in a Dubnoff shaker with a constant temperature bath or a stationary incubator, Precision Scientific Company.

Methods

Treatment of Animals

All surgical operations were performed in this laboratory;

castration *via* scrotal incision and adrenalectomy *via* two dorsal lateral incisions. Some animals were subjected to a double operation, adrenalectomy and castration. Mock surgeries were performed using the methods described without removal of the organs. The operated rats were allowed a minimum of ten days for recovery from surgical trauma.

Injections of phenobarbital were given subcutaneously, 8–10 mg/100 g body weight per day in 0.9% saline; testosterone propionate was given either subcutaneously or intramuscularly, 1 mg/100 g body weight in corn oil. A mixture of hydrocortisone and corticosterone, a total of 0.2 mg/day in a ratio of 1:2 (w/w), was given intramuscularly as a suspension in 0.9% saline.

Preparation of Liver Microsomes

The rats were stunned with a blow to the head, exsanguinated by severing the carotid arteries, the livers removed, chilled in cold potassium phosphate buffer (0.15 M, pH 7.5), weighed and homogenized in cold phosphate buffer, 5 ml per gram of liver. To pellet cellular debris, nuclei and mitochondria, the homogenate was centrifuged in the cold at 8000 x g for 15 minutes. The supernatant fraction was centrifuged for 90 minutes at 105,000 x g in a Spinco Model L or L-265 ultracentrifuge using a 30 rotor. Then the microsomal pellet was resuspended in the same volume of fresh cold phosphate buffer and recentrifuged for 60 minutes at 30,000 rpm. This wash was repeated once, the buffer drained and the final microsomal pellet stored at -20° C overnight. Before use, the pellet was thawed in an ice bath and the microsomes resuspended by homogenization in tris-HCl buffer (0.05 M, pH 7.5), 1 ml of suspension

equivalent to the microsomes from 1 g of liver wet weight. The microsomal suspension was used immediately.

Assay of Microsomal Drug Hydroxylation

A reaction system consisting of 0.1 ml microsomal suspension (approximately 1 mg protein), 11 mM $MgCl_2$, 43 mM KCl, 21 mM nicotinamide, 850 mM tris (pH 7.5) a NADPH generating system (5 mM glucose-6-phosphate, 0.16 mM NADP, 0.40 I.U. glucose-6-phosphate dehydrogenase) and either of the following substrates, 2.5 mM codeine, 2.5 mM aminopyrine or 8mM aniline in a total volume of 2 ml was used. The reaction was initiated by the addition of microsomes and carried out at 37° C in a Dubnoff metabolic shaker. When codeine or aminopyrine were the substrates, reaction tubes were inactivated after 5, 10, 15 and 30 minutes by the addition of 0.2 ml 20% $ZnSO_4$ and 0.5 ml saturated $Ba(OH)_2$. After centrifugation for 15 minutes at 2600 rpm, aliquots were taken and the formaldehyde formed from codeine or aminopyrine measured by a modification of the Nash method (95). Reaction systems with aniline were incubated and stopped after 15 and 30 minutes by the addition of 0.5 ml 35% TCA and centrifuged 15 minutes at 2600 rpm; aliquots were taken and the product, p-hydroxyaniline, was measured by a modification of Axelrod's procedure (81).

Lipid peroxidation was measured using the same incubation systems as above but without substrate. After inactivation with 1.0 ml 35% TCA, thiobarbituric acid (2.0 ml) was added, and the tubes heated for 15 minutes in a boiling water bath. Them 2.0 ml 70% TCA was added and after centrifugation for 15 minutes at 2600 rpm, the tubes were read at 532 mμ (99).

Determination of Cytochromes

The contents of both cytochrome P-450 and cytochrome b_5 in the microsomes used in enzymic assays were determined from difference spectra (19). Cytochrome b_5 was measured by taking a 1.0 ml aliquot of the microsomal suspension used in hydroxylation experiments and adding 9.0 ml of a 1/1 (v/v) mixture of glycerol and potassium phosphate buffer, pH 7.5 (151). After thorough mixing, 2.0 ml of the solution were transferred to each of two cuvettes, 1 cm path length, which were placed in the reference and sample positions of a Cary recording spectrophotometer. Approximately 4 mg of crystalline sodium dithionite was added to the sample cuvette and after thorough mixing, the difference spectrum scanned in the visible range from 600-400 mμ. The amount of cytochrome b_5 was determined by the difference spectrum between 424 mμ and 410 mμ using an extinction coefficient of 160 mM^{-1} cm^{-1} (7).

To measure cytochrome P-450, dithionite was added to both the reference cuvette and sample cuvette and carbon monoxide bubbled slowly for 1.5 minutes through the solution in the sample cuvette. The difference spectrum was again measured between 600-400 mμ. Cytochrome P-450 was determined by the difference spectrum between 500 mμ and 450 mμ, using the extinction coefficient 91 mM^{-1} cm^{-1} as described by Omura and Sato (19).

Preparation of Histones

Nuclei from liver cells were isolated by the method of Blobel and Potter (102), with a recovery of over 90% and very little cytoplasmic contamination. Chromatin was separated by the method of Paul and Gilmour

(103), and histones by a modification of the technique of Bonner and colleagues (100).

Polyacrylamide Gel Electrophoresis

Electrophoresis was performed in the urea gel system described by Bonner, et al. (100) in 5 x 120 mm tubes using 7.5% acrylamide gels containing 6 M urea. They were prepared by mixing 1 volume of TEMED (48 ml of 1 N KOH, 17.2 ml glacial acetic acid, 4 ml of N,N,N'N'-tetramethyl ethylene diamine, glass distilled water to 100 ml), 2 volumes acrylamide (30 g acrylamide, 0.8 ml of N,N' methylene-bis-acrylamide, double distilled water to 100 ml) and 5 volumes 0.2% (w/v) ammonium persulfate in 10 M aqueaous urea solution. Then 0.0004 g potassium ferric cyanide per 32 ml was added to slow polymerization (101). These gels had a pH of 4.5. Tray buffer was made up as follows: 31.2 g ß-alanine, 8 ml concentrated acetic acid, and water to 1.0 liter. These gels were used for histone separations.

All gels were routinely stained with Coomassie brilliant blue (0.25% Coomassie brilliant blue and 5:5:1 water methanol, acetic acid v/v/v) or amido schwartz (1% Buffalo Black, 7% acetic acid). Twelve percent trichloroacetic acid was used to set protein in the gels.

The gels were subjected to preliminary electrophoresis for one hour at 6 mamps tube then removed, drained of tray buffer and electrophoresis carried out for six hours at 1.5 mamps per tube. A long 22 gauge needle attached to a syringe was inserted between the gel and tube wall and water pressure applied to remove the gels from the tubes. The gels were destained with 7% acetic acid for several days. The

number of protein bands and the proportional amount of protein in each band were determined by recording at a wave length of 500 mμ for Buffalo Black or 540 mμ for Coomassie blue on a Gilford 2400 gel scanning spectrophotometer.

Protein Determination

Protein determinations for all assays and procedures were performed by the method of Lowry, et al. (126).

Statistical Analyses

The Dunnet's test (104) was used in this study for statistical analysis. This test was decided on after consultation with the Department of Biostatistics. These data were set up to compare several experimental groups with one control group. It was determined that the proper statistical test to use in this case was the Dunnet's test.

CHAPTER III

RESULTS

Microsomal Hydroxylation of the Control and the Experimental Rat

Comparative studies were performed to determine the effects on liver microsomal hydroxylation of feeding rats a diet which had a low content of vitamin E (experimental animals) with those fed the chemically defined diet supplemented with vitamin E (control animals). That the experimental diet was truly inadequate in α-tocopherol was documented by the observation that the testes of the rats fed this diet became degenerate; otherwise, the animals appeared to be in good health and their growth rates were identical with those of the controls.

Liver weight to body weight ratios were determined for animals in each diet group. Rats fed a diet deficient in α-tocopherol were observed to have larger livers per gram body weight than control animals (Table 1). Recovery of microsomal protein was routinely measured and it would appear that the recoveries in the two diet groups were identical (Table 1). When comparing recoveries with those reported by others (74,86), the values reported here are smaller. These lower recoveries are probably due to the procedure for microsomal purification used in this study in which the microsomes were washed twice after isolation.

TABLE 1

HYDROXYLATION RATES AND HEMOPROTEIN CONTENTS

OF THE MALE RAT LIVER MICROSOMES

	Control[a]	Experimental[a]
Number	(79)	(59)
Hydroxylation		
Codeine[b]	12.93 ± 0.28	7.40 ± 0.20†
Aminopyrine[b]	10.09 ± 0.27	6.51 ± 0.22†
Aniline[c]	0.71 ± 0.02	0.44 ± 0.02†
Hemoprotein[d]		
Cytochrome P-450	1.57 ± 0.03	1.38 ± 0.04
Cytochrome b_5	0.80 ± 0.01	0.73 ± 0.02
Cytochrome P-450/ Cytochrome b_5	1.96	1.89
Chromagen[1]	0.015 ± 0.000	0.299 ± 0.014†
LW/BW[2]	0.039 ± 0.000	0.043 ± 0.000†
Microsomal protein[3]	1.08 ± 0.02	1.00 ± 0.02

a All values are the mean ± S.E.
b mμmoles HCHO/mg microsomal protein/min.
c mμmoles p-OH-aniline/mg microsomal protein/min.
d mμmoles cytochrome/mg microsomal protein.
1 ΔOD at 532 mμ/mg microsomal protein/30 min.
2 g liver/g body weight.
3 mg microsomal protein/0.1 g liver
† = $p<0.01$

The rates of microsomal hydroxylations and the concentrations of microsomal pigments for control and experimental rats are shown in Table 1. Microsomes of the control animals metabolized codeine and aminopyrine, both "type I" substrates, at a significantly faster rate than those from experimental rats. Aniline, a "type II" substrate, was also metabolized significantly faster by the control microsomes than the experimental microsomes. Thus it appears that the effect of vitamin E is not specific for either type of substrate used in this study.

The specific activities shown in the table represent the initial velocities. Rates of microsomal drug hydroxylation by rat liver microsomes are linear during the first 15 minutes of reaction, and then the rate slows down in both the control and experimental (Figure 1). Gram and Fouts (138) also observed this and noted that hydroxylation rates using rabbit liver microsomes are linear for 30 minutes.

No significant difference in content of cytochrome P-450 was observed between the control and the experimental animal, which is in contrast to the data of Murty, et al. (70). They have reported that the content of cytochrome P-450 and b_5 in the vitamin E-supplemented rat is 100% greater than that of the vitamin E-deficient rat. The significance of their observations will be discussed later. The fact that no difference was observed may be important since cytochrome P-450, the oxygen activating component of the microsomal electron transport system, binds the substrate which is to be metabolized; however, it should be noted that although the content is not different this does not imply that the structure of the molecule remains unchanged in the vitamin E-deficient animal.

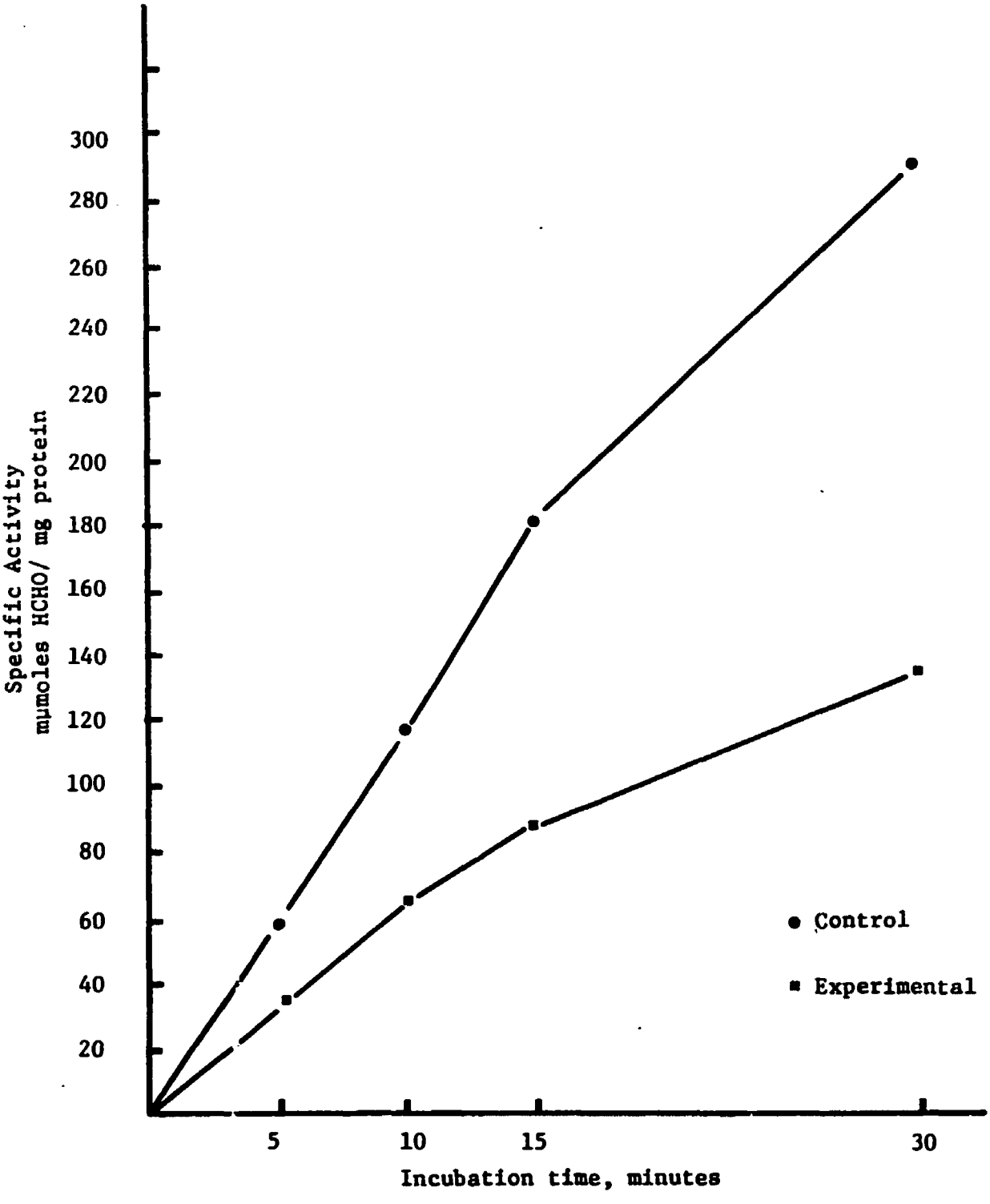


Figure 1. Rates of microsomal enzyme hydroxylation for control and experimental animals.

Cytochrome b_5, another hemoprotein associated with the microsomes, was also routinely measured. This heme protein is a very stable component of the microsomal membrane, and the content remains very constant from animal to animal. Because of the unchanging content of this cytochrome, it is a convenient molecule with which to compare cytochrome P-450. Data are presented in Table 1 which give the ratio of cytochrome P-450 to cytochrome b_5.

Tissues isolated from vitamin E-deficient animals when exposed to oxygen, peroxidize more readily than those from supplemented animals. Lipid peroxidation by the control and experimental microsomes was routinely measured. Little or no lipid peroxidation occurs in the control microsomes; a significant amount is observed in the experimental microsomes (Table 1). Again it is important to note the observations of Carpenter (72), that the in vitro addition of α-tocopherol caused the cessation of lipid peroxidation without an increase in drug hydroxylation. These studies were performed with no addition of substrate to the incubation system used to study lipid peroxidation. In these studies the effect of α-tocopherol on the microsomal hydroxylating system cannot be explained as that of an antioxidant. Carpenter (82) has shown that when codeine is added to the lipid peroxidation system that lipid peroxidation remains the same as that observed without addition of substrate, while the addition of aminopyrine caused a fifty percent reduction of lipid peroxidation. There was no chromagen formation when aniline was added to the incubation system.

Animals of different age groups were used for the experiments presented in this study. Mature animals were used for surgical mani-

pulation, allowed an 8-14 day minimum recovery time, then in some cases were not sacrificed until a few months after surgery. For these reasons, it was necessary to determine the effects of age on hydroxylation rates. Published data (142,146) indicate low rates in young immature animals, higher rates in adult animals, and a gradual decrease in activity of the microsomal hydroxylase system in old animals. Table 2 presents data which confirm that the liver microsomes from immature animals have a lowered rate of hydroxylation; moreover, the rate increases with age to a maximum that remains constant for all older age groups presented. This is in agreement with the data of Das and Ziegler (110). The reason for the apparent discrepancy in the 17-20.5 week age group is unknown. The purpose of presenting the data in Table 2 was to demonstrate at approximately what age microsomal activity reached optimal activity and if older animals exhibited a decreased enzyme activity. For this purpose a statistical test for significant differences was not necessary.

Effect of Castration on Microsomal Hydroxylation

Studies by Kato et al. (73,74) have indicated a sex difference in the metabolism of various drug substrates by rat liver microsomes. Reports show lower rates in females, castrated males, and prepubertal males, than in adult males; moreover, administration of androgens to the first three groups results in an increase in liver microsomal hydroxylating activity. Carpenter (82) has shown that the ability of liver microsomes from the female rat to metabolize most drug substrates is generally lower than observed in the male. It was also observed that response to α-tocopherol was the same as observed in males i.e., a decreased enzyme activity in the absence of vitamin E.

Vitamin E-deficient male rats exhibit a degeneration of the testes. Since this appears to cause no change in the production of androgens (169), but the liver microsomal hydroxylation rates are different between the vitamin E-supplemented and vitamin E-deficient rats, it was of interest to determine the possible relationship between vitamin E and androgens. Steroid hormones are normal *in vivo* substrates for this hydroxylation system, suggesting that there might be a relationship between α-tocopherol and androgens in the regulation of this enzyme system.

Preliminary work on the effect of castration was performed using animals fed the commercial stock diet (Table 3). The data presented confirm similar data in the literature, and show a lowered rate of hydroxylation due to castration. Moreover, it was determined from these studies and those published by others (169), that a recovery time was required to insure that the observations made were an effect of hormone deletion and not surgical trauma. Data showing the results of both sham-castration and castration of the stock-fed rats are shown in the table. The data from the sham-castrated rats show that a recovery time is required to overcome surgical trauma. Moreover, the data for the 1-3 day castrate is in close agreement with that for the 1-3 day sham castrate. Animals allowed 8 or more days for recovery had an increased microsomal hydroxylating activity; however, in agreement with the literature (74), this specific activity was lower than that observed in the unoperated rat. Kato and Onoda (74) found a decreased content of cytochrome P-450 after castration.

TABLE 3

EFFECT OF CASTRATION ON MICROSOMAL HYDROXYLATION
FROM LIVER OF STOCK-DIET RATS

	Treatment		
	Untreated[a]	Sham Castrate[a] (1-4 days)	Castrate[a] (1-3 days)
Number	(9)	(6)	(9)
Hydroxylation			
Codeine[b]	7.63 ± 0.44	3.61 ± 0.38 †	3.65 ± 0.15 †
Aminopyrine[b]	8.36 ± 0.51	4.03 ± 0.28 †	3.64 ± 0.29 †
Aniline[c]	0.50 ± 0.09	0.53 ± 0.08	0.47 ± 0.05
Hemoprotein[d]			
P-450	0.73 ± 0.09	0.92 ± 0.08	0.67 ± 0.13
b_5	0.58 ± 0.13	0.43 ± 0.07	0.53 ± 0.08
P-450/b_5	1.53	2.5	1.27

a mean ± SE
b mμmoles HCHO/mg microsomal protein/min.
c mμmoles p-OH-aniline/mg microsomal protein/min.
d mμmoles cytochrome/mg microsomal protein.
† $p<0.01$ (untreated)

The effects of castration of control and experimental rats are shown in Table 4. Castration of the control rats was without effect on hydroxylation activity and cytochrome content. This observation indicates an important difference, presumably due to α-tocopherol, between these data and those of others (73,74) where castration effected a decrease in hydroxylation. Testosterone, the major steroid produced by the testis, was given as the testosterone propionate (T.P.) derivative in corn oil from 5-8 days as a hormone replacement to the castrated rat. This treatment caused neither a difference in the hydroxylation activity nor the cytochrome content, an observation which was also different from those published by others (74,170). Corn oil controls were done (data not shown) indicating that there was no effect on this system due to corn oil injection.

Unlike the control rat, castration of the experimental resulted in a decreased hydroxylation of codeine and aminopyrine (Table 4). There was no change in cytochrome content. In this situation, as opposed to that observed in the vitamin E-supplemented rats, a change due to castration is apparent, indicating that in the absence of vitamin E, testosterone may be important in regulation of enzyme activity. The administration of testosterone propionate (T.P.) (8-14 days) brought about an increase in hydroxylation activity, again indicating that testosterone may function to regulate hydroxylation in the experimental animals. When the experimental, castrated animals were placed on an α-tocopherol-supplemented diet (α-T.) for a period of time ranging from 7-67 days, the enzymic activities increased; however, the specific activities were not comparable with that of the control, vitamin E-

TABLE 4

EFFECT OF CASTRATION ON MICROSOMAL HYDROXYLATION

Treatment	Number	Hydroxylation[a]			Hemoprotein[a,d]		
		Codeine[b]	Aminopyrine[b]	Aniline[c]	P-450[d]	b_5[d]	P-450/b_5
Control	79	12.93 ± 0.27	10.09 ± 0.27	0.71 ± 0.02	1.57 ± 0.03	0.80 ± 0.01	1.96
Control Castrate	10	12.06 ± 0.72	10.26 ± 0.77	0.77 ± 0.11	1.55 ± 0.06	0.90 ± 0.03	1.72
Control Castrate + T.P.	4	10.70 ± 1.31	9.53 ± 1.27	0.47 ± 0.07	1.59 ± 0.27	0.87 ± 0.22	1.82
Experimental	59	7.40 ± 0.20	6.51 ± 0.22	0.44 ± 0.02	1.38 ± 0.04	0.73 ± 0.02	1.89
Experimental Castrate	12	5.29 ± 0.44††	5.33 ± 0.38	0.52 ± 0.06	1.25 ± 0.06	0.65 ± 0.03	1.93
Experimental Castrate + T.P.	7	8.60 ± 1.07	8.07 ± 1.02	0.59 ± 0.10	1.45 ± 0.10	0.73 ± 0.04	1.98
Experimental Castrate + α-T.	12	7.69 ± 0.77	7.80 ± 0.71	0.53 ± 0.07	1.45 ± 0.16	0.84 ± 0.06	1.72

a mean ± S.E.
b mμmoles HCHO/mg microsomal protein/min.
c mμmoles p-OH-aniline/mg microsomal protein/min.
d mμmoles cytochrome/mg microsomal protein
†† = $p < 0.01$ (experimental).

supplemented animal. It should be noted here that both testosterone propionate and α-tocopherol restored the level of activity in the experimental, castrated to that in the unoperated, experimental rat. Carpenter (60) has shown that in 24-48 hours after α-tocopherol administration, the hydroxylating activity in the experimental rat is equal to that of the control. After castration, the liver microsomal enzyme system of the experimental rat could not be induced to a level comparable to the control enzyme activity.

Lipid peroxidation of the microsomes as measured by thiobarbituric acid chromagen, did not change after castration or hormone treatment. In the presence of α-tocopherol, lipid peroxidation is abolished, an observation seen using microsomes from an experimental, castrated rat administered vitamin E. Total microsomal protein remained unchanged in both the control and experimental animals after castration.

The possible regulation of the vitamin E effect on microsomal hydroxylation by androgens has been studied. It would appear that in the presence of vitamin E, castration does not affect enzyme activity, however this is valid only if α-tocopherol is present before and after castration. In the vitamin E-deficient state, castration partially blocks the response to α-tocopherol, suggesting that after prolonged vitamin E-deficiency androgens are a requirement for induction by α-tocopherol.

Effect of Adrenalectomy on Microsomal Hydroxylations

Castration resulted in decreased activity in the experimental animal but not in the control. This suggests that the effect of α-

tocopherol on this mixed function oxidase system is independent of androgen. Adrenalectomies were performed to ascertain the role of the adrenal in regulating the liver microsomal hydroxylase activity of the control and experimental animals. Data have been published showing adrenalectomy elicits a decrease in liver microsomal hydroxylation (93). Mock-adrenalectomies were performed on control rats and the specific activity for codeine was 7.47 mμmoles HCHO/mg protein/min. This activity remained low for 10-14 days before returning to the normal rate of 12-14 mμmoles. Margolius et al. had also observed that 10-14 days were required to overcome surgical trauma (147). In the present study, young adult animals which ranged from eight to ten weeks of age at the time of adrenalectomy, were used. They were sacrificed after varying periods of time post surgery (14-150 days) but no difference in rates of microsomal hydroxylation was observed due to age, an observation which paralleled that observed in the unoperated animals.

That adrenalectomy depressed the rates of microsomal drug hydroxylations by liver microsomes of both controls and experimentals is shown in Table 5. Liver microsomes from the control rats exhibited a decrease in the hydroxylation of the three substrates tested, while the content of cytochrome P-450 remained unchanged. Since removal of the adrenal glands resulted in hormone deletion, steroid hormone replacement therapy seemed appropriate to determine if the decrease in microsomal hydroxylation was a result of inadequate steroid hormone levels. Corticosterone (Cort.) and hydrocortisone (H.C.) are the major steroids of the rat adrenal and are found in the approximate ratio of 2:1. Therefore, a suspension of a mixture of these hormones was

administered to rats intramuscularly (3-13 days). This treatment brought about no change in the activity of the control liver microsomal hydroxylase system, and no change in the amount of cytochrome P-450, suggesting that the α-tocopherol effect is dependent on the presence of intact adrenal glands. However, it does not appear that the steroids produced by the adrenal glands are the only molecules important to the activity of the liver hydroxylase system as it is known that the adrenal gland produces hormones other than steroids.

Adrenalectomy of the experimental rats also caused a decrease of microsomal hydroxylase activity. The decreased microsomal hydroxylation in the vitamin E-deficient animal was limited to codeine and aminopyrine, aniline hydroxylation being unaffected after adrenalectomy. Although there appears to be a decrease in the content of cytochrome P-450 in the vitamin E-deficient, adrenalectomized rats, this is not significant at the p value tested. Treatment of the vitamin E-deficient adrenalectomized rats with the mixture of corticosterone and hydrocortisone resulted in an increase in enzyme activity while not altering the content of cytochrome P-450. These observations indicate that adrenal steroids have a basic role in the regulation of liver mixed function oxidase activity that is independent of cytochrome P-450 and which does not depend on α-tocopherol. The adrenal steroids were also administered to unoperated experimental animals and there was no effect on either drug hydroxylation or cytochrome P-450 content.

When the experimental, adrenalectomized rats are transferred to a vitamin E-supplemented diet (15-51 days), the microsomal hydroxylation activities for codeine and aminopyrine increased significantly compared

a mean ± S.E.
b mμmoles HCHO/mg microsomal protein/min.
c mμmoles p-OH-aniline/mg microsomal protein/min.
d mμmoles cytochrome/mg microsomal protein.
† = $p<0.01$ (control).
†† = $p<0.01$ (experimental).

TABLE 5

EFFECT OF ADRENALECTOMY ON MICROSOMAL HYDROXYLATION

Treatment	Number	Hydroxylation[a]			Hemoprotein[a,d]		
		Codeine[b]	Aminopyrine[b]	Aniline[c]	P-450	b_5	P-450/b_5
Control	79	12.95 ± 0.27	10.09 ± 0.27	0.71 ± 0.02	1.57 ± 0.03	0.80 ± 0.01	1.96
Control Adrenalectomy	51	8.43 ± 0.29†	7.55 ± 0.27†	0.55 ± 0.03†	1.46 ± 0.04	0.84 ± 0.03	1.84
Control Adrenalectomy + HC + Cort.	15	9.39 ± 0.72†	8.33 ± 0.75	0.58 ± 0.06	1.51 ± 0.10	0.76 ± 0.05	1.99
Experimental	59	7.40 ± 0.20	6.51 ± 0.22	0.44 ± 0.02	1.38 ± 0.04	0.73 ± 0.02	1.89
Experimental Adrenalectomy	39	5.48 ± 0.22††	4.90 ± 0.18††	0.42 ± 0.02	1.20 ± 0.05	0.73 ± 0.02	1.64
Experimental Adrenalectomy +HC + Cort.	8	8.01 ± 0.91	6.93 ± 0.63	0.70 ± 0.11††	1.28 ± 0.13	0.76 ± 0.05	1.66
Experimental Adrenalectomy + α-T.	6	6.98 ± 0.86	6.32 ± 1.02	0.43 ± 0.08	1.58 ± 0.06	0.63 ± 0.06	2.18

to those observed for the experimental, adrenalectomized animal. Three groups of rats which have similar hydroxylation rates are control adrenalectomized, experimental, adrenalectomized plus steroid hormones, and experimental, adrenalectomized plus α-tocopherol.

No changes in either the control or experimental liver microsomes due to adrenalectomy or other manipulations were seen in regard to total microsomal protein content of the microsomes and chromagen formation.

The control, adrenalectomized rat does not respond to steroid hormones but the experimental, adrenalectomized rat does; moreover, the hydroxylation activities observed in the experimental, adrenalectomized animal administered the steroid hormone mixture are comparable to the activity seen in the control-adrenalectomized rat. Vitamin E and adrenal steroids may function at a similar site but the effects are not additive. It is possible that α-tocopherol may have an important role in the regulation of steroid biosynthesis.

The effects of either castration or adrenalectomy on the experimental animals are similar. Administration of the appropriate steroid hormone to the surgically treated rats enhances the activity to a level comparable to that of the experimental, unoperated rat but not to the control activity. Administration of α-tocopherol to the vitamin E-deficient operated rats (adrenalectomy or castration) also restores activities and P-450 content to the level of the vitamin E-deficient unoperated rat.

A comparison has been made between the control and experimental rats of the effects of either castration or adrenalectomy along with

the administration of the appropriate hormone or α-tocopherol in the case of the vitamin E-deficient rat. While castration had no effect on control activities, it lowered the microsomal hydroxylating activity in the experimental rat. Administration of testosterone propionate to the castrated rats had no effect on control animals but did bring about an increased activity in the experimental rat. Adrenalectomy, in contrast to castration, caused a decrease in microsomal hydroxylation activity in both the control and vitamin E-deficient rats; however, administration of hydrocortisone and corticosterone enhanced rates of hydroxylation in the experimental animals but did not in the control animals.

The administration of α-tocopherol to either the castrated or adrenalectomized experimental rats enhanced enzyme activity. These results would then seem to indicate that the effects of α-tocopherol were only partially blocked by steroid depletion, but the levels of activity never reached that of the vitamin E-supplemented rats.

Cytochrome P-450 content in the control rats remained very constant throughout these studies, an observation not seen in the experimental rats where a decrease in content is observed due to both castration and adrenalectomy. The fact that α-tocopherol administration to either experimental, operated animal increased the cytochrome P-450 content may be an indication of its function.

Effect of Adrenalectomy and Castration on Control and Experimental Rats

Either castration or adrenalectomy have an effect on the microsomal hydroxylase system. Both the testes and the adrenal glands

produce androgens. The adrenal is known to undergo hypertrophy after castration (108). To evaluate the role of androgen and -tocopherol in the regulation of microsomal hydroxylations, a double operation, i.e., adrenalectomy and castration was performed to produce "androgen-free" rats.

Andrenalectomy plus castration produced a sharp decrease in the hydroxylation activity of the control rat and not significantly lowered levels of cytochrome P-450 (Table 6). These data indicate that steroid hormones are important in regulating the activity of the rat liver microsomal drug hydroxylating system. The activity measured for the double operated control is comparable to that observed in the unoperated, untreated, experimental rat.

The double operation also results in a depression of microsomal hydroxylation rates in the experimental rats. Rates of hydroxylation for codeine and aminopyrine were noticeably decreased as was the content of cytochrome P-450. Although liver microsomal enzyme activities for both control and experimental rats were decreased to the same extent after adrenalectomy and castration, the activities of the adrenalectomized-castrated control were still higher than those observed in the adrenalectomized-castrated experimental animals.

Administration of corticosterone and hydrocortisone (8-18 days) to the control resulted in an increased hydroxylation rate but no significant increase in cytochrome P-450. Increased rates of microsomal hydroxylation in the experimental rats were not nearly so pronounced as observed in control animals due to the steroid hormone administration (8-11 days). Only two animals were used in the experimental, adrenal-

a mean ± S.E.
b mμmoles HCHO/mg microsomal protein/min.
c mμmoles p-OH-aniline/mg microsomal protein/min.
d mμmoles cytochrome/mg microsomal protein.
† = $p<0.01$ (control).
†† = $p<0.01$ (experimental).

TABLE 6

EFFECT OF ADRENALECTOMY AND CASTRATION ON MICROSOMAL HYDROXYLATION

Treatment	Number	Hydroxylation[a]			Hemoprotein[a,d]		
		Codeine[b]	Aminopyrine[b]	Aniline[c]	P-450	b_5	P-450/b_5
Control	79	12.93 ± 0.27	10.09 ± 0.27	0.71 ± 0.02	1.57 ± 0.03	0.80 ± 0.01	1.96
Control A.& C.	7	6.26 ± 0.43†	5.32 ± 0.43†	0.40 ± 0.08†	1.29 ± 0.16	0.84 ± 0.07	1.51
Control A.& C. + HC + Cort.	5	10.10 ± 0.55†	8.06 ± 1.12	0.62 ± 0.07	1.28 ± 0.17	0.84 ± 0.08	1.52
Experimental	59	7.40 ± 0.20	6.31 ± 0.22	0.44 ± 0.02	1.38 ± 0.04	0.73 ± 0.02	1.89
Experimental A.& C.	7	4.10 ± 0.62††	4.38 ± 0.49	0.34 ± 0.06	1.10 ± 0.10	0.71 ± 0.03	1.53
Experimental A.& C.+ HC + Cort.	2	5.25 ± 0.95††	5.05 ± 0.75	0.65 ± 0.15	1.05 ± 0.15	0.65 ± 0.05	1.35
Experimental A.& C.+ α-T	7	6.17 ± 0.70	6.09 ± 0.09	0.67 ± 0.09	1.46 ± 0.09	0.88 ± 0.08	1.66

ectomized castrated group to which corticosterone and hydrocortisone were administered. In conjunction with the above experiment, analyses were also performed of two unoperated vitamin E-deficient rats. The results obtained were identical to those shown in the table for the vitamin E-deficient animal.

When experimental castrated and adrenalectomized animals were transferred to the control diet (20-50 days), the activities increased to a level comparable to the control rat which had been doubly operated; thus deletion of the steroid hormones did not block increased activity by α-tocopherol. It is also noted that the content of cytochrome P-450 increased.

Neither total microsomal protein nor lipid peroxidation were different in either the vitamin E-supplemented or the vitamin E-deficient animals after castration-adrenalectomy.

These data suggest that there is a steroid hormone requirement for α-tocopherol to achieve its maximum inductive effect. However, the data also clearly show that in all animals there is an enhanced rate of hydroxylation in the control compared to the experimental, regardless of surgical or hormone manipulation. This would indicate that the effect of α-tocopherol is not totally dependent on steroid presence. Steroid hormones show an independence from vitamin E in that they can bring about enhancement in the experimental surgically treated animals.

Figure 2 summarizes the results of castration, adrenalectomy and the double operations. Data obtained from the control (+E) animals indicate that while there is not a pronounced reduction of activity due to castration (Cast.) there is with adrenalectomy (Adren.). This latter

observation supports a definite role for the adrenal gland in the control of liver microsomal hydroxylating activity. The fact that there is not a return to control levels of activity with administration of the deleted adrenal steroid hormones, hydrocortisone and corticosterone (H.C.+ C.), a situation not observed in the castrated animal receiving a testicular steroid hormone (T.P.), would indicate that perhaps other adrenal hormones or adrenal products are important in regulation of the liver microsomal hydroxylating system. Data presented on the control, castrated-adrenalectomized (A.& C.) rat show a marked reduction of hydroxylating activity. Administration of the adrenal steroid hormones partially restores activity but again not to control levels. Preliminary results indicate that administration of testosterone (T.P.) also brings about an enhancement of activity in the doubly operated animal but again not to control levels.

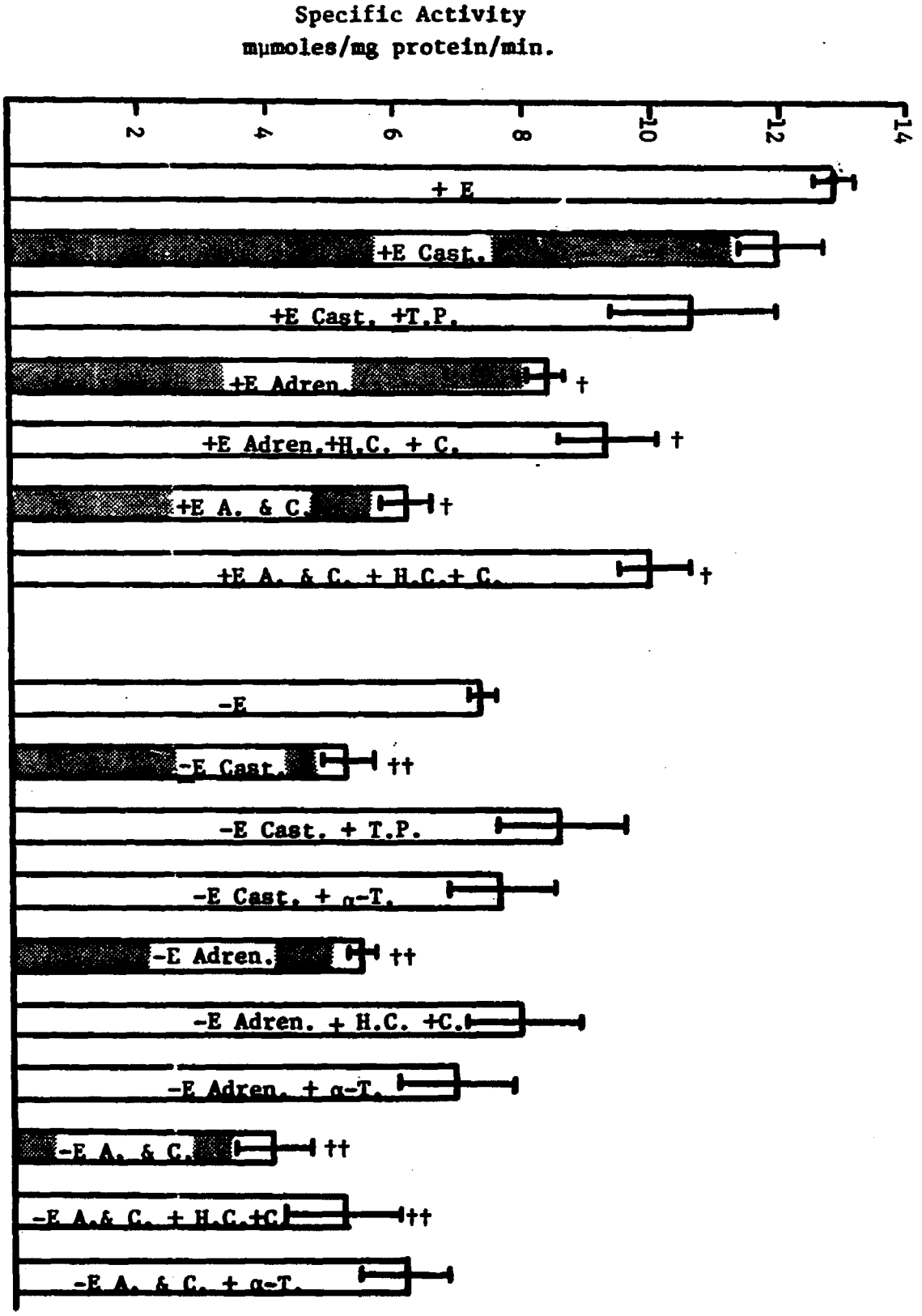
The data on the control (+E) animals then indicate that while intact testes may not be necessary for optimal activity of the liver microsomal hydroxylations that the intact adrenal glands are necessary.

Observations of the experimental animal indicate that in the absence of vitamin E (-E), steroid hormones are important to the liver microsomal electron transport system. Testosterone (T.P.) was able to restore activity in the vitamin E-deficient castrate (Cast.). This indicates it is an important molecule in regulation of the liver hydroxylating system during vitamin E deprevation. Similarly, as was observed in the control animals, an intact adrenal (Adren.) is necessary for optimal activity. In the experimental animal, however, there is restoration of activity to the unoperated level with adminis-

Figure 2. **Effects of Castration, Adrenalectomy and Castration-Adrenalectomy on Microsomal Hydroxylation in control and experimental rats.**

† = p 0.01<(control)
†† = p 0.01<(experimental)

I = Mean ± S.E.


Specific Activity
mμmoles/mg protein/min.
2
4
6
8
10
12
14
+ E
+E Cast.
+E Cast. +T.P.
+E Adren.
+E Adren.+H.C. + C.
+E A. & C.
+E A. & C. + H.C.+ C.
-E
-E Cast.
-E Cast. + T.P.
-E Cast. + α-T.
-E Adren.
-E Adren. + H.C. +C.
-E Adren. + α-T.
-E A. & C.
-E A.& C. + H.C.+C.
-E A. & C. + α-T.

tration of adrenal steroid hormones (H.C.+ C.).

The doubly operated experimental animal also responded in a manner similar to that observed in the control. Adrenal steroid administration to the castrated-adrenalectomized (A.& C.) animal enhanced the liver microsomal activity, again indicating some control independent of α-tocopherol. α-Tocopherol (α-T.) was capable of enhancing activity in all experimental operated groups. In the case of both castration and adrenalectomy, the activity was restored to approximately the unoperated level of activity while its effect on the doubly operated experimental animal was not quite so pronounced. The experimental animal which was castrated-adrenalectomized and given vitamin E was the only one of the experimental group which had an activity similar to that observed in the control animals which received the same operation.

Phenobarbital Induction of Microsomal Hydroxylation

Phenobarbital is a known inducer of the liver cytochrome P-450 microsomal hydroxylase system. α-Tocopherol also appears to function as an inducer of the system. Orrenius has reported that phenobarbital will not induce the hydroxylating system in an animal which has been both adrenalectomized and castrated unless the animal is treated with androgen (98). This indicates that the induction by phenobarbital is dependent on androgen. In the studies reported here α-tocopherol had a positive effect on microsomal hydroxylations by vitamin E-deficient animals that were castrated, adrenalectomized or castrated and adrenalectomized. It was of interest then to determine whether phenobarbital would induce in such animals. Parallel studies were done on the effect

of phenobarbital on the vitamin E-supplemented animals that were castrated, adrenalectomized or castrated-adrenalectomized.

The results of phenobarbital studies are presented in Table 7. Phenobarbital readily induced the control animal; both specific activities and cytochrome P-450 content were increased. The induction process appeared to be limited in the castrated adrenalectomized rat, which indicated that phenobarbital induction is partially dependent on steroid hormones. Cytochrome P-450 in the castrated-adrenalectomized control, however, was induced, an observation which reiterated that there was not a direct relationship between microsomal enzyme activity and the amount of cytochrome P-450.

Since there were no differences between the control and experimental animals after phenobarbital induction, this induction process appeared not to be dependent on α-tocopherol. The data indicate that the inductive effect of phenobarbital was not enough to restore levels of activity in the vitamin E-deficient, surgically manipulated animals to that of the phenobarbital experimental rat. There was not a significant decrease in the cytochrome P-450 content of the experimental adrenalectomized and castrated-adrenalectomized as compared to the unoperated phenobarbital experimental animal. These results indicate that in the absence of vitamin E, steroid hormones may be very important in the mediation of phenobarbital induction.

Total microsomal protein is increased after phenobarbital induction in both vitamin E-supplemented and vitamin E-deficient rats, a fact also observed after hormone deletion and phenobarbital treatment. There were no differences in total microsomal protein between any of the

TABLE 7

EFFECT OF PHENOBARBITAL ON MICROSOMAL HYDROXYLATION AFTER ADRENALECTOMY AND CASTRATION

Treatment	Number	Hydroxylation[a]			Hemoprotein[a,d]		
		Codeine[b]	Aminopyrine[b]	Aniline[c]	P-450	b_5	P-450/b_5
Control	3	20.33 ± 0.56	20.53 ± 3.07	1.30 ± 0.15	3.43 ± 0.24	1.03 ± 0.12	3.40 ± 0.32
Control Castrated	4	17.70 ± 2.25	16.28 ± 2.06	1.28 ± 0.17	3.08 ± 0.29	1.10 ± 0.15	2.83 ± 0.12
Control Adrenalectomy	2	20.40 ± 2.80	18.15 ± 2.75	1.15 ± 0.25	2.85 ± 0.45	0.90 ± 0.10	3.15 ± 0.15
Control Adrenalectomy and Castrated	6	15.80 ± 1.40†	17.88 ± 1.41	1.22 ± 0.17	3.20 ± 0.39	1.15 ± 0.14	2.78 ± 0.13
Experimental	3	19.50 ± 1.40	17.07 ± 2.52	1.40 ± 0.06	2.73 ± 0.20	1.00 ± 0.00	2.73 ± 0.20
Experimental Castrated	5	13.58 ± 0.85††	17.42 ± 1.09††	1.00 ± 0.12	3.22 ± 0.38	1.16 ± 0.06	2.72 ± 0.20
Experimental Adrenalectomy	2	12.45 ± 0.65††	11.75 ± 0.05††	0.85 ± 0.15	2.20 ± 0.00	0.90 ± 0.10	2.50 ± 0.03

TABLE 7 continued

Treatment	Number	Hydroxylation			Hemoprotein[d]		
		Codeine[b]	AP[b]	Aniline[c]	P-450	b_5	P-450/b_5
Experimental Adrenalectomy and Castrated	2	12.55 ± 0.25††	11.70 ± 0.70††	1.05 ± 0.05	2.30 ± 0.00	1.10 ± 0.40	2.40 ± 0.90

a mean ± S.E.
b mμmoles HCHO/mg microsomal protein/min.
c mμmoles p-OH-aniline/mg microsomal protein/min.
d mμmoles cytochrome/mg microsomal protein.
† = $p<0.01$ (control).
†† = $p<0.01$ (experimental).

groups studied after phenobarbital treatment. Microsomal lipid peroxidation was unaffected by phenobarbital treatment.

The effects seen are very complicated in that although phenobarbital has induced the levels of activity and cytochrome P-450 in the surgically treated over that seen in the animals not administered phenobarbital, the levels are not the same as that observed in the unoperated phenobarbital treated experimental animal.

This experiment was designed to answer two questions: Are the effects of phenobarbital and α-tocopherol additive? Does the phenobarbital effect depend on androgen? The data suggested that the effect of phenobarbital and α-tocoperol were not additive. Microsomal hydroxylation in both the vitamin E-supplemented and vitamin E-deficient animals was induced to the same level of activity. Liver microsomal hydroxylation in androgen depleted animals could be induced which indicated that the phenobarbital effect was not dependent on androgens, however there would seem to be a steroid hormone requirement for optimal induction.

Effect of α-tocopherol on Histones

From the above studies it would appear that vitamin E acts as an inducer in the microsomal hydroxylation system. Moreover, Carpenter has shown that the effect of vitamin E can be blocked if actinomycin D is administered prior to α-tocopherol treatment. These data suggest that a primary site of action for vitamin E is at or in the nucleus. In the past few years several investigators have studied nucleoprotein changes in regard to observed protein inductions. The studies have in-

dicated that differences may exist in the amount or species of nucleoproteins after the administration of protein inducers. In view of these observations, it is possible that vitamin E could cause changes in nuclear protein content or species. A comparative study of liver nuclear histones was undertaken to determine if differences could be observed between vitamin E-supplemented and vitamin E-deficient rats.

The purified histones were subjected to disc gel electrophoresis, stained with either Coomassie blue or Buffalo Black (Amido Schwartz), and measured for content and species using a Gilford Scanning Spectrophotometer. Both staining procedures indicated four major bands in addition, 8-10 minor bands were resolved with Coomassie blue. The four major bands were quantified using Buffalo Black and the results are presented in Table 8. The data are expressed as a percentage of the total area for all bands. No significant differences were noted between the control and experimental animals. Figure 3 represents a typical histone separation on polyacrylamide gel stained with Coomassie blue. There appear to be no obvious qualitative differences between the control and experimental animals.

The results of the histone analyses indicate that the effect of α-tocopherol is not mediated through changes in content or species of these nuclear proteins.

TABLE 8

COMPARISON OF HISTONES STAINED WITH AMIDO SCHWARTZ

	Control	Experimental
Number	(17)	(14)
Major band 1*	7.24 ± 0.74	5.64 ± 0.66
Major band 2*	21.04 ± 0.78	19.82 ± 1.00
Major band 3*	55.57 ± 1.56	58.06 ± 1.00
Major band 4*	18.62 ± 0.80	18.25 ± 0.59

*percent of total area ± S.E.

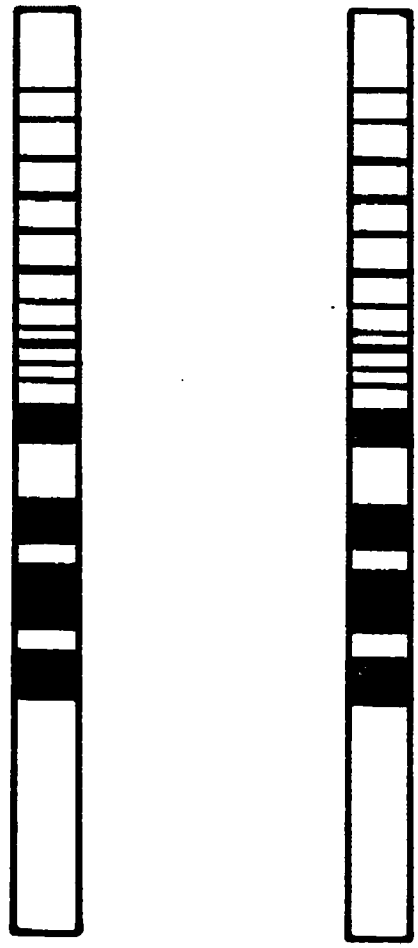


Figure 3. Polyacrylamide disc gel patterns of control and experimental histone stained with coomassie blue.

CHAPTER IV

DISCUSSION

Drug metabolism by liver microsomes is known to be influenced by many factors which include age, sex, species, strain, nutritional status and hormone levels. Vitamin E, a nutritional requirement and constituent of cell membranes has been implicated as a possible regulator in the drug hydroxylation system. Very significant differences were observed in hepatic drug metabolism activity between the vitamin E-supplemented and vitamin E-deficient rats. The effect of α-tocopherol can be blocked with actinomycin D, indicating protein synthesis is involved. This latter observation by Carpenter and those of Green and Bunyan (107) as well as Olson and Carpenter (96) suggest that α-tocopherol may be involved in microsomal protein regulation.

Liver microsomal drug hydroxylation in the vitamin E-supplemented rat is significantly higher than in the vitamin E-deficient rat, while the content of cytochrome P-450 is the same in both animals. These data suggest that α-tocopherol is an important physiological regulator of the liver microsomal hydroxylase system and that the observed difference in enzyme activity is not dependent on the content of cytochrome P-450.

Steroid hormones appear to be very important as regulators of the microsomal hydroxylase system, as well as a host of other hepatic enzymes (74,77,80,87,105,106,125). To study the possible interrelationships between androgens and vitamin E, animals of both diet groups were castrated.

Castration of the vitamin E-supplemented animal had no effect on either rates of hydroxylation or cytochrome P-450 content. Administration of testosterone to the castrated control was without effect on rates of hydroxylation and the amount of cytochrome P-450. In contrast, castration of the experimental resulted in decreased hydroxylation, but no change in content of cytochrome P-450. Administration of either testosterone or α-tocopherol to the castrated experimental caused an increase in microsomal hydroxylation but no change in content of cytochrome P-450. These data indicate that α-tocopherol can replace androgens as a regulator of this enzyme system, and in the absence of α-tocopherol, androgens appear to be regulators of the enzyme system.

Kato and Gillette (86) observed that castration of the male rat caused a lowered demethylation of aminopyrine. Their animals received an apparently adequate amount of vitamin E in the diet. The response to castration is similar to that observed in this study for deficient rats, but not to the control animals fed a diet high in α-tocopherol. Booth and Gillette (84) observed that administration of androgens to female rats increased drug metabolism, supporting the importance of androgens as possible regulators of this enzyme system. Finally, Kato and Onoda (74) were able to show that castration lowered activity, testosterone increased activity and estrogens inhibited the effect of

androgens in the male.

After adrenalectomy, hydroxylation by the control microsomes decreased, but the cytochrome P-450 content remained unchanged. Adrenal steroid hormones were ineffective in restoring enzymatic hydroxylation in the control, adrenalectomized animal. Similar decreases of hydroxylation were observed in the microsomes of the experimental rats after adrenalectomy, while the content of cytochrome P-450 was unchanged, but this animal responded to either adrenal hormones or α-tocopherol. Nebert and Gelboin (64) in studies on the effects of drug and hydrocarbon administration to normal and adrenalectomized rats, also observed that adrenalectomy caused a lowered rate of hydroxylase activity.

Several laboratories have reported a host of changes to specific enzyme activities due to adrenalectomy, ranging from a loss of glycogen to losses of nucleotides (DNA, RNA and coenzymes) (86,93,111-115). Marshall and McLean reported that either castration or adrenalectomy caused a loss of microsomal activity while the content of cytochrome P-450 was unchanged (109). These data agree with my observations.

The results of the present studies show that an intact adrenal gland is necessary for optimal activity of the liver microsomal hydroxylase system. The adrenal requirement for the activity of the microsomal hydroxylase system appears to be more than just the role of adrenal steroids, since the administration of the latter did not restore a complete recovery of activity in the adrenalectomized control administered adrenal hormones.

The adrenal is known to produce hormones other than steroid hormones, be important in ion balance and perform other functions in

the body. This work neither encompassed a complete study of all the effects incurred by deletion of the adrenal gland nor an evaluation of the effects of other adrenal products in relation to the liver microsomal hydroxylase system. The experimental, adrenalectomized rat did respond to adrenal steroid hormones. One explanation could be that the animal is so depleted of essential physiological molecules that administration of any physiological substrate would elicit a response. Because adrenal steroids did not cause a significant response in microsomes from the control, adrenalectomized animal does not mean that they are not important as possible regulators of the liver microsomal hydroxylation system.

When the animals were castrated and adrenalectomized, there was a fifty percent reduction of microsomal enzyme activity in both the control and experimental animals, while there was no change of cytochrome P-450 content in either diet group. Administration of adrenal steroid hormones had a positive effect in that it partially restored activity in both diet groups. The results of the work on the castrated, adrenalectomized control rats are intriguing. Since there was no effect on enzyme activity after castration and there was after adrenalectomy, one might expect to see enzyme activity in the double operated animal which was similar to that observed in the adrenalectomized rat. The enzyme activity dropped below that expected, indicating that in the absence of an intact adrenal gland, castration did have an effect. This reiterated the apparent requirement for steroid hormone substrate to maintain enzyme activity above a constituitive level.

Phenobarbital induces microsomal enzyme activity and cytochrome P-450 content in both the control and experimental animals. In regard to the control, neither castration or adrenalectomy inhibit this induction, however, the double operation does partially inhibit the phenobarbital induction. Cytochrome P-450 is induced comparably in all three groups of operated animals. The situation in the experimental animal is different in that castration, adrenalectomy and castration-adrenalectomy all cause some inhibition of enzyme induction by phenobarbital. Induction of cytochrome P-450 content, however, is not influenced by the various surgical procedures. The observations made in the phenobarbital studies indicate that in the absence of vitamin E, steroid hormones are important for phenobarbital induction. Moreover, some level of steroid hormone would appear to be important for optimal phenobarbital induction regardless of nutritional state.

These studies clearly indicate that phenobarbital induction is independent of α-tocopherol and steroid hormones. Orrenius, et al. (97) stated that the castrated-adrenalectomized rat could not be induced with phenobarbital unless adrogen was administered first. The data presented in the present study are clearly different and do not support the observations of Orrenius.

Nebert and Gelboin (64) observed that after administration of either phenobarbital or methylcholanthrene, an induction of enzyme activity occurred. The same percentage induction was observed in the adrenalectomized animals treated with either phenobarbital or methylcholanthrene as was seen in the controls.

In considering the function of α-tocopherol in the regulation of liver microsomal drug hydroxylation, an obvious parameter to look at closely is the well known antioxidant effect of this molecule. Lipid peroxidation *in vitro* is impaired in the presence of vitamin E. The possibility that the role of vitamin E in this system is that of an antioxidant has been suggested (62,121-124). During the course of the studies reported in this work, measurements of microsomal lipid peroxidation were performed routinely. These measurements showed no differences in lipid peroxidation by microsomes of the vitamin E-deficient animals regardless of hormone manipulation. Little lipid peroxidation was observed by preparations from the supplemented controls regardless of hormone status. These observations as well as the failure of administration of other antioxidants by Carpenter (82) *in vivo* or *in vitro* to influence the rate of microsomal drug metabolism but to eliminate lipid peroxidation do not support a direct correlation between enzymatic hydroxylation and lipid peroxidation.

The effect of α-tocopherol is related to the enzyme activity found in the liver endoplasmic reticulum. In trying to determine whether this effect was on rates of protein synthesis, turnover or differences in species, the work of Tsai (163) is of interest. She studied protein synthesis and turnover *in vivo* and observed that the rates of total microsomal protein synthesis and turnover were the same in the vitamin E-supplemented and vitamin E-deficient animals. She also reported that after phenobarbital administration, the initial incorporation *in vivo* of labeled amino acid into microsomal membrane was the same for both diet groups.

Tsai (163) then undertook a project to determine possible quantitative or qualitative differences of microsomal proteins by use of polyacrylamide disc gel electrophoresis. In these studies, it was determined that there was no differences in species of proteins between the α-tocopherol supplemented animal and the α-tocopherol deficient animal, however, it was observed that out of eighteen bands or protein species, six were quantitatively different between the two diet groups. A similar observation was made after phenobarbital treatment.

Cytochrome P-450 is the active oxygenase in this system. It would therefore seem probable that regulation of microsomal hydroxylation would involve this cytochrome. Since the liver microsomal hydroxylase system is dependent on cytochrome P-450 for activity, it is pertinent to consider a possible role of α-tocopherol in relation to heme synthesis.

Murthy, et al. (69) and others (70,116-119,121) have suggested that vitamin E is involved in heme syntehsis. Cytochrome P-450 has been studied extensively in the past few years. There now seems to be good evidence that multiple forms of the cytochrome exist (143-145). The different forms of cytochrome P-450 are preferentially induced by specific agents. Phenobarbital induces the "native" form of cytochrome P-450 while 3-methylcholanthrene induces a new form (cytochrome P_1-450). This latter data was obtained by comparing spectral properties which were different for P-450 and P_1-450. Data obtained using substrate binding as an index also indicate different forms of the cytochrome exist. Nair reported a two-fold difference in levels of both cytochrome

b_5 and P-450 between vitamin E-supplemented and vitamin E-deficient rats. He suggests that the rates of δ-aminolevulenic acid synthetase activity in bone marrow and δ-aminolevulenic acid dehydratase in liver are depressed in vitamin E-deficient rats and that lowered heme synthesis is responsible for lowered levels of heme containing cytochromes. Since cytochrome P-450 is the binding site for substrate, Nair maintains that this reflects the difference seen in drug hydroxylation between the two diet groups. Several hundred measurements of cytochrome P-450 content in the microsomes of the two diet groups have been made in this laboratory and do not indicate there are real differences in heme protein content. Nair used a different strain of rats and different assay conditions for measurements of cytochrome P-450, which may account for the difference between his observations and ours. The present studies do not indicate a new species of cytochrome P-450 with different spectral properties is found. These measurements, however, do not tell us whether a conformational change has occurred.

One approach in elucidating the primary site of action of α-tocopherol would be to look at some excellent work concerning another lipid soluble vitamin, which is also considered a hormone by many scientists. Grey and DeLuca (63) have been involved for quite some time in establishing the primary site of action and active form of vitamin D. They have shown that vitamin D is initially converted to the 25-hydroxy derivative in the kidney, then transported to the liver where it undergoes yet another hydroxylation at the one position. The 1,25-dihydroxy derivative then seems to be the active molecule. DeLuca has submitted evidence that the 25 hydroxy derivative enters the

nucleus and causes formation of proteins which then further metabolizes the molecule. Vitamin D is actively involved in the uptake of calcium but is active only as a derivative other than that taken into the body. Further work now shows the existence of a carrier protein in the cell for vitamin D binding and transport to other parts of the cell (127).

It is very tempting to postulate a similar arrangement for α-tocopherol. At the nucleus, vitamin E could be involved in the initiation of some component of the microsomal hydroxylase system, either structural or enzymic. This mechanism would appear then to be hormone-like.

Another approach to understanding the effects of α-tocopherol and its interrelationships with steroid hormones would be to look at some of the current concepts of androgen action. The function of α-tocopherol in liver mixed function hydroxylation appears to be hormone-like.

Liver microsomal hydroxylation in the vitamin E-supplemented rat was unaffected by either androgen deletion or addition, suggesting that the effect of α-tocopherol is not mediated via androgen. Results obtained from vitamin E-deficient livers suggest that in the absence of α-tocopherol, androgens are important as regulators of this hydroxylating system; moreover, when the experimental castrated animals are placed on a vitamin E diet, the response is inhibited by androgen deletion. These studies indicate that α-tocopherol can replace androgens in this system. However, one must be careful not to extrapolate too far concerning vitamin E replacement of androgens. When vitamin E is given to an unoperated vitamin E-deficient animal, one observes an

"induction" of enzyme activity to the level of the vitamin E-supplemented but after removal of the testes, enzyme activity returns only to the level of the unoperated vitamin E-deficient in response to α-tocopherol.

Androgens have been implicated in two roles; the first being that of a coenzyme which serves as an allosteric effector, the second being a reaction with repressors to initiate protein synthesis at the transcriptional level (105). In consideration of an allosteric effector, one would expect to see immediate effects in the cell on a particular target enzyme. Androgens reacting with repressors of protein synthesis at the transcriptional level would require more time for an effect to be observed. Many different systems have been studied which are readily responsive to steroid hormones, especially androgens; moreover, in several species, administration of androgens to females can enhance a multitude of enzyme activities. In contrast, the administration of estrogens to males results in a decrease, or in some examples, cessation of particular enzyme activities (74).

In the past few years, a molecule that at first was considered an artifact of isolation but later proved to exist within the cell has come into prominance as a "hormone-like" molecule itself. This molecule, which often is called the second messenger, is 3',5'-cyclic AMP. Certainly the most interesting aspect of cyclic AMP is its ability to bring about a host of responses depending on the particular tissue being discussed or whether one is looking at an <u>in vivo</u> situation. The brilliant work of Sutherland in the illucidation of the function of c-AMP has suggested that it may mediate almost any response one wants

to observe. The present idea concerning its mechanism of action appears to be one of activating specific phosphorylases which activate an enzyme, or to phosphorylate histones which facilitate DNA transcription. This latter event would seem to require a chain of events.

Singhal, et al. (14) reported that in rat seminal vesicles and prostate gland, the addition of testosterone propionate to immature and castrated rats produced increased levels of c-AMP. Paradoxically, Tsang, et al. (120) presented data using adrenal slices in vitro in which c-AMP stimulated corticosterone synthesis. These two observations point out all too well that the c-AMP story is very complicated and what one sees in vitro or in vivo as well as under "normal" and stress conditions must be interpreted with caution. Grannier, et al. (76) has reported induction of tyrosine transaminase in HTC cells by adrenocorticosteroids which indicates that mediation of the response is not through c-AMP. Even more intriging are reports that steroidogensis is in fact initiated by c-AMP (128-129).

The observation which is gaining more prominance suggests that cyclic AMP is very intricately involved in phosphorylation of histones to facilitate transcription (130,131). Here cyclic AMP becomes the mediator of hormones in the inductive process. A recent publication suggests that an adenyl cyclase is located in the nucleus (132), indicating that cyclic AMP levels in the cell need not be affected to show induction via cyclic AMP through inducer molecules such as vitamins or hormones.

Williams-Ashman has suggested that steroids are involved in the induction of protein synthesis at the transcriptional level, an effect

blocked by actinomycin D (133). In support of Williams-Ashman's idea, reports from another laboratory (134) present data which reveal that both estrogens and androgens are involved in stimulation of both RNA synthesis, RNA polymerase activity, and that this is a long term effect involving enhancement of both ribosome and endoplasmic reticulum production.

Tomkins, et al. (136) has suggested a model for the regulation of protein synthesis by steroid hormones. Working with HTC cells in culture and the tyrosine aminotransferase enzyme, he found that adrenal cortical steroids stimulated enzyme activity. This stimulation appeared to be a post transcriptional process because chemical agents used to block transcription did not inhibit, whereas those which blocked at later steps of protein synthesis, were inhibitory. Tomkins then suggested that an RNA repressor was produced from a regulator gene which bound to a constituitive m-RNA produced by the structural gene to block post transcriptional process. Steroids bound to this repressor molecule and prevented its binding to RNA produced by the structural gene. C-AMP is not involved in Tomkins system since neither adenyl cyclase activity nor c-AMP have been found in the HTC cells. Since the work of Tomkins in 1970, Manganiello and Vaughan (91) have observed that adenyl cyclase activity is present in HTC cells. Moreover they were able to stimulate this activity with epinephrine and dexamethasone.

Dinning (49) has reported that due to lack of a needed control mechanism, DNA replication is much greater in the vitamin E-deficient animal as opposed to the supplemented animal. This occurs in many

tissue types and both DNA and RNA levels are increased. Dinning then suggests that α-tocopherol exerts a controlling influence on the rate of DNA biosynthesis. Kassenaar, et al. (137) reported a similar situation in castrated rats in which an elevated concentration of DNA in the seminal vesicles was observed in the absence of testicular steroids.

In light of Dinning's observations and other work which has suggested that hormones and some vitamins may exert their effect at the nuclear level, nuclear proteins were studied as a possible point of affect. There were neither qualitative nor quantitative differences of histones between vitamin E-supplemented and vitamin E-deficient animals. It has been observed by many people that histones tend to aggregate during isolation and that these aggregations do not separate on polyacrylamide gel electrophoresis. Therefore, it is not possible at this time to make a conclusive statement that no differences in histones exist between the two diet groups. There is now a growing body of evidence that acidic nuclear proteins (ANP) are involved in the regulation of many enzyme systems. This area of nuclear protein involvement and its possible consequences is now being studied in the laboratory.

Murthy et al. (50), using adrenalectomized rats, have reported an increased rate of RNA synthesis and histone phosphorylation after hydrocortisone administration, however, they found the rate of histone synthesis was unchanged. They also observed that the rate of turnover of DNA and histones was the same. These data then suggest that the stimulation of RNA and then protein synthesis may be independent of

histone levels or states. However, studies by Burdon and Pierce (51) have shown that a variety of steroid hormones inhibit the action of c-AMP phosphodiesterase. This would suggest that steroid hormones are able to mediate modification of histones for RNA synthesis. However, a report by Arnaud et al. (52) suggests that a nuclear protein-hormone receptor complex can directly stimulate the activity of RNA polymerase.

From the observations made in my work, it appears that there is a constituitive level of enzyme activity in the liver microsomes and that this constituitive level exists after castration, adrenalectomy and vitamin E deprivation. The site of regulation of this enzyme system by vitamin E may be at the level of transcription. I would like to propose a model adapted from Ruddon and Rainey (53) to explain a possible control mechanism for the liver microsomal hydroxylase system. The model (figure 4) proposes that α-tocopherol enters the nucleus via a carrier protein from the cytoplasm and interacts with a specific acidic nuclear protein (ANP) receptor. This α-tocopherol nuclear protein then becomes a substrate for a specific phosphorylation reaction, mediated by cyclic AMP. This phosphorylation activates the tocopherol ANP in such a way that it can actively initiate RNA polymerase activity of a particular gene site. Once the initiation has occurred, the phosphorylated, vitamin ANP would be released, dephosphorylated and recycled, thus maintaining a particular level of inducer (α-tocopherol) in the nucleus. However, it should be noted that most of the aforementioned complex should be metabolized so as to provide for a shut off of activity. This metabolism could then be a signal for the cytoplasm

to make new carrier protein, a second point of regulation. In this model chemical alteration of histone by either acetylation or phosphorylation as a concomitant, permissive event allows for the local "melting out" of the DNA chain which would be involved in RNA synthesis. This is not to say that histone is released. The role of hormones may be thought of in terms of the hypothesis proposed by Burdon (51) in which they inhibit c-AMP phosphodiesterase. However, steroid hormones may also follow the pattern proposed for vitamin E.

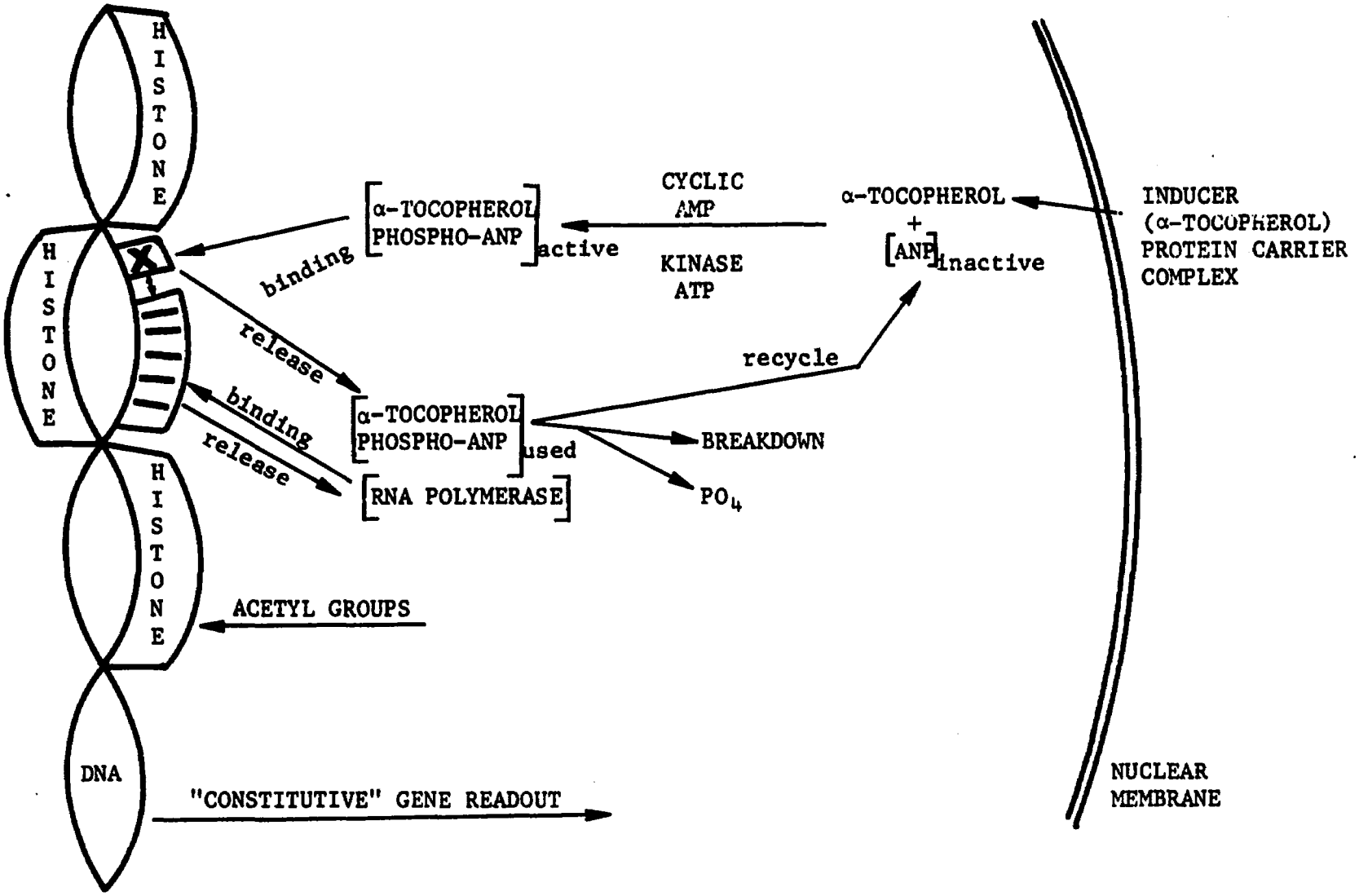


Figure 4. Proposed scheme for α-tocopherol regulation.

CHAPTER V

SUMMARY

The regulatory role of α-tocopherol on liver microsomal enzyme hydroxylation has been studied in vitamin E-supplemented and deficient animals. The rates of microsomal hydroxylation in the control animals are about twice that observed in the experimental. Furthermore, castration of the control animal has no effect on microsomal hydroxylation while it causes a reduction in the experimental. In the experimental, the effect of castration can be reversed with either α-tocopherol or testosterone, indicating that these two molecules can replace each other in this system. The latter statement can also be applied to the control situation.

Adrenalectomy causes the same decrease of enzyme activity in both the control and experimental animals. The effect in the control animal cannot be reversed by administration of adrenal steroid hormones. The effect of adrenalectomy in the experimental animal can be partially reversed with adrenal steroid hormones or α-tocopherol. These observations indicate that an intact adrenal gland is necessary for optimal enzyme activity.

Castration and adrenalectomy causes a fifty percent drop of enzyme activity in either the control or experimental. Administration

of adrenal steroid hormones partially restores activity in either the control or experimental animals while α-tocopherol partially restores activity in the experimental.

The content of cytochrome P-450 is unaffected in all of the above mentioned manipulations, i.e. castration, adrenalectomy, castration-adrenalectomy, etc.

Induction of this enzyme system by phenobarbital has also been studied. The vitamin E state of the animal does not affect the induction of either the enzyme activity or content of cytochrome P-450. This would indicate a role of phenobarbital independent of vitamin E. The operated animals were also administered phenobarbital and were induced. Although hormone deletion did not block the induction by phenobarbital, it would appear that in the absence of hormones, α-tocopherol is required for optimal induction.

Vitamin E is proposed to regulate this enzyme system at the transcritional level of protein synthesis.

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