OXIDATIVE METABOLISM OF ESTRONE BY RAT LIVER PREPARATIONS

by

Neil J. Moir, B.S., M.S.

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

May 1968

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

To Kathy

ACKNOWLEDGEMENTS

The author gratefully acknowledges the instruction, advice and encouragement given by Doctor A. Colás, Professor in charge of this thesis. Appreciation is expressed to Drs. Edward S. West and Richard T. Jones, former and present Chairmen of this Department. The author greatly appreciates the technical assistance provided by Mr. Dennis Partipilo and Mr. Don Gregonis and the secretarial help given by Mrs. Mary Buck.

Permission to use major equipment items was given kindly by Drs. C. Beatty, M.A.Greer, R.B. Lyons and H.S. Mason.

TRIVIAL AND SYSTEMATIC NAMES

cholesterol - cholest-5-en-3β-ol

progesterone - pregn-4-en-3,20-dione

deoxycorticosterone - 21-hydroxypregn-4-en-3,20-dione
cortisol - 11β,17α,21-trihydroxypregn-4-en-3,20-dione

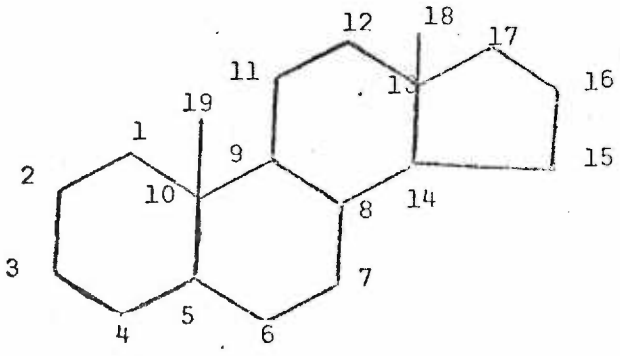
DHA, dehydroepiandrosterone - 3β-hydroxyandrost-5-en-17-one
7α-OH-DHA - 3β,7α-dihydroxyandrost-5-en-17-one
16α-OH-DHA - 3β,16α-dihydroxyandrost-5-en-17-one

androstenedione - androst-4-en-3,17-dione
testosterone - 17β-hydroxyandrost-4-en-3-one

E_1, estrone - 3-hydroxyestra-1,3,5 (10) trien-17-one
2-OH-E_1, 2-hydroxyestrone - 2,3-dihydroxyestra-1,3,5 (10)-trien-17-one
4-hydroxyestrone - 3,4-dihydroxyestra-1,3,5 (10)-trien-17-one
6α-hydroxyestrone - 3, 6α-dihydroxyestra-1,3,5 (10)-trien-17-one
6β-hydroxyestrone - 3,6β-dihydroxyestra-1,3,5 (10)-trien-17-one
15α-hydroxyestrone - 3,15α-dihydroxyestra-1,3,5 (10)-trien-17-one
16α-hydroxyestrone - 3,16α-dihydroxyestra-1,3,5 (10)-trien-17-one
2-methoxyestrone - 2-methoxy-3-hydroxyestra-1,3,5 (10)-trien-17-one

E_2, estradiol - estra-1,3,5 (10)-trien-3,17β-diol
2-OH-E_2, 2-hydroxyestradiol - estra-1,3,5 (10)-trien-2,3,17β-triol
6α-hydroxyestradiol - estra-1,3,5 (10)-trien-3,6α,17β-triol
6β-hydroxyestradiol - estra-1,3,5 (10)-trien-3, 6β,17β-triol
7α-hydroxyestradiol - estra-1,3,5 (10)-trien-3,7α,17β-triol
15α-hydroxyestradiol - estra-1,3,5 (10)-trien-3,15α,17β-triol
2-methoxyestradiol - 2-methoxyestra-1,3,5 (10)-trien-3,17β-diol

E_3, estriol - estra-1,3,5(10)-trien-3,16α,17β-triol
2-hydroxyestriol - estra-1,3,5(10)-trien-2,3,16β,17α-tetraol
epiestriol - estra-1,3,5(10)-trien-3,16β,17α-triol



Steroid ring system

TABLE OF CONTENTS

LIST OF TABLES AND ILLUSTRATIONS

Statement of the Problem:

The level of thyroid activity in humans modifies the pattern of estradiol-^{14}C metabolism *in vivo* (46,47). The formation of 2-hydroxy derivatives is increased at high levels of thyroid function so that they become the major products of estrogen metabolism; in instances of hypothyroid activity the amount of these derivatives is diminished. Estriol formation (16α-hydroxylation) changes in the opposite direction, that is, with increasing levels of thyroid activity the rate of 16α-hydroxylation decreases. These workers have concluded that hydroxylations at C-2 and at C-16 of estrone are competitive reactions for the available substrate.

The original intent of this work was to test the conclusions from the *in vivo* work of Fishman, et al. (46, 47) with rat preparations *in vitro*. Using liver slices, 10,000 x *g* supernatant or microsomes from normal, thyroidectomized, and thyroxine treated rats I was unable to demonstrate a thyroid-dependant competition between the 2-and 16α-hydroxylation reactions.

My attention then turned to finding differences in estrogen hydroxylase activities so that efforts could be made to classify them as a first step in the isolation and purification of the components involved in these reactions.

I. INTRODUCTION

A. Effects of thyroid function on steroid metabolism

The functional state of the thyroid gland exerts profound effects on steroid metabolism. Two main approaches to study these effects have been (1) *in vivo* in humans and (2) *in vitro* with tissues from animals with altered thyroid hormonal activity.

The interrelationships between thyroid activity and various steroid transformations have been summarized in Table 1.

One of the earliest noticed effects of thyroid function on steroid metabolism was that in cases of human thyrotoxicosis there was an increased rate of disappearance of administered cortisol over that observed in normal subjects (19, 93, 112, 113). The studies of McGuire and Tompkins (98) suggest more than one mechanism is responsible for such an acceleration. They gave thyroid hormone to immature rats and noted that there was an increased rate of reduction of the 4-5 double bond of Δ^4-3-ketosteroids, which was a reflection of an increase in both the available NADPH, the rate limiting reactant in this process, and the microsomal 5α-reductase activity. This work was confirmed by the results of Bradlow et al. (6) who found that thyroid hormone affects primarily the reductive pathways of testosterone and dehydroepiandrosterone *in vivo*.

Continuing their study of the 5α-reductive pathways, Bradlow et al. (7) were unable to detect any substantial difference between the metabolic patterns of ^{14}C-progesterone produced by myxedemic and normal patients. By contrast there was a shift towards the increased production

Table No. 1. A summary of thyroid hormone effects on steroid metabolism.

SUMMARY OF THYROID EFFECTS ON STEROID HORMONE METABOLISM

Steroid	Reaction	Type of Study	Hyperthyroidism	Hypothyroidism
Cholesterol	7α-Hydroxylation	Mitochondria*	Normal	Not Tested
Cortisol	11β-OH dehydrogenation	*in vivo*	Increases	Decreases
Progesterone	5α-Reduction	*in vivo*	Increases	Normal
11β-Hydroxyandrost-4-ene-3,17-dione	5α-Reduction C-3-reduction	*in vivo*	Normal Normal	Decreases Increases
Dehydroepiandrosterone	7α-Hydroxylation	Homogenate*	Decreases	Not Tested
Testosterone	5α-Reduction	*in vivo*	Increases	Decreases
Estradiol	2-Hydroxylation 16α-Hydroxylation	*in vivo*	Increases Decreases	Decreases Increases

* animals pretreated with thyroxine

of 5α-reduced metabolites in hyperthyroid subjects.

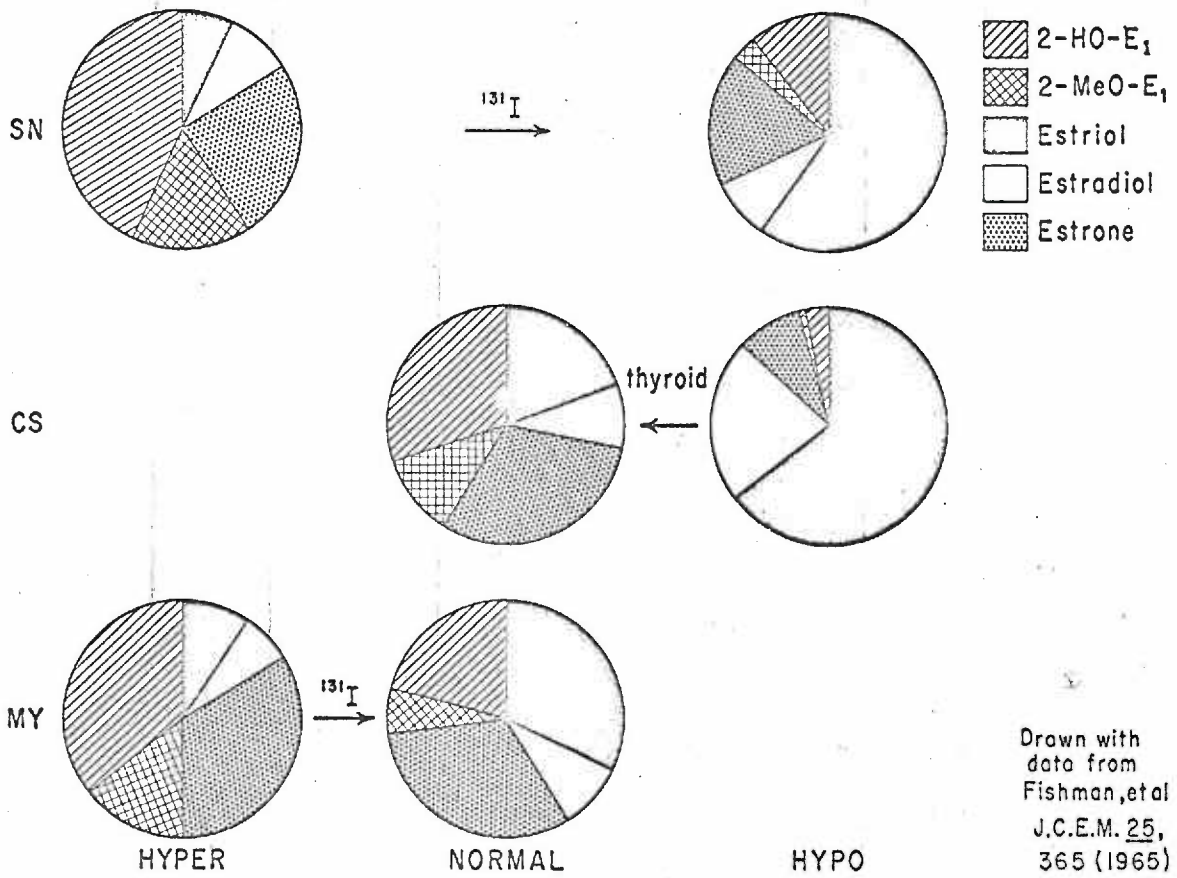
Rat liver 11ß-hydroxysteroid dehydrogenase activity has been reported to decrease upon pretreatment of the animals with thyroxine and increase upon thyroidectomy (82). Addition of thyroxine to liver homogenates from thyroidectomized rats has no effect on this dehydrogenase activity.

Besides the aforementioned reductive pathways, thyroid hormonal levels also influence a number of steroid hydroxylase activities. Recently Sulcova, et al. (119) reported that thyroid hormone administration suppresses 7α-hydroxylation of dehydroepiandrosterone by rat liver preparations. This report contrasts, however, with the observations of Mitropoulos and Myant (101) who have concluded that thyroxine pretreatment in rats has little or no effect on mitochondrial 7α-hydroxylation of cholesterol.

Fishman et al. (46, 47) have concluded that a competition between hydroxylation of estrone at C-2 and C-16 exists in humans which is partially controlled by the level of thyroid hormones. As thyroid activity increases (either artificially or as a result of a pre-existing condition) there is an increased urinary excretion of 2-hydroxylated estrogens when tracer ^{14}C-estradiol-17β is administered. This increase is accompanied by a decrease in 16α-hydroxylated products. Furthermore, ^{131}I treatment or conditions of hypothyroidism shift the metabolic patterns to more 16α- and less 2-hydroxylated products. For a summary of these results see figure 1.

Figure No. 1. Thyroid function and estrogen hydroxylation *in vivo*.

THYROID FUNCTION AND ESTROGEN HYDROXYLATION *IN VIVO*



Drawn with data from Fishman, et al J.C.E.M. 25, 365 (1965)

In a strain of guinea pigs characterized by a high urinary production of polar corticosteroids, Burstein and Fajer (20) have been able to induce further increases in the 2α- and 6β-hydroxylase activities with massive doses of thyroxine. Thyroxine is without effect however in guinea pigs with a low production of polar corticosteroids.

Since a number of other cellular components involved in oxidation reduction reactions can influence steroid metabolism either directly or indirectly, a short summary of thyroid effects on some of these constituents may be useful. Treatment of rats with high doses of thyroxine leads to an increase in the levels of succinic dehydrogenase, coenzyme Q, and cytochrome oxidase as well as a decrease in NADH: cytochrome *c* oxidoreductase activity present in liver homogenate (102). Raw and DaSilvia (114) treated rats for 3 to 4 weeks with thyroxine and noted decreases in both mitochondrial and microsomal NADH: cytochrome *c* oxidoreductase. Microsomal cytochrome b_5 was decreased to 40 percent of the control. In this same group of animals there was a 3-fold increase in the amount of microsomal cytochrome P-450. In contrast to these findings, Alvares and Mannering report that thyroxine pretreatment decreases the level of cytochrome P-450 and certain N-demethylating enzymes (1).

Most recently Suzuki et al. (124) have reported that thyroidectomy in rats decreases the rate of rat liver microsomal NADPH oxidation, an effect which can be reversed upon triiodothyronine treatment

(124). These same workers find an increase in the content of cytochromes b_5 and P-450 10-15 days after thyroidectomy. These increases are reversed by triiodothyronine treatment.

A synthesis of the effects of thyroid hormone on steroid metabolism cannot be made at present because of insufficient information concerning its mechanism of action in regard to steroid metabolism. The need to explore in detail some of these mechanisms was one of the factors which induced me to undertake this project.

B. Hydroxylations of estrogens *in vitro*

Many years were spent in the isolation of the three classic estrogens-estrone, estradiol-17β and estriol. E. A. Doisy, in the United States, E. Laqueur in Holland and A. Butenandt in Germany independently isolated estrone in the years 1929-1930. Estradiol-17β was isolated by Doisy and his colleagues a few years later. In this same period estriol was isolated by G. F. Marrian in England. For about twenty years no new estrogens were discovered. A summary of the recent literature involving estrogen hydroxylation reactions is given in Tables 2, 3 and 4.

The interconversion of estrone and estradiol-17β catalyzed by a steroid 17β-hydroxysteroid dehydrogenase has been well documented in both *in vitro* and *in vivo* systems in many animals (36). The enzyme has been purified by Langer & Engel (90). Quantitative measurements made in recent years have shown humans metabolize estrone almost identical to the manner in which they metabolize estradiol 17β (3, 4, 18).

Table No. 2. Estrogen hydroxylations demonstrated in *in vitro* rat liver preparations.

RAT LIVER HYDROXYLATIONS *IN VITRO*

Position	Substrate	Product Isolated	Preparation	Reference
2	Estriol	2-Hydroxyestriol 2-Methoxy estriol	Slices	75, 74
2	Estriol	2-Estriol	Microsomes	76
2	Estrone	2-Hydroxyestrone 2-Methoxy estrone	8,000 x g Supernatant	97
2	Estrone	2-Hydroxyestrone	Microsomes	58, 99
4	Estrone	4-Hydroxyestrone	Homogenate Microsomes	58, 99
6	Estrone	6(ξ)-Estrone	Slices	9
6	Estradiol	6(ξ)-Estradiol	Slices	12
6	Estradiol	6α-Hydroxyestradiol 6β-Hydroxyestradiol	Microsomes	15
10	Estradiol	17β-OH-estra-p quinol-10β	Microsomes	53, 54, 57
10	Estrone	17-Oxo-estra-p quinol-10β	Microsomes	56, 60, 58
16	Estradiol	Estriol	Brei	51

Continued

Position	Substrate	Product Isolated	Preparation	Reference
16	6-OH-estradiol	6-OH-estriol	Slices	12
16	Estradiol	Estriol	Slices	9, 10
16	Estrone	16α-Hydroxyestrone 16β-Hydroxyestrone	Microsomes	58

Table No. 3. Estrogen hydroxylation occurring in human liver *in vitro*.

HYDROXYLATION BY HUMAN LIVER *IN VITRO*

Position	Substrate	Product Isolated	Preparation	Reference
2	Estrone	2-Methoxy estrone	Slices	16
6	Estrone	6α-OH-estriol	Slices	16
6	Estradiol	6α-OH-estradiol 6α-OH-estriol	Slices	16
6	Estradiol	6α-OH-estradiol	Slices	11, 13
7	Estrone	7α-OH-estradiol	Slices	16
15	Estradiol	15α-OH-estradiol	Slices	16
15	Estradiol	15α-OH-estradiol 15α-OH-estrone	Perfusion	119
16	Estrone	16α-OH-estrone Estriol Epiestriol	Slices	16
16	Estradiol	16α-OH-estrone Estriol	Slices	16
16	Estradiol	Estriol	Slices	40
16	Estradiol	Estriol Epiestriol		42

Table No. 4. Estrogen hydroxylation reaction demonstrated in humans *in vivo*.

HYDROXYLATION REACTIONS DEMONSTRATED IN HUMANS *IN VIVO*

Position	Substrate	Product	Reference
2	Estradiol-17β	2-Methoxy estrone	45, 46, 47
	Estradiol-17β	2-Methoxy estrone	85
	Estradiol-17β	2-Methoxy estrone	39, 41
15	Estrone Estradiol	15α-Hydroxyestradiol	119
15	Estrone sulfate	15α-Hydroxyestrone	72
16	Estradiol-17β	Estriol	41, 45, 46, 47
16	Estrone, Estradiol-17β	Estriol	3, 4
16	Estradiol-17β	16-Keto-Estradiol-17β	94
16	Estrone	Estriol	112

Fishman, et al. (45), using an isotope ratios technique, noted that the conversion of estradiol-17β to estrone must proceed *in vivo* more rapidly than the reduction of estrone to estradiol-17β. These workers concluded that 16- and 2-hydroxylation products formed by incubating either substrate took place with estrone rather than estradiol-17β.

1. *In vitro* 2-hydroxylation of estrogens

After the isolation of 2-methoxylated derivatives of estrogen from human urine by Kraychy and Gallagher (84, 85) and Engel (38) and subsequent identification of 2-hydroxyestrone as a metabolite of estradiol in man by Fishman, et al. (45), the question as to the site of 2-hydroxylation became important. King (75, 76) was the first to demonstrate *in vitro* 2-hydroxylation of estriol by rat and rabbit liver homogenates. He noted that 2-hydroxylation occurs in the microsomal fraction and requires either $NADH_2$ or $NADPH_2$ and possibly a folic acid derivative, and there is no sex difference. This worker found no 2-hydroxylase activity in homogenates of kidney, ovary and uterus.

Marks and Hecker (99) have confirmed King's results using radioactive estradiol-17β and estrone, except that they noted a sex difference in that male rats produce more 2-hydroxylated products than female rats. In addition, Hecker, et al. have noted that incubations of microsomes in Tris buffer stimulates the production of 2-hydroxylated products over that observed in phosphate buffer. These results are different from King's in that he observed no 2-hydroxylation of estriol in Tris buffer. Hecker and Marks have found intensive formation of 2-hydroxyestrone only if incubations with microsomes are fortified with

additions of soluble supernatant or tetrahydrofolic acid. These additions were seen to inhibit other hydroxylations.

In vitro 6-hydroxylation of estrogens

Mueller and Rumney (103) were the first to demonstrate the *in vitro* conversion of estradiol-17β to 6-hydroxylated products by mouse liver microsomes incubated under aerobic conditions. The absolute configuration was not determined for the reference compounds at that time and the products were tentatively identified as 6'β'-hydroxyestrone and 6'β'-hydroxyestradiol-17β and later correctly identified as 6α-epimers. Breuer (8) subsequently identified the same metabolites from incubation of estradiol-17β with rat liver slices. The configuration of the 6-hydroxyl groups in the neutral steroids at this time was well established (2), so the configuration of the 6-position in estrogens was determined by incubating 6α-hydroxy-4-androstene-3,17-dione with placental microsomes (15). The product obtained was identical with 6'α'-hydroxyestrone and 6β-hydroxy-4-androstene-3,17-dione yielded 6β-hydroxyestrone. Breuer also demonstrated that rat liver slices contain a 6α- and a 6β-hydroxysteroid oxidoreductase. Later experiments have demonstrated that both 6α- and 6β-hydroxylase are localized in the microsomal fraction of the cell (5). Breuer, et al. (81, 17) have also isolated 6-hydroxylated estrogens from human urine and Cedard and Knuppen (25) from human placental incubations *in vitro*.

In vitro 16-hydroxylation of estrogens.

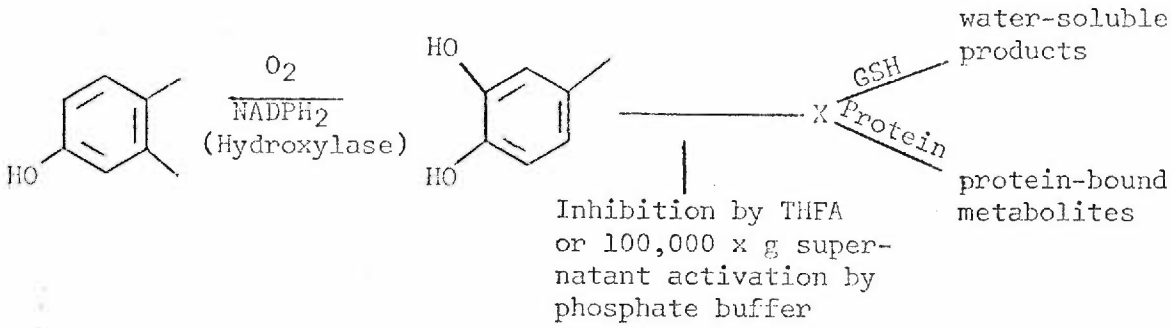
Estriol, isolated by Marrian in 1930, was the first 16-hydroxylated estrogen identified. As much as 45% of an administered dose of

either radioactive estradiol-17β or estrone is excreted by man as estriol (14, 18). Fishman, et al., by using estradiol labeled in the α-position by tritium has shown that *in vivo* most of the estradiol is converted to estrone before 16α-hydroxylation takes place (45). Pangel and Breuer (110) have shown with rat liver that the 16α-hydroxylase is located in the microsomes and requires $NADPH_2$ as a cofactor. For a more complete discussion of 16-hydroxylation see the review by Breuer (14).

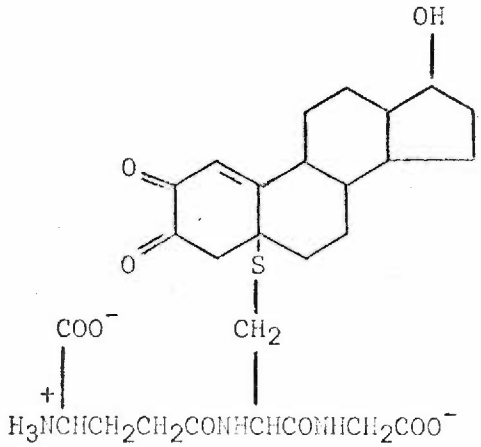
C. Formation of protein-bound and ether-insoluble, water-soluble products

Since 1934, when Zondek (132) demonstrated that minced liver could rapidly inactivate estrogens, many workers have attempted to study the nature of the inactivation products (14). More recently, Valcourt, et al. (126) found that, of the estrone-^{14}C administered to rats and recovered from the bile and urine, only one-third was extractable with ether even after enzymatic hydrolysis. Estrogens are inactivated largely by oxidative enzyme systems since cyanide, azide and carbon monoxide inhibit the inactivation processes. Knowing that plant phenolases could also inactivate estrogens (49, 128) Jellinck (69) compared plant and liver inactivation processes and demonstrated differences in the nature of the products formed by mushroom tyrosinase and rat liver. Inactivation products can be divided into two groups - protein-bound (precipitated by 10% trichloroacetic acid) and ether-insoluble, water-soluble products. Riegel and Mueller (117) and Hecker and Zayed (55) have excluded conjugation with sulfuric or glucuronic acid as possibilities

since these products cannot be released by procedures which cleave these linkages. Recently these workers have suggested that the water-soluble products are formed by combination with naturally occurring sulfhydryl compounds, for example, glutathione (59, 70, 71, 99). This suggestion is supported by the fact that the addition of glutathione or cysteine to rat liver microsomes incubated with estrone-^{14}C produces a marked increase in the amount of ^{14}C recovered in the aqueous fraction after ether extraction. Lazier and Jellinck (91) have noted that 2-hydroxyestrone and 2-hydroxyestradiol are potent inhibitors of the conversion of estrone to water-soluble metabolites while estrogens substituted in the 6, 10 or 16 positions are not. Marks and Hecker (99) have recently noted that by using Tris instead of phosphate buffer there was a marked increase in the amount of 2-hydroxyestrone that could be formed from estrone by rat liver microsomes. This increase was concomitant with a decrease in the amount of protein-bound and water-soluble metabolites formed. These two workers postulate that estrone is first 2-hydroxylated and then converted to a compound X which then reacts with glutathione or protein to become either a water-soluble or protein-bound metabolite. They further postulate that the conversion of 2-hydroxyestrone to X is inhibited by tetrahydrofolic acid or 100,000 x *g* supernatant and activated by phosphate ions:



Jellinck has recently isolated by means of Sephadex chromatography a glutathione-estrogen complex after incubation of estradiol-14-C with rat liver microsomes in the presence of the cell sap or with glutathione (71). The addition of albumin to the same system resulted in the formation of a radioactively labeled albumin complex. These observations support in part the postulate of Marks and Hecker (99). Jellinck has further postulated that the formation of the protein-bound metabolite involves reaction of a sulfhydryl group with the number 5 carbon of the 2-hydroxylated estrogen as shown below:



D. Involvement of cytochrome P-450 in steroid metabolism

The presence of a CO-binding pigment in liver microsomes was first observed by G. R. Williams (129) in 1958. Klingenberg (77) later examined NADH reduction of this compound and its lability in the presence of certain detergents. Omura and Sato have originated the name cytochrome P-450 (107) to describe this unique hemoprotein which in its reduced form exhibits an absorption maxima at 450 mμ after exposure to carbon monoxide. They (108) later described the conversion of P-450 in the presence of detergents to its inactive form P-420.

The parallel increase in the amount of P-450 and NADPH-dependent, drug-metabolizing and steroid-hydroxylation activities following administration of certain drugs (i.e., phenobarbital) to rats (115, 29) has suggested close relationship between the two.

In 1957, Ryan and Engel (118) observed a decrease in 21-hydroxylation of 17-hydroxyprogesterone by beef adrenal microsomes in the presence of carbon monoxide. Following up this work a few years later, Estabrook's group (43) provided evidence for the involvement of P-450 in this reaction by using a photochemical action spectrum technique. They observed that light having the wave length of 450 mμ was the most effective in relieving the CO inhibition.

Cooper, et al. (32,33), studying the CO sensitivity of codeine *N*-demethylation, aminopyrine *O*-demethylation, and acetaniline *p*-hydroxylation reactions, reported a competition between carbon monoxide and oxygen. More recently, Conney, et al. (30, 89) have extended these studies to steroids and reported that carbon monoxide inhibits the

hydroxylation of testosterone, estradiol, progesterone and deoxycorticosterone by rat liver microsomal enzymes. They have shown that hydroxylations at some positions are more sensitive to the presence of CO than others; for example, the ratio of CO/O_2 needed for 50% inhibition of testosterone hydroxylation in the 16α, 6β and 7α positions was 0.88, 1.59 and 2.53, respectively. In addition, a photochemical action spectrum revealed maximum release of inhibition at 450 mμ. On the basis of these results they proposed the presence of one or more cytochrome P-450's in liver microsomes that participate in the above hydroxylation reactions.

Reconstitution experiments carried out by Omura et al. (109), with sonicated adrenal mitochondria, indicate that non-heme iron is an essential component in the 11β-hydroxylation of deoxycorticosterone. The inability to detect non-heme iron in liver microsomes points out that one must be careful in comparing adrenal mitochondrial and liver microsomal hydroxylation systems. The possible existence of more than one form of cytochrome P-450 (66, 68, 122) and the lack of CO inhibition in placental 19-hydroxylation clearly indicates the need for much more work in the area of mixed function oxidase reactions with purified systems.

II. MATERIALS AND METHODS

A. Animals.

Sprague Dawley rats were purchased from Berkeley Pacific Laboratories, Berkeley, California. Except for special studies male rats 8-11 weeks of age were used. The surgically thyroidectomized rats used in these studies were purchased from this source. Thyroxine pretreatment consisted of daily intraperitoneal injections of 100 µg of thyroxine in an aqueous suspension for seven days, the last injection being the day prior to the experiment. The suspension was prepared essentially as described by McGuire et al. (98). The animals were fed Purina rat chow and maintained by the Animal Care Department until used.

B. Preparation of Tissues.

1. Slices

Rats were killed by a blow on the head and the livers removed, blotted, weighed and perfused with 0.25 *M* sucrose solution. Slices were cut with a Stadie-Riggs hand microtome to a thickness of approximately 0.5 mm.

2. 10,000 x *g* Supernatant

Livers were treated as above but chopped into small pieces with scissors and homogenized in 0.88 *M* sucrose solution (1/3; w/v) with a teflon pestle inside a smooth glass homogenizer (1.25×10^{-2} mm of clearance).

Nuclei and mitochondria were removed by centrifugation at 10,000 x *g* for 35 minutes in a Lourdes refrigerated centrifuge.

The supernatant was then recentrifuged at 10,000 x *g* for 25 minutes after the addition of 5 volumes of 0.04 *M* KCL. This supernatant will be henceforth called the 10,000 x *g* supernatant.

3. Crude Microsomes

The above 10,000 x *g* supernatant was centrifuged at 78,000 x *g* ($r_{ave.}$ in a Spinco #30 rotor at 30,000 rpm) for 60 minutes. The pellet obtained was then resuspended in 0.1 *M* phosphate buffer, pH 7.4 (1/1; v/fresh tissue weight) for biuret determinations. This suspension was then diluted tenfold with Krebs-Ringer phosphate buffer, pH 7.4. Approximately 1 milligram of microsomal protein was used in each incubation flask.

For the experiments designed to test the thermal stability of the enzymatic activity, microsomes were diluted to give a concentration of protein of about 10 mg/ml in phosphate buffer, pH 7.4. About 20 ml of the diluted solution was placed in a 50 ml erlenmeyer flask and was subsequently heated in a water bath with constant swirling. Samples were taken at specified intervals and diluted in ice-cold Krebs-Ringer phosphate buffer to give an approximate concentration of microsomal protein of 2 mg/ml. For subsequent incubations and cytochrome P-450 measurements, 0.5-ml aliquots were used.

In the experiments where microsomes were treated with pancreatic trypsin, crude microsomes were diluted in phosphate

buffer, pH 7.4, to a concentration of microsomal protein of 10 mg/ml.

Trypsin[1] was then added to the microsomal suspension to give a ratio of 0.2 mg of trypsin to 200 mg of microsomal protein (0.1% by weight) and incubated at 32°C. Under the conditions described, not optimal for tryptic action, the rate of hydrolysis was slow enough to allow for several samples to be taken before a total loss of hydroxylase activity occurred. Aliquots of the digestion mixture were then removed at specified intervals and placed into ground-glass, stoppered test tubes containing soybean trypsin inhibitor[2] and ice-cold Krebs-Ringer phosphate buffer. The amount of soybean inhibitor was twice that of trypsin present in the aliquot on a weight basis. After dilution, the treated microsomes were then used in incubation experiments and cytochrome P-450 determinations.

The effectiveness of the soybean inhibitor in stopping tryptic hydrolysis of the microsomal protein is shown in Table 5. The soybean inhibitor itself has little or no effect on estrogen hydroxylase activities (comparing the control values

[1] Worthington Biochemical Corp., Freehold, New Jersey, Lot #TRL 6JA.

[2] Worthington Biochemical Corp., Freehold, New Jersey, Lot #S1 61B.

Table No. 5. The effect of trypsin inhibitor on estrone metabolism by rat liver microsomes in the presence and absence of trypsin.

EFFECT OF TRYPSIN AND TRYPSIN INHIBITOR ON ESTRONE* METABOLISM

	2-OH-E_1	16α-OH-E_1	Water Soluble Products	Protein-Bound Metabolites
	10^4 dpm/mg protein/min.			
Controls	1.38	0.61	0.27	0.31
	1.45	0.59	0.30	0.31
Inhibitor	1.40	0.59	0.26	0.31
	1.36	0.59	0.27	0.34
Trypsin + inhibitor	1.31	0.59	0.29	0.35
	1.36	0.54	0.30	0.31

*Specific activity of ^{14}C-E_1 was 50 µg E_1/1.1 x 10^6 dpm; 4 µmoles NADPH added; incubation time 10 minutes.

to those obtained with soybean trypsin inhibitor present). The inhibitor is capable of almost complete inhibition of tryptic hydrolysis, as shown by comparing control values with those for microsomes plus trypsin and soybean trypsin inhibitor.

Carbon monoxide has been reported to inhibit steroid hydroxylation by competing with oxygen, presumably at the level of cytochrome P-450 (30). The following experiment was designed to test whether carbon monoxide can inhibit estrone metabolism, and whether varying the ratios of the concentrations of carbon monoxide and oxygen will affect the individual hydroxylase enzymes in the same or a different manner. Air and CO at varying flow rates were admitted into a small mixing jar and the O_2 and CO concentrations were quantitated by means of flow meters. The system contained microsomes, substrate and buffer and the incubation flasks were painted black to prevent light interference. The flasks were flushed with a minimum of 2 liters of the appropriate gas mixture over a period of two minutes prior to incubation, stoppered with rubber vial stoppers, and placed in the incubation bath for temperature equilibration. The reactions were started two minutes later by the injection of NADPH into the incubation media and stopped by rapid cooling in a dry ice-ethanol bath.

C. Chromatography.

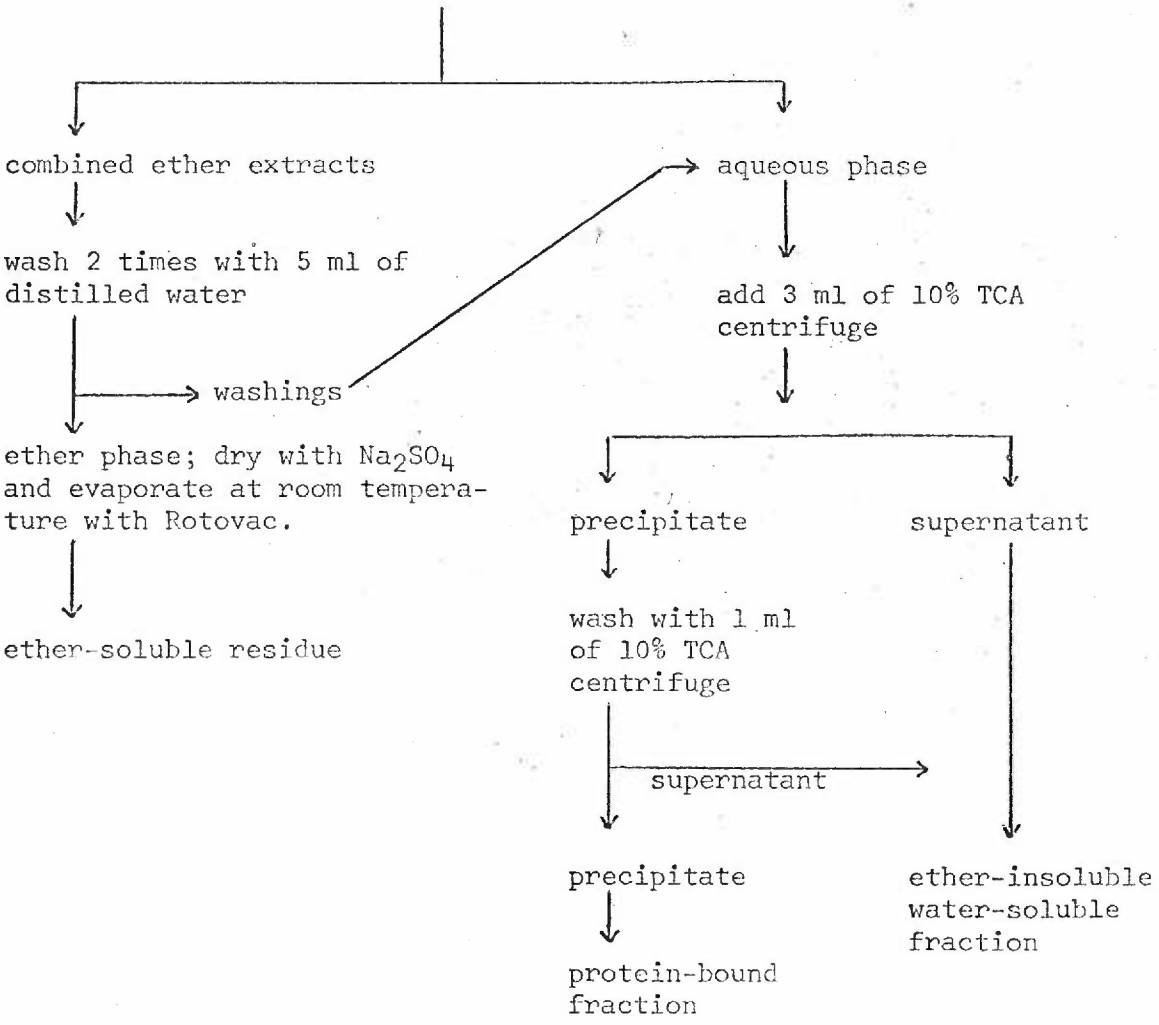
A diagram illustrating the procedure followed for extraction of the incubation mixtures is given in Figure 2.

Aliquots of the ether-soluble residue dissolved in methanol were applied with the use of Hamilton micro syringes to either Whatman #2 or #42 chromatography paper, which had been previously washed for 48 hours with methanol. The dimensions of the paper were 18 x 46 cm and the starting line was located 11 cm from the top. In order to cut down the time necessary for equilibrium in the chromatography tanks, the papers were impregnated with a mixture of the stationary phase and acetone (17%, by vol.) prior to chromatography in the following manner. After applying all the samples to a chromatogram, it was dipped by the top or starting end in the impregnating mixture which was allowed to move slowly toward the solvent front to within 0.3 - 0.5 cm of the starting line. The front was then allowed to advance 0.1 - 0.2 cm beyond the starting line by capillary penetration and the sheet was then rapidly withdrawn. The sheet was then dipped into the impregnating mixture by the other end and the same process repeated. The paper was then allowed to dry in a vented cabinet for 1-5 minutes (until the smell of acetone and the gross moisture had disappeared) and hung in the tank. The mobile phase was then added 15 to 20 minutes later closing the lid. The timing of drying after impregnation is critical, since if the paper is too dry, equilibration is incomplete in the next 15 to 20 minutes and uneven solvent fronts are observed (outer edges tend to move faster). Also, if the paper is too wet, the solvent will run either

Figure No. 2. Procedure for extraction of estrone metabolites from incubation mixtures and fractionation of ether-insoluble, water-soluble, and protein-bound products. Greater than 95% recovery is usually attained using this extraction procedure.

Extraction Procedure

1. After incubation and prior to extraction, 20 µg each of 2-OH-E_1, 16α-OH-E_1 and E_2 were added as carriers.
2. Extract four times with 20 ml of ethyl ether.



very slowly or unevenly, although good separations usually occur.

The glass chromatography tanks used for the paper chromatography (11-1/2 x 23 inches) were lined with five sheets of Whatman #1 chromatography paper (46 x 57 cm) to increase the rate of saturation of the tank. Mobile phase was placed in a 600 ml petri dish containing a filter paper cylinder to facilitate equilibration of the mobile phase within the tank. The tanks were equilibrated for at least 24 hours prior to use. The solvent systems utilized in these studies were of the Bush type (volatile stationary phase) (23, 79). They have been designated B-1, B-2 and B-14, and consisted of: benzene, petroleum ether, methanol and water (70:30:40:60; by volume); toluene, methanol and water (2:1:1; by volume); and benzene, methanol and water (100:55:45; by volume) respectively. The solvents were mixed in large separatory funnels. After 24 hours the lower (stationary) and upper (mobile) phases were separated for use within the chromatography tanks.

R_f values for each of the steroids used in this study for each of the chromatography systems are given in Table 6.

D. Radiochemical Techniques.

The radioactive estrone (estrone-4-^{14}C) used in these studies was purchased from Nuclear Chicago. The specific activity of this isotope was 29.4 mc/m mole. Prior to usage this material was checked for isotopic purity by paper chromatography in system B-1 and subsequent scanning with a Vanguard Model 880 strip scanner. If the steroid was

Table No. 6. R_f values of estrone and estrone metabolites in several paper chromatography systems.

R_f VALUES FOR ESTRONE AND METABOLITES FROM PAPER CHROMATOGRAPHY SYSTEMS

Steroid	Systems*		
	B-1	B-2	B-14
	R_f values ± 1 S. D.		
Estrone	0.65 ± 0.04	0.88 ± 0.02	0.88 ± 0.04
2-Methoxyestrone	0.79 ± 0.05	-	-
2-Hydroxyestrone	0.21 ± 0.02	0.62 ± 0.02	0.69 ± 0.02
6α-Hydroxyestrone	-	0.22 ± 0.01	-
16α-Hydroxyestrone	0.11 ± 0.01	0.48 ± 0.02	0.61 ± 0.02
Estradiol	0.32 ± 0.02	0.74 ± 0.02	0.76 ± 0.03
Y	-	0.37 ± 0.02	-
Estriol	0.01	0.04 ± 0.01	0.08 ± 0.01

* For composition, see text (p. 32).

judged less than 98 percent pure, it was then purified by chromatography in system B-1 and subsequent elution of the radioactive estrone from the paper using the technique of Kornel (83).

In the earlier experiments presented in this thesis (those up to and including studies with 10,000 x *g* supernatant), the amount of radioactivity incorporated into a specific metabolite was quantitated by running the chromatographic strip through a Model 880 Vanguard strip scanner. The amounts of isotope were chosen so that at least 4,000 dpm of each metabolite were present in each zone. The scanner was adjusted for optimum resolution and long enough counting times so that adequate precision in the number of counts was obtained. In order to determine the counting efficiency for this technique, known amounts of radioactivity were spotted on Whatman #42 filter paper and the strips were then scanned. In order to determine the counts per minute (cpm) of each zone the following relationship was employed:[1]

$$\text{cpm} = \frac{\text{area on graph} \times \text{conversion factor}}{\text{residence time}}$$

where $$\text{residence time} = \frac{\text{slit width}}{\text{strip speed}}$$

and $$\text{conversion factor} = \frac{\text{linear range}}{\text{chart speed} \times \text{chart width}}$$

For a slit width of 5 mm and a strip speed of 12 in/hr. (5.08 mm/min.) the residence time is 0.98 minutes. The linear range is defined as the number of cpm required for full scale deflection. Under normal

[1] Packard operation mannual for model 7201 Radiochromatogram Scanner.

conditions the following settings were used:

slit width = 5.0 mm

strip speed = 5.08 mm/min.

chart width = 114 mm

linear range = 1000 cpm

and a time constant of 10 seconds. Under these conditions and using Whatman #42 chromatography paper a counting efficiency carbon-14 of 27.5 $\pm$ 2.2. percent was obtained. During actual scanning, standards were placed at appropriate points.

In all the other experiments the amounts of radioactivity incorporated into each of the estrogens was determined by cutting out the zone of paper containing the radioactivity and placing it directly into scintillation vials. In order to check the recovery, known amounts of ^{14}C-labeled estrogens from incubations experiments were placed in scintillation vials and on chromatography paper which was allowed to stand overnight. The following day the zones containing the radioactivity were cut out and placed in scintillation vials. The vials were then filled with scintillation fluid (A) [toluene solution of 5 grams of P.P.O. (2.5-Diphenyloxazole)[1] and 0.3 grams of dimethyl P.O.P.O.P. (1,4-bis-2-4-methyl-5-phenyloxazolyl-benzene) per liter] inverted several times, and counted one hour later. The results given in Table 7 show that there is no difference between the count rate of radioactivity in the presence and in the absence of filter paper.

Protein-bound and ether-insoluble, water-soluble metabolites

Table No. 7. Comparison of counting rates obtained for ^{14}C-estrone placed directly into scintillation vials and of that allowed to dry on chromatography paper which was then placed into a scintillation vial for subsequent counting. The data indicate that ^{14}C-estrone was eluted from the chromatography paper since there was no decrease in counting rates when the activity was present on the paper. The efficiency of counting in each case was 70%.

RECOVERY OF ^{14}C-ESTRONE FROM PAPER CHROMATOGRAMS

Vol. of Std. Soln.*	(n)	A** cpm	B** cpm	B as % of A
10 µl	(5)	10423	10460	100
20 µl	(5)	21181	20930	99
30 µl	(5)	31993	31562	99
40 µl	(5)	42787	42930	100

* 14000dpm/µl

** A: Radioactive samples placed directly into vial.
B: Paper strip placed in vial.

were radioassayed by means of one of two Packard Tricarb Liquid Scintillation Spectrometers, either a model 3000 or a model 3315.[1] Both instruments automatically count groups of samples and print out the sample number, the number of counts recorded in each channel and the counting time. The two models differ in that the 3000 has two channels, while the 3315 has three, a better counting efficiency, and lower background.

Protein-bound products were solubilized in hyamine hydroxide 10-X[1] prior to counting. Ether-insoluble, water-soluble metabolites were counted after neutralization of the excess trichloroacetic acid with sodium hydroxide. Aqueous samples were counted using the following liquid scintillation fluid (B); 5 grams of P.P.O. (2.5-Diphenyloxazole)[1] and 0.3 grams of dimethyl P.O.P.O.P. (1,4-bis-2-4-methyl-5-phenyloxazolyl-benzene)[1] per liter of ethanolic toluene (2:3 v/v) solution. The determination of the radioactivity present in nonaqueous media was performed using scintillation media A.

The simultaneous determination of tritium and carbon-14 radioactivities was accomplished using the model 3315 spectrometer using the following settings: 2% gain and 50-1000 window in the carbon-14 channel (channel 1) and 70% gain and 35-1000 window in the tritium channel (channel 2). With the above settings and the scintillation media A the following counting efficiencies were obtained: 54% for

[1] Packard Instrument Company, Inc., Downers Grove, Illinois.

carbon and <0.01% for tritium in channel 1; and 18% for carbon and 38% for tritium in channel 2. The true counting rates were determined by using a discrimination ratios technique (106).

In order to establish the identity and purity of the radioactive zones separated by paper chromatography the labeled steroids were first located by scanning and subsequently eluted from the paper. Several zones from different experiments were then pooled and later rechromatographed in the same system. They were then located by running the appropriate standards and eluted. The second running was used to remove the estrone which is normally present in small amounts in all zones and which is corrected in actual experiments. The conditions of elution are important since microamounts of steroid appear to be easily oxidized on the paper. In order to decrease such oxidation the steroids were chromatographed and the paper removed and immediately placed in a foil packet and taken to the cold room. There the reference strips were removed and the reference steroids located by dipping in 1% $FeCl_3/KFe(CN)_4$ solution. The corresponding zones were then cut from the sample strips and eluted with 95% methanol while the paper was still damp with stationary phase. The eluted steroids were then pooled with nonradioactive carrier and crystallized to constant specific activity. The results of such experiments are given in Table 8.

In order to establish the identity of the radioactive zone tentatively designated X a small amount of this material was reduced

Table No. 8. Crystallization of 2-methoxyestrone, estradiol-17β, 2-hydroxyestrone and 16α-hydroxyestrone to constant specific activities after the addition of nonradioactive carrier.

CRYSTALLIZATION OF 2-METHOXYESTRONE, ESTRADIOL-17β (E_2), 2-HYDROXYESTRONE AND 16α-HYDROXYESTRONE TO CONSTANT SPECIFIC ACTIVITY

Purification Step	Specific Activity dpm/mg			
	2-MeO-E	E_2	2-OH-E	16α-OH-E
Paper chromatography*	6514	4917	4350	2575
1st crystallization	5972	4583	3958	2454
2nd crystallization	5861	4621	3948	2430
3rd crystallization	5851	4589	3984	2514

* A known amount of nonradioactive carrier was added to the residue from the paper.

with potassium borohydride and chromatographed on thin layer chromatography [system D of Lisboa (95)]. Non-reduced X and 6α-hydroxyestradiol were included as reference standards. Radioactive X and reduced X were located on thin layer plate (R_f values of 0.40, and 0.23, respectively) by scraping 1-cm horizontal sections of the silica gel and counting them by means of a liquid scintillation counter. The standard 6α-hydroxyestradiol had an R_f value of 0.25. Lisboa reports R_f values of 0.35 and 0.23 for 6α-hydroxyestrone and 6α-hydroxyestradiol, respectively, in this system. The R_f value of reduced X is consistent with the value quoted by Lisboa. The presence of a carbonyl group was also confirmed by running X and reduced X in the paper chromatography system B-2. To test whether the location of the carbonyl group was at position C-17, a micro Zimmerman reaction was carried out (34, 130). The unknown X exhibited a maximum at 520 mμ which was consistent with values obtained simultaneously for estrone and 2-hydroxyestrone, and is characteristic of a number of 17-ketosteroids.

The presence of a 17-carbonyl group suggested that X is either a very polar monohydroxylated derivative or a dihydroxylated derivative of estrone. The latter possibility was judged unlikely because of the relatively large amounts of this compound and the short time interval of substrate incubation.

Positions 2 and 16 as possible hydroxylation sites were already eliminated and of the others (see Tables 2 and 3) 6, 7 and 15

seemed most likely. On the basis of polarity 15α-hydroxylation has been excluded, as X is more polar than 15α-hydroxyestrone on paper chromatography. That is, in system B-2 the R_f of 15α-hydroxyestrone is 0.35, whereas the R_f of X was 0.18. Knuppen et al. (81) have reported the conversion of 6α- and 6β-hydroxyestrone to 6-dehydroestrone by treatment of the respective hydroxy compounds with 1 *N* methanolic HCl for 24 hours. Using their conditions we were able to convert approximately 70 percent of the radioactivity present in zone X to a compound which had an R_f value almost identical to that of estrone (R_f = 0.75 in system B-2). In this system 6-dehydroestrone and estrone are reported to have similar R_f values (79). The remainder of the radioactivity migrated with an R_f value of 0.20 which suggests two possibilities: (1) that the reaction was incomplete and complete conversion of 6α- or 6β-hydroxyestrone to 6-dehydroestrone was not achieved, or (2) that the remainder of the untransformed radioactivity was some other product, possibly 7α-hydroxyestrone since its polarity is reported to be the same in several chromatographic systems (79).

To determine the amount of 6α-hydroxyestrone present in this zone, radioactive X was added to carrier 6α-hydroxyestrone and was crystallized to constant specific activity. Because of the small amount of carrier 6α-hydroxyestrone used and the difficulty encountered in crystallizing this compound, the results were not conclusive; however, they indicate that 6α-hydroxyestrone is likely to be the major component.

The identity of the metabolite of estrone designated as Y has not been established. The following tentative evidence suggests that at least part of it is the 10β-hydroxy derivative of estrone: (1) partial conversion to estrone by overnight treatment with zinc dust in aqueous acetic acid (23); (2) reduction by borohydride to a more polar compound; (3) presence of hydroxyl group, as shown by increased Rf after acetylation.

E. Syntheses of 6α-Hydroxyestrone, 16α-Hydroxyestrone, and ^{3}H-2-Hydroxyestrone

1. Preparation of 6α-Hydroxyestrone.

The first step in synthesizing 6α-hydroxyestrone was to introduce a keto group into the 6 position of estradiol diacetate. The method utilized was similar to that followed by Longwell and Wintersteiner (96). Estradiol diacetate was dissolved in acetic acid and chromium trioxide in aqueous acetic acid was added dropwise. The mixture was allowed to stand overnight at room temperature. Excess chromic acid was reduced with ethanol. The reaction mixture was then extracted with ether. Acidic reaction products were then removed by extraction with saturated bicarbonate solution, followed by extraction with a 3:1 mixture of 1 *N* sodium carbonate and saturated sodium bicarbonate solutions, washed with water and dried with sodium sulfate. The ether was evaporated in a rotovac at room temperature and the residue fractionated with Girard's Reagent T. The ketonic fraction was then acetylated with acetic anhydride and crystallized first in

absolute, then in 90% ethanol. From 2 grams of estradiol diacetate a total of 0.30 grams of crystals were obtained having a melting point of 174-176.5°C (reported: 173-175°C).

In order to convert the 6-ketoestradiol diacetate to 6α-hydroxyestradiol 3, 17β-diacetate, the keto compound was reduced with sodium borohydride according to the method of Wintersteiner and Moore (131) which, according to Breuer (15), gives 6α-hydroxy-estradiol as the primary product. The 6-ketodiacetate was dissolved in 30 ml of methanol and 0.293 mg of sodium borohydride, dissolved in 23 ml of methanol, was added dropwise to the steroid. After two hours the solution was acidified with 10% acetic acid and the solution evaporated. The residue was then extracted with ether and hydrolyzed in 5% methanolic potassium hydroxide solution under nitrogen for 20 hours. Crystals, 260 mg, having a melting point of 246-249°C (reported for 6α-hydroxyestradiol 249-251°C) were separated from an acetone solution.

Selective benzoylation of 260 mg 6α-hydroxyestradiol with benzoylchloride in the 3 and 6β positions was then done according to the technique of Knuppen and Breuer (78). Upon crystallization in methanol and acetone the product yielded 120 mg of crystalline material melting at 206-213°C (reported value 213-215°C).

The 3, 6α dibenzoate of estra-1,3,5(10)-trien-3,6α,17β-triol was then oxidized with chromium trioxide as described by Knuppen and Breuer (78) and the product hydrolysed in 5% aqueous potassium hydroxide

under a nitrogen atmosphere. A total of 51 mg of 6α-hydroxyestrone was isolated, having a melting point of 225-229°C (reported 220°C). Knuppen and Breuer (78) have reported the log of the extinction coefficient at 272 mμ to be 3.35, the value we obtained was 3.37. We also obtained the minimum they report at 247 mμ. An infrared spectra of this compound is given in the appendix.

2. Synthesis of 16α-Hydroxyestrone.

At the start of this work 16α-hydroxyestrone was not commercially available, so it was synthesized from estrone. The synthesis as described below was basically that used by Leeds et al. (92) and Biggerstaff et al. (5). Five (5) grams of estrone was refluxed with isopropenyl acetate in the presence of sulfuric acid which served as a catalyst. The resulting solution was diluted with ether, which was subsequently washed with ice-cold sodium bicarbonate solution and dried with anhydrous sodium sulfate. The ether was evaporated and the residue taken up in 3-1/2 liters of petroleum ether and this solution passed through a short column of alumina (5 grams). The petroleum ether was evaporated and the residue, crystallized from ether, yielded 3.2 grams of the enol acetate, estra-1,3,5(10),16 tetraen-3,17β-diol diacetate, melting point of 147-149°C. From the mother liquor an additional 1.6 grams was obtained [m.p. 144-147°C, reported in the literature 149-150°C (92)].

Enol acetate (4.74 grams) was added to 500 ml of 0.05 *M* perbenzoic acid in a benzene solution and allowed to react at room

temperature overnight. The resulting mixture was then washed with 100 ml of ice-cold 0.5 N NaOH and then two times with ice-cold 1% sodium chloride solution. The solvent was then dried over sodium sulfate and evaporated. The product, 16α,17αepoxy-estra-1,3,5(10)trien-3,17β-diol diacetate (3.2 grams) was crystallized from an acetone-petroleum ether mixture and had a melting point of 148.5-153°C; the reported value was 150-152°C (92).

The epoxy compound (3 grams) was then dissolved in 450 ml of methanol and 75 ml of 6 N sulfuric acid and was allowed to stand at room temperature for three days. The solution was then concentrated to one-third its original volume and chilled in an ice-bath and filtered. The residue was washed with distilled water and dried in a vacuum oven. Crystallization from methanol yielded 1.4 gm of 3,16α-dihydroxy-estra-1,3,5(10)-trien-17-one (m.p. 212-214°C). In the literature the value reported is 205-206.5°C (5), but that sample was crystallized from petroleum ether-acetone. An infrared spectrum was later shown to be identical to that of reference 16α-hydroxyestrone and the maxima were those reported by Biggerstaff. The spectrum was also compared with that of 16-oxo-estradiol-17β and found to differ at several wave lengths. The infrared spectra of these two compounds are included in the appendix.

3. Preparation of ^{3}H-2-hydroxyestrone.

One millimole of ^{3}H-estrone (500 μc/m mole) was incubated in 10 ml of Krebs-Ringer phosphate, containing 3 mg of microsomal protein,

10 moles of NADPH and 10^{-3} *M* ascorbic acid, for 30 minutes. The incubation mixture was then extracted as described before and the ketonic fraction was separated from other lipids by the Girard technique (23). The ^{3}H-2-hydroxyestrone was then purified by chromatography in the paper systems B-1 and B-2, in that order. After chromatography, the ^{3}H-2-hydroxyestrone was then checked for purity by adding carrier and subsequent crystallization to constant specific activity and by chromatography on the paper in system B-14. By means of these criteria the purity was judged greater than 95%.

F. Miscellaneous Procedures

Melting points (uncorrected) were obtained with a Kofler hot-stage microscope. Infrared spectra of steroids in KI or KBr pellets were determined using a Perkin-Elmer Model 221 infrared spectrophotometer. Steroids were acetylated at room temperature in 0.1 ml of acetic anhydride and 0.1 ml of pyridine.

The protein content of the microsomal preparations was determined using the biuret method (48). The crude microsomal suspensions (0.2 ml) were incubated in the presence of 0.2 ml of 2% sodium deoxycholate for two hours at 37°C; 0.9 percent NaCl was then added to bring the volume of the samples to 1 ml. After the addition of 4.0 ml of biuret reagent the samples were allowed to stand at room temperature for 30 minutes. The samples were read at 540 mμ in a Zeiss PMQ II spectrophotometer against a blank which was treated as above except that 0.45 *M* NaOH was added instead of the biuret reagent. Simultaneous

duplicate samples containing 4.0 mg of serum albumin[1] were used as reference standards in the calculations of the amount of microsomal protein.

Cytochrome P-450 was quantitated by pipetting 1 ml of a microsomal protein solution containing 2 to 3 mg/ml of protein in two 0.5 x 1.0 x 4.0 cm quartz cuvettes which were then placed into a Cary Model 14 recording spectrophotometer. This instrument was equipped with a scattered transmission attachment and a high-intensity quartz lamp and operated at 70 volts.

After the absorbancy difference had been adjusted to zero, carbon monoxide was bubbled into the sample solution for 30 seconds and a few crystals of sodium dithionite dissolved in both solutions. The difference spectrum was recorded from 500 to 400 mμ and the absorbancy at 450 mμ minus that at 490 mμ and the molar extinction coefficient (91 $cm^{-1}{}_{m}M^{-1}$) were used to calculate the content of cytochrome P-450 (35, 107).

[1] Mann Research Laboratories, Inc., New York, N. Y.

III. RESULTS

This part has been divided into two sections: (1) Effect of thyroidectomy and thyroxine treatment on estrone metabolism, and (2) The comparison of estrone hydroxylase activities. In the experiments described in the first section the purpose was to seek a suitable system to study the effects of thyroid hormonal alterations on estrone metabolism. It was the intent to duplicate the work on clinical material of Fishman et al., using rat liver preparations. The results of these studies prompted the second series of experiments.

A. Effect of thyroidectomy and thyroxine pretreatment on estrone metabolism

1. Liver Slices.

The results given in Table 9 show that liver slices from each of the groups of animals can readily metabolize estrone. Similar preparations from thyroxine-treated animals are, in general, less active in carrying out the various reactions under consideration. Thyroidectomy also decreased most hydroxylase activities, but to a lesser extent than thyroxine treatment.

Liver slices from both thyroidectomized and thyroxine-treated animals show diminished 2- and 16α-hydroxylation as compared to the control. There is no decrease in the amount of 2-methoxyestrone formed in slices from thyroidectomized animals nor an increase in 2-methoxylation in thyroxine-treated animals as might have been

Table No. 9. Conversion of estrone-4-^{14}C to 2-methoxyestrone, 2-hydroxyestrone, estradiol-17β, and 16α-hydroxyestrone by liver slices from normal, thyroidectomized and thyroxine-treated rats. The results are expressed as the percent of substrate transformed to the corresponding product.

CONVERSION OF ESTRONE-4-^{14}C TO 2-METHOXYESTRONE, 2-HYDROXYESTRONE, ESTRADIOL-17β, AND 16α-HYDROXYESTRONE BY LIVER SLICES FROM NORMAL, THYROIDECTOMIZED AND THYROXINE-TREATED RATS

	Recovery of ^{14}C-labeled product (% of substrate added)				
Condition of the Animal	2-Methoxy-estrone	2-OH-Estrone	16α-OH-Estrone	Estradiol-17β	Unaccounted for
normal (5)	6.1 ± 1.2	6.5 ± 1.1	4.6 ± 2.5	10.0 ± 1.4	73.0
thyroidectomized (5)	6.3 ± 1.1	4.7 ± 1.4*	2.8 ± 1.0	8.3 ± 2.3	78.0
thyroxine treated (5)	4.7 ± 0.8	2.9 ± 0.7 **	2.3 ± 0.5	5.4 ± 1.0**	84.8

The experimental conditions are given in the Methods section. Values shown are the mean ± S.D. The numbers in brackets represent the number of animals used in each series of experiments.

* Significant difference between normal and thyroidectomized at $p < 0.05$.

** Significant difference between normal and thyroxine treated at $p < 0.01$.

expected from the results of Fishman et al. (46, 47). The fraction "unaccounted for" includes remaining substrate and unidentified products. About 50% of the substrate remained unchanged in the few instances where an approximate estimation was made using separate scannings at different amplifications.

2. 10,000 x *g* Supernatant.

Thyroidectomy markedly decreases the ability of the 10,000 x *g* supernatant to convert estrone to hydroxylated products (see Table 10). There seems to be no specific effect on either the 2- or 16α-hydroxylase enzymes as we had expected. No significant changes in estrone hydroxylase activities were observed when animals were pretreated with thyroxine daily for seven days. These results differ from those obtained with slices where both thyroxine treatment and thyroidectomy decreases the hydroxylase activities. The extent of 2- and 16α-hydroxylation by the thyroxine-treated animals does not differ significantly from control values. Both 2- and 16α-hydroxylation are significantly decreased in thyroidectomized animals.

The amount of radioactivity incorporated into the protein-bound, and ether-insoluble, water-soluble fractions is not significantly altered by thyroxine pretreatment (see Table 11). Thyroidectomy, however, significantly decreases the ability of the 10,000 x *g* liver supernatant to incorporate radioactivity into these fractions.

3. Microsomal Preparations.

Lazier et al. (91) and Marks and Hecker (99) have provided strong indirect evidence that 2-hydroxylation of estrogens is an obligatory step toward the formation of protein-bound and ether-insoluble,

Table No. 10. Conversion of estrone-4-^{14}C to 2-methoxyestrone, 2-hydroxyestrone, 16α-hydroxyestrone and estradiol-17β y the 10,000 x *g* supernatant from livers of normal, thyroidectomized and thyroxine-treated rats.

CONVERSION OF ESTRONE-4-^{14}C TO 2-METHOXYESTRONE, 2-HYDROXYESTRONE, 16α-HYDROXYESTRONE AND ESTRADIOL-17β BY THE 10,000 x *g* SUPERNATANT FROM NORMAL, THYROIDECTOMIZED AND THYROXINE-TREATED RATS

Treatment	Recovery of ^{14}C-labeled product (% of substrate added)				
	2-Methoxy-estrone	2-Hydroxy-estrone	16α-Hydroxy-estrone	Estradiol-17β	Unaccounted for
normal (5)	5.9 ± 1.1	12.2 ± 4.7	8.7 ± 0.8	4.0 ± 1.0	69.2
thyroidectomized (5)	3.4 ± 0.7*	6.2 ± 1.0*	3.4 ± 1.0*	4.9 ± 1.1	82.3
thyroxine treated (5)	4.8 ± 1.0**	12.8 ± 0.8	7.0 ± 0.9*	6.1 ± 0.7*	69.4

The experimental conditions are given in the text. Values are the mean ± S.D. The numbers in brackets represent the number of animals used in each series of experiments.
Values different from normal at $p < 0.01$ (*)
Values different from normal at $p \leq 0.05$ (**)

Table No. 11. Effect of thyroid hormone on the formation of ether-insoluble, water-soluble, and protein-bound metabolites of estrone-4-^{14}C by the 10,000 x *g* supernatant fraction from rat livers from normal, thyroidectomized and thyroxine-treated rats.

EFFECT OF THYROID HORMONE ON THE FORMATION OF ETHER-INSOLUBLE, WATER-SOLUBLE, AND PROTEIN-BOUND METABOLITES OF ESTRONE-4-^{14}C BY THE 10,000 x g FRACTION FROM RAT LIVER

State of the Animal	Percent of Added Radioactivity	
	Water-Soluble Products	Protein-Bound Products
A. Normal (5)	6.50 ± 0.75 *	7.22 ± 0.90**
B. Thyroxine treated (5)	4.95 ± 0.50	6.71 ± 1.12
C. Thyroidectomized (5)	3.82 ± 1.41 *	4.24 ± 0.91**

* Significant difference between A and C at $p < 0.02$.
** Significant difference between A and C at $p < 0.01$.

water-soluble products. With this assumption in mind I examined the kinetics of protein-bound and ether-insoluble, water-soluble product formation in normal, thyroidectomized and thyroxine-treated animals. The velocity, expressed as μmoles x liter^{-1} x min^{-1}, was plotted vs. μmolar concentration of the substrate. Figures 3 and 4 give rates of formation of ether-insoluble, water-soluble products from estrone. The rate of formation of these products is seen to be decreased by both thyroidectomy and thyroxine treatment.

The apparent KM value found in these reactions (5×10^{-5} *M*) is of the same order of magnitude as the values obtained by Lazier (91) with female rats (1×10^{-5} *M*). From the graphs in Figure 4 and 6, it is apparent that alteration of the thyroid activity has the greatest effect on the rate of these reactions rather than on the apparent Km.

Figures 5 and 6 give the rates of conversion of estrone to protein-bound metabolites. As in the case of the formation of ether-insoluble, water-soluble products, there is a decrease in the relative rate at which these products can be formed by thyroidectomized animals but little or no change in the apparent Km value for these reactions. Thyroxine treatment seemed to increase slightly the formation of protein-bound products, which is in contrast to the results for the ether-insoluble, water-soluble fraction in the same animals.

In order to check the effect of thyroxine treatment on thyroidectomized rats the following experiments were carried out.

Figure No. 3. Conversion of estrone to ether-insoluble, water-soluble products by liver microsomes from normal, thyroidectomized and thyroxine-treated rats. The units of velocity are expressed as μmoles of product formed $liter^{-1}$ x min^{-1}. The thyroxine-treated animals received 100 μg of hormone for seven days. The thyroidectomized rats were used two weeks after surgery. An equivalent of 0.2 grams of liver was incubated for 15 minutes in the presence of 4 μmoles of NADPH. The maximum standard error was ± 2%.

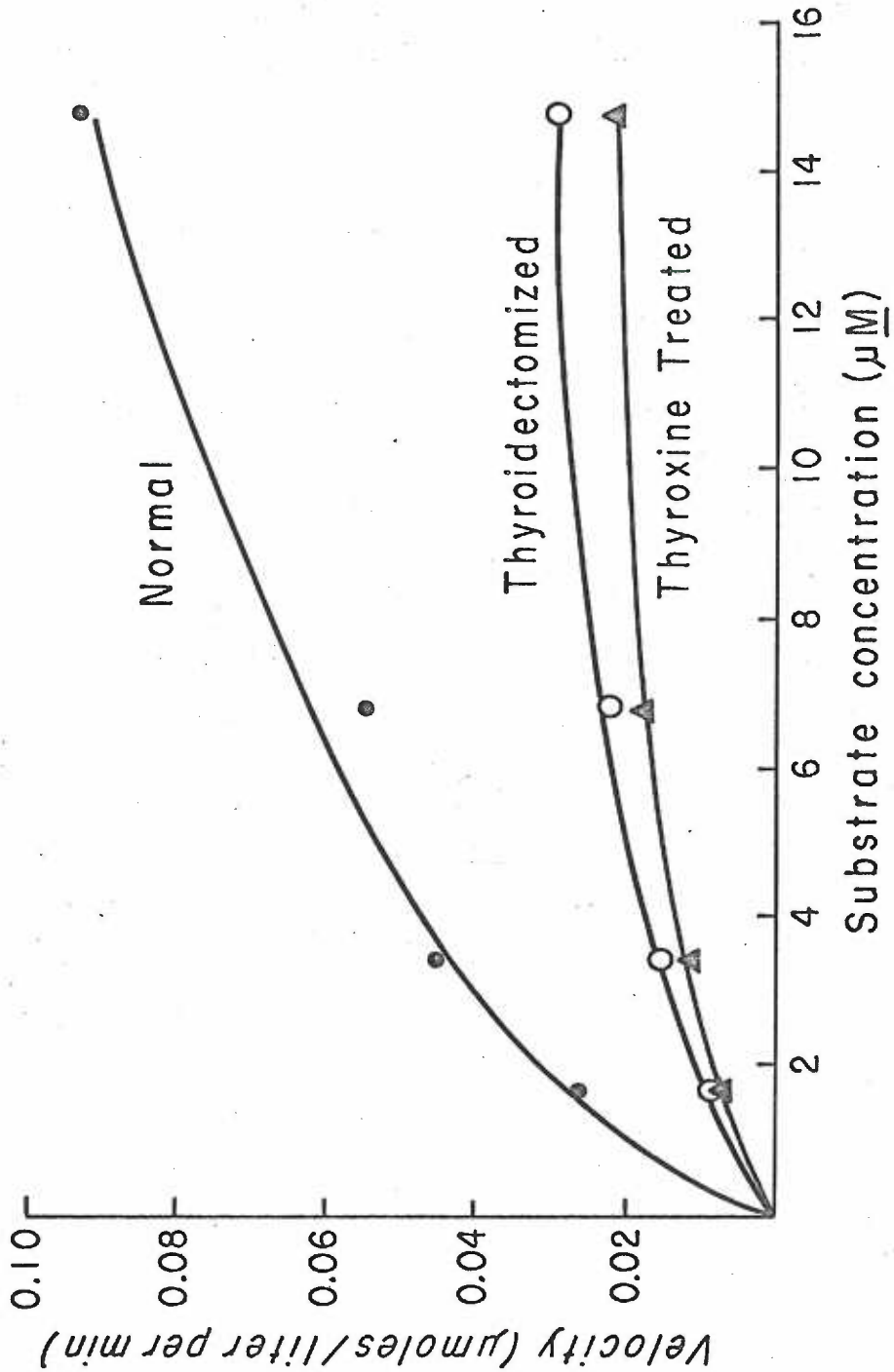

Normal
Thyroidectomized
Thyroxine Treated
Velocity (μmoles/liter per min)
0.10
0.08
0.06
0.04
0.02
2
4
6
8
10
12
14
16
Substrate concentration (μM)

Figure No. 4. Effect of thyroidectomy and thyroxine treatment on the formation of ether-insoluble, water-soluble products by rat liver microsomes. Same data as in Figure 3.

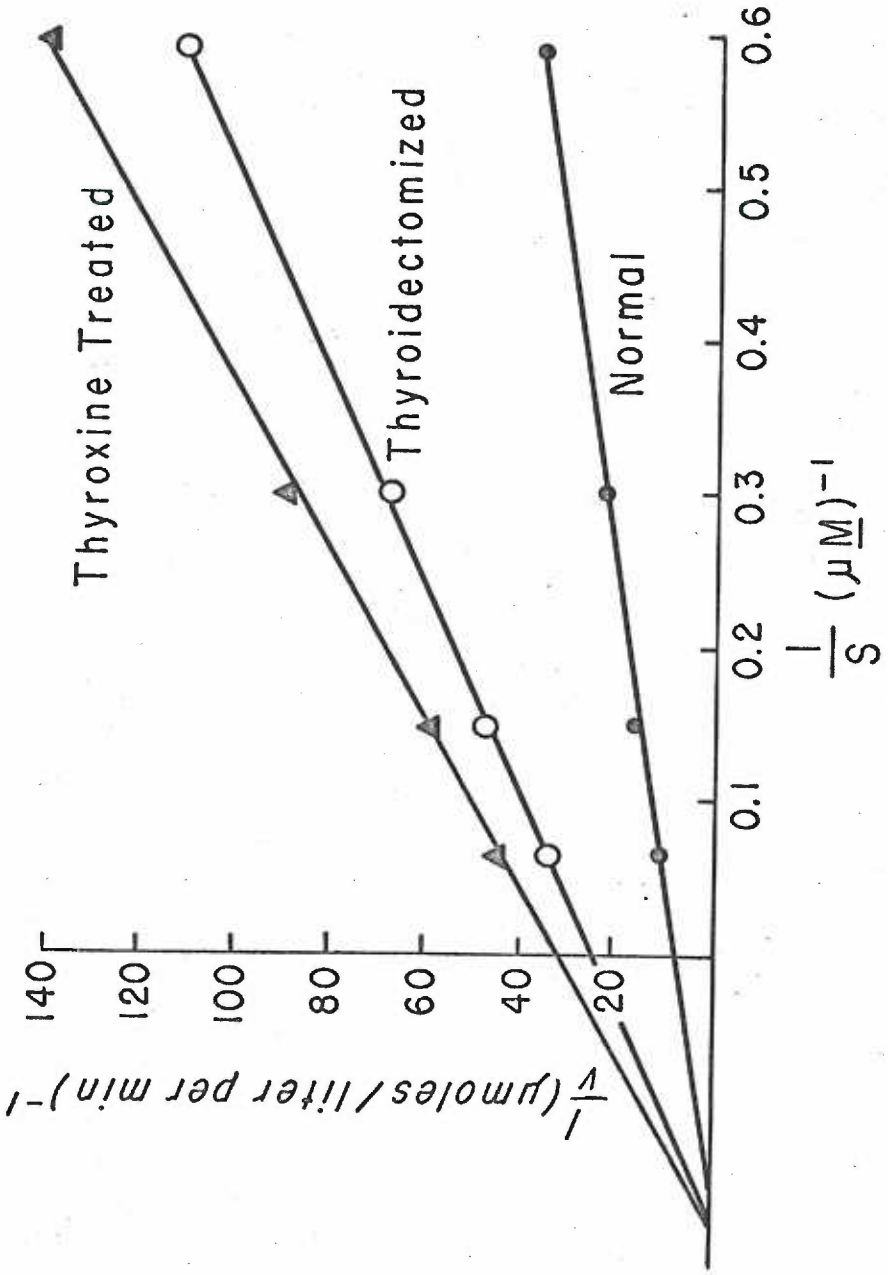

Thyroxine Treated
Thyroidectomized
Normal
140
120
100
80
60
40
20
0.1
0.2
0.3
0.4
0.5
0.6
1/v (μmoles/liter per min)⁻¹
1/S (μM)⁻¹

Figure No. 5. Effect of thyroidectomy and thyroxine treatment on the formation of protein-bound products from ^{14}C-estrone. Same conditions used as in Figure 3. The maximum standard error was ± 2%.

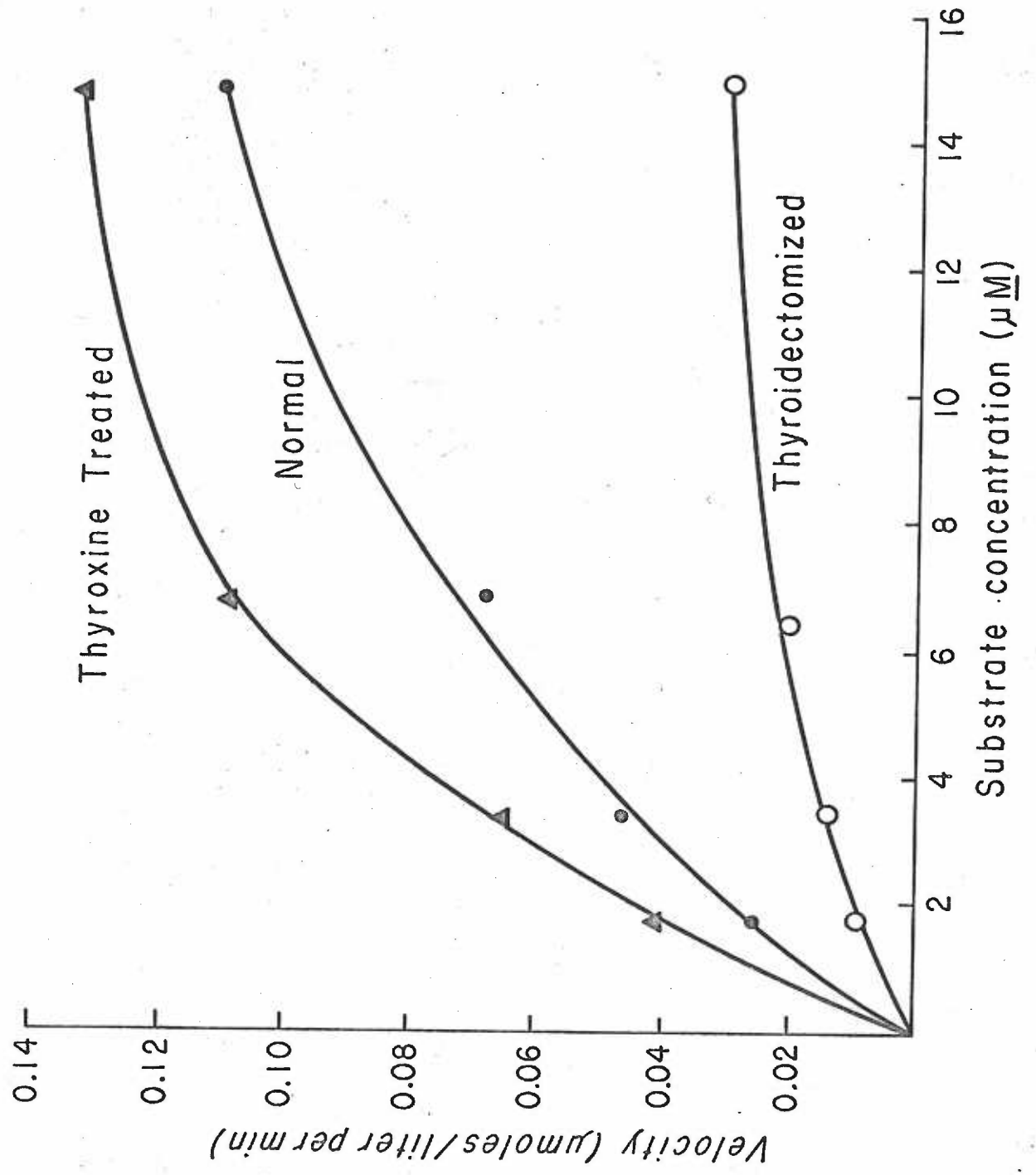
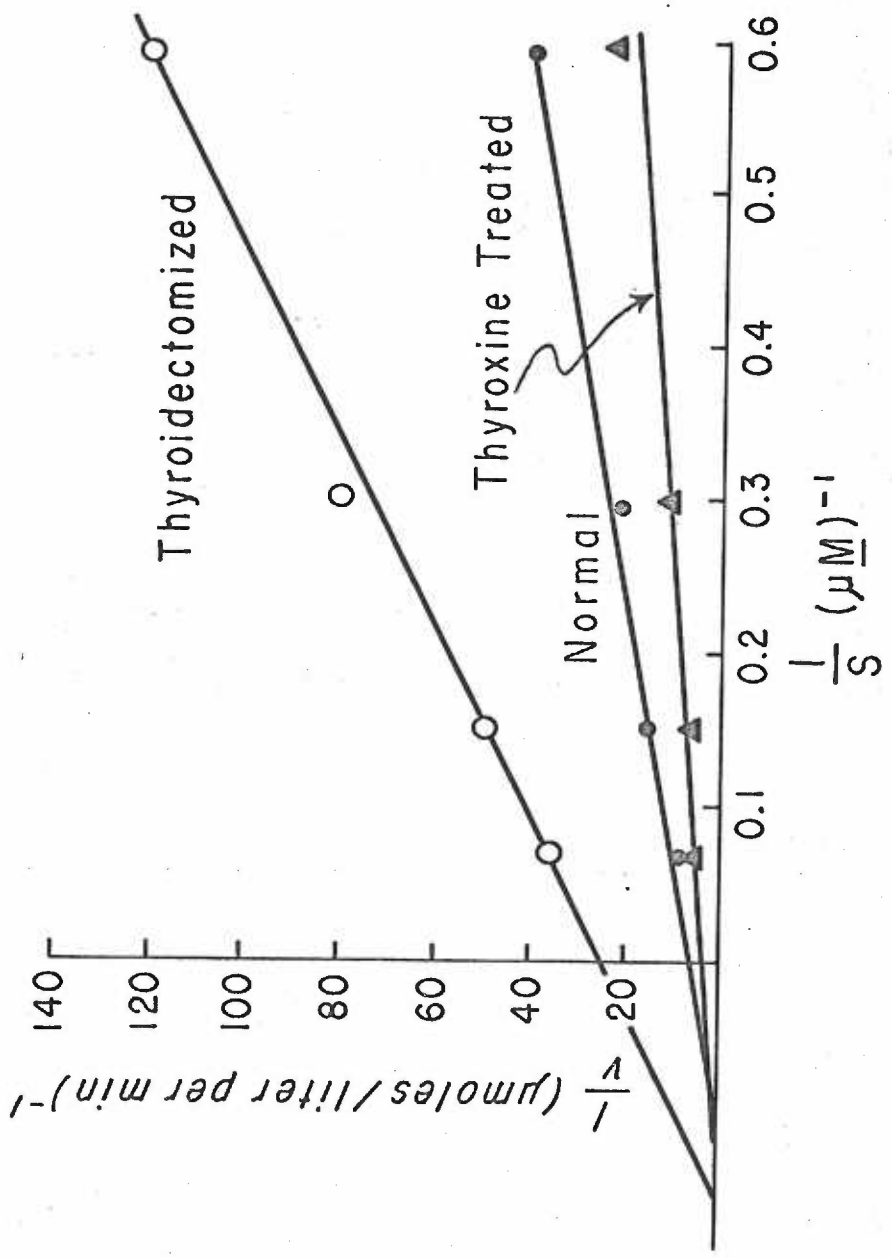

Thyroxine Treated
Normal
Thyroidectomized
Velocity (μmoles/liter per min)
Substrate concentration (μM)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
2
4
6
8
10
12
14
16

Figure No. 6. Effect of thyroidectomy and thyroxine treatment on the formation of protein bound products from ^{14}C-estrone.


Thyroidectomized
Thyroxine Treated
Normal
140
120
100
80
60
40
20
0.1
0.2
0.3
0.4
0.5
0.6
1/v (μmoles/liter per min)⁻¹
1/S (μM)⁻¹

Thyroidectomized rats, both male and female, were treated two and one-half weeks after surgery with thyroxine, 100 μg per day for four days. On the fifth day the rats were sacrificed and microsomes prepared and incubated with estrone as usual. The results of these incubations are given in Table 12. It is seen that thyroxine causes an increase in the concentration of microsomal protein per gram of liver in both male and female rats. Also apparent is a much greater hydroxylase activity present in the microsomes from male rats than from females; for example, there is four times more 2-hydroxylase activity and nine times more 16α-hydroxylase activity. Treatment with thyroxine at this dosage level seems to have little influence on the net formation of either 2- or 16α-hydroxyestrone in female or male rats. There is possibly a slight stimulation of the formation of ether-insoluble, water-soluble product formation in the case of female rats after thyroxine treatment. A decrease in the formation of ether-insoluble, water-soluble product formation, but no change in protein-bound metabolites, is observed after thyroxine treatment in male rats. A greater 17β-hydroxysteroid dehydrogenase activity is present in microsomes from female rats. The small amount of radioactivity which was unaccounted for probably represents the formation of products more polar than 16α-hydroxyestrone.

B. Comparison of estrone hydroxylase activities.

1. Kinetic Conditions.

In order to compare 2- and 16α-hydroxylase activities it is desirable to choose the conditions of incubation so that the rates of reaction will be linear with respect to time. In Figure 7 the net

Table No. 12. The effect of thyroxine treatment on estrone metabolism by liver microsomes from thyroidectomized rats. Results are expressed as dpm of product formed per milligram of microsomal protein.

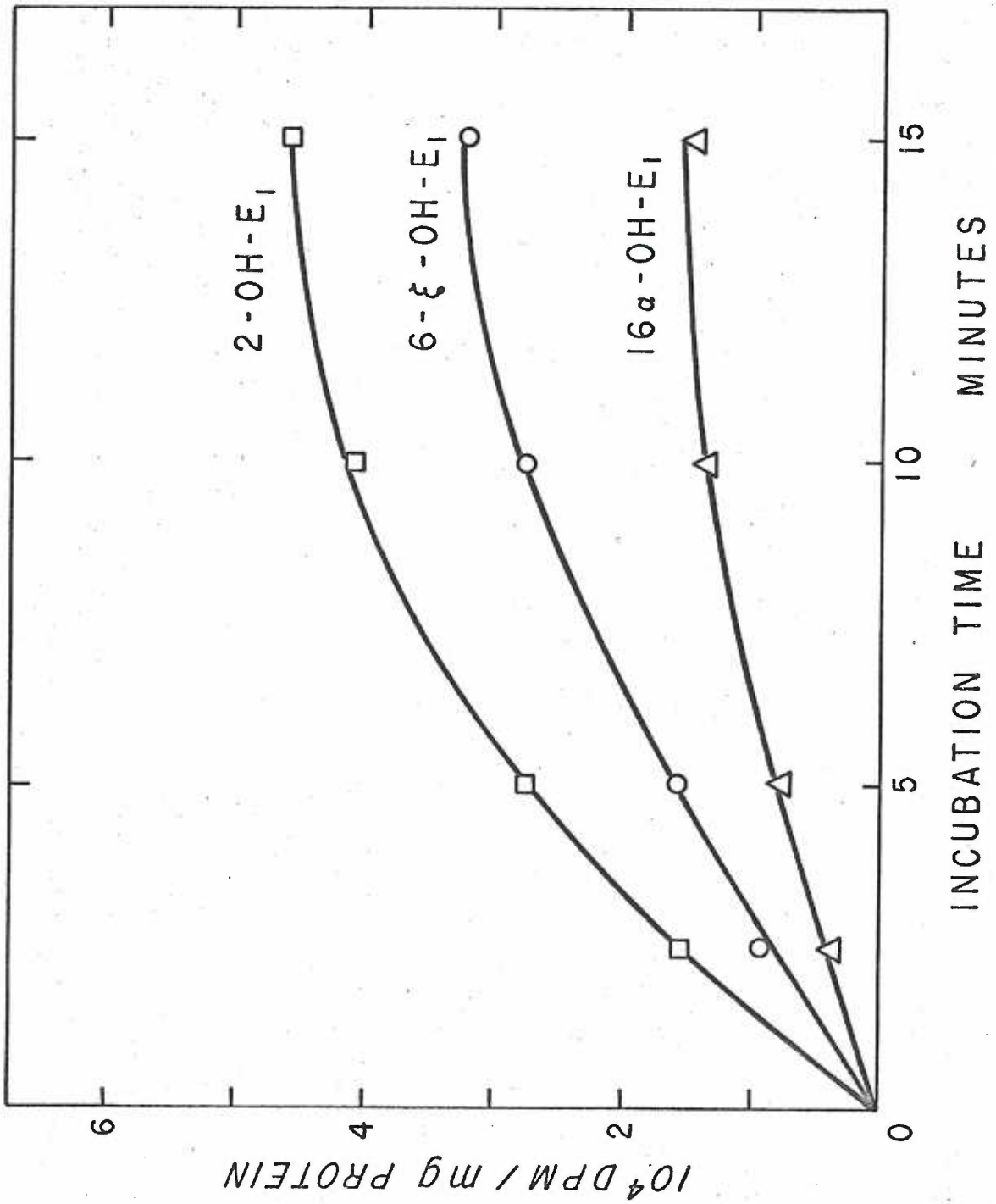
EFFECT OF THYROXINE TREATMENT ON THYROIDECTOMIZED RATS

(Mg protein/ gram liver)	Sex	Treatment	10^3 dpm/mg(**) microsomal protein in estrone metabolites after incubation						
			E_1	E_2	2-OH-E_1	16α-OH-E_1	H_2O sol. products	Protein bound	Unaccounted for
7.2	♀	-	621	37±2	24±2	6.3±0.3	1.6±0.6	3.4±0.5	0
11.2	♀	thyroxine*	534	36±3	22±1	6.4±0.3	3.5±0.5	5.3±0.1	14
7.7	♂	-	240	23±3	109±4	53 ±3	44 ±3.8	25 ±1.9	27
9.3	♂	thyroxine*	309	24±1	100±5	45 ±3	24 ±3.4	29 ±2.8	22

+ 3 rats used in each experiment.

* 100 µg/day for four days prior to experiment.
** ± 1 S.E.

Figure No. 7. Net formation of 2-, 6ξ- and 16α-hydroxyestrone as a function of time. Conditions: Incubations at 37° of a mixture of estrone (1.1×10^6 dpm ^{14}C-estrone 100 µg), 4 µmoles of NADPH, 1 mg microsomal protein in a final volume of 5 ml. Values are expressed as the mean and the range. Liver microsomes pooled from 3 rats, triplicate samples for each point.


2-OH-E1
6-ξ-OH-E1
16α-OH-E1
0
2
4
6
10⁴ DPM / mg PROTEIN
0
5
10
15
INCUBATION TIME MINUTES

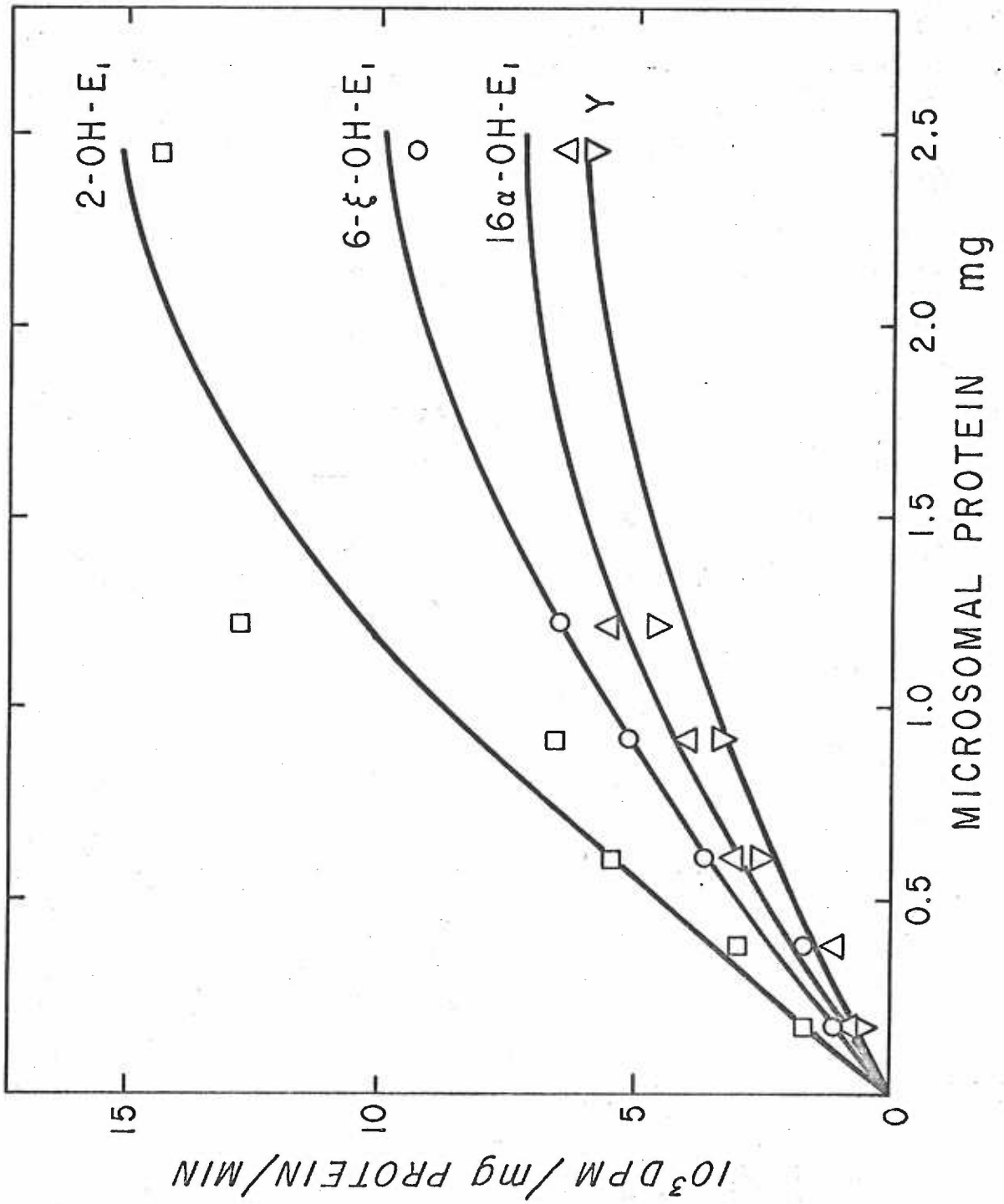
rates of formation of 2-hydroxyestrone, 16α-hydroxyestrone and 6ξ-hydroxyestrone are given as a function of time. The results indicate that in order to achieve linear rates the incubation period should not exceed ten minutes.

The rates relative to each other remain fairly constant for the whole time interval studied. That is, the net amount of 16α-hydroxyestrone is about one-third the net amount of 2-hydroxyestrone present at all times studied.

Since the amount of microsomal protein present in rat liver varies from animal to animal it was necessary to establish how the net rates of estrone hydroxylation varied with respect to the amount of microsomal protein present in the incubation flasks. The results given in Figure 8 are expressed as 10^3 dpm/mg x min^{-1}. The flasks contained 0.5 μc of ^{14}C-estrone and enough cold carrier to bring the concentration up to 74 μmolar. Four μmoles of NADPH were added as before and the flasks were incubated for 10 minutes. The results indicate that the net rates of formation are essentially linear with respect to protein concentration up to 1.25 milligrams per flask. After this point the rates fall off rapidly, possibly due to excess substrate or cofactor consumption.

Since it is probable that 2-hydroxyestrone is converted to protein-bound and ether-insoluble, water-soluble products, it was desirable to know how this series of reactions might be influenced by the concentration of microsomal protein present in the incubation

Figure No. 8. Net rate of formation (10^3 dpm/mg/min) of 2-, 6ξ-, 16α-hydroxyestrone and Y from estrone as a function of protein concentration. Conditions: Ten minute incubations at 37°C of a mixture of ^{14}C-estrone (1.1×10^6 dpm, 100 µg), 4 µmoles NADPH and 0.3 - 2.4 mg of microsomal protein in a total volume of 5 ml.


2-OH-E1
6-ξ-OH-E1
16α-OH-E1
Y
0
5
10
15
0.5
1.0
1.5
2.0
2.5
MICROSOMAL PROTEIN mg
10³DPM /mg PROTEIN/MIN

mixture. The results given in Figure 9 are derived from the previous experiment. The same units of expressing rates are used as in the previous experiment to facilitate comparisons. At the lowest concentration of microsomal protein utilized, the rates of protein-bound and ether-insoluble, water-soluble product formation are too low for accurate determination. Between 0.4 mg of microsomal protein to 1.25 the rates are linear. At concentrations up to 2.5 milligrams of protein the increase in the rate of protein-bound product formation remains constant; whereas, the increase in the rate of ether-insoluble, water-soluble product formation declines markedly.

I originally choose 4 µmoles of NADPH as the amount of cofactor for each flask since similar amounts were used by other investigators using comparable systems. Since this amount of cofactor might not be enough in our preparation we tested this point in the following manner. Each flask in each group contained 0.5 µc of ^{14}C-estrone (74 µmoles) and approximately 1 milligram of microsomal protein. The first group of flasks were incubated for 7-1/2 minutes and contained 4 µmoles of NADPH. The last group of flasks were incubated for 15 minutes but 4 µmoles of NADPH were added at the beginning and another 4 µmoles at 7-1/2 minutes. The results given in Table 13 clearly show that the decrease in rate observed for 2-hydroxy and 6-hydroxyestrone is not due to a decrease in cofactor present in the mixture. For economy 4 µmoles was chosen as the amount to be used in all other experiments.

Figure No. 9. Net rates of formation of protein-bound and ether-insoluble, water-soluble products as a function of protein concentration. Conditions are the same as in Figure 8.

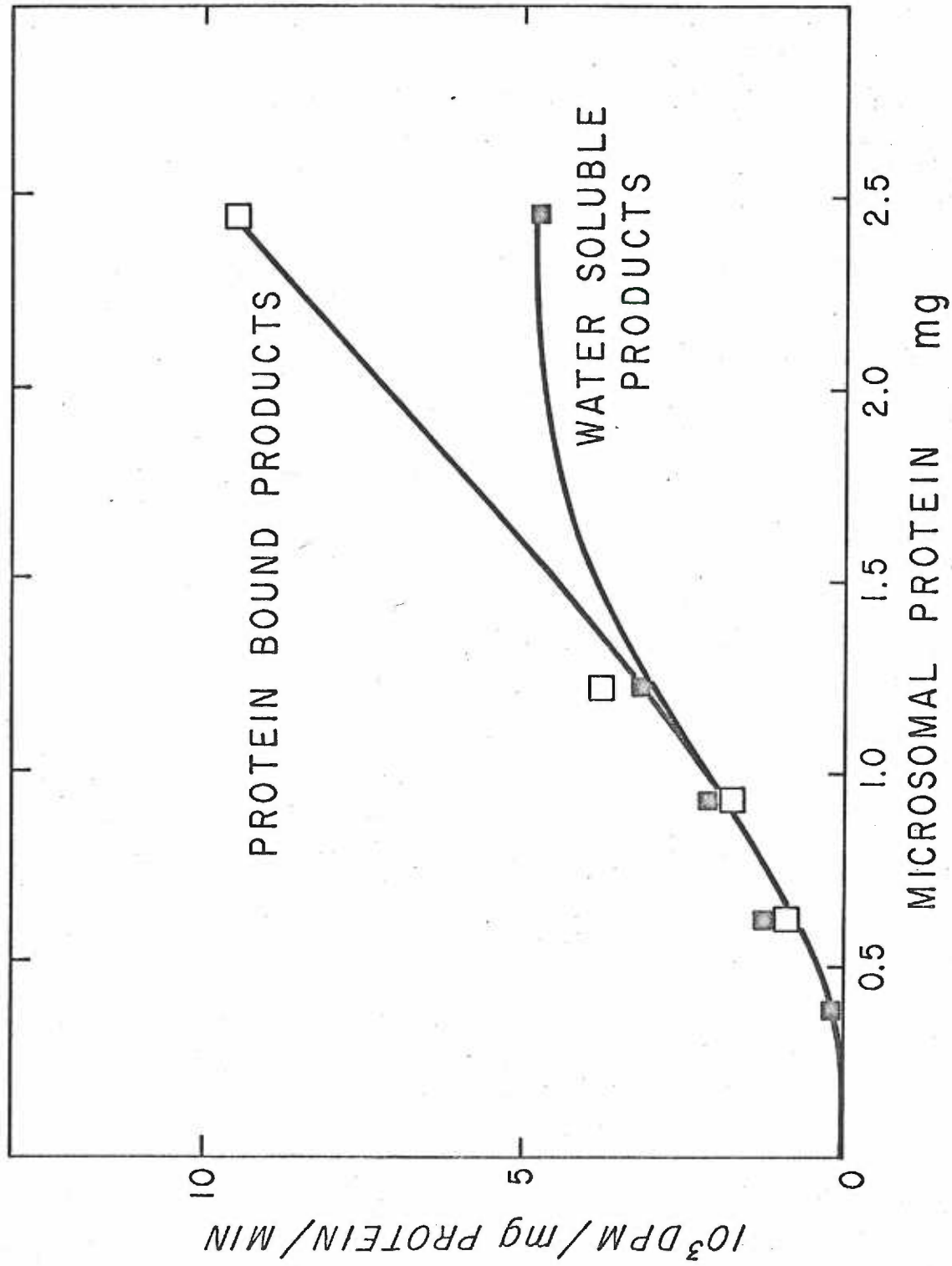

PROTEIN BOUND PRODUCTS
WATER SOLUBLE PRODUCTS
MICROSOMAL PROTEIN mg
0.5
1.0
1.5
2.0
2.5
0
5
10
10³DPM/mg PROTEIN/MIN

Table No. 13. The effect of additional NADPH on estrone metabolism by rat liver microsomes. Results expressed as dpm ($\bar{x} \pm 1$ S.E.) of product formed per milligram of microsomal protein.

EFFECT OF NADPH* ADDITION ON ESTRONE** METABOLISM

Incubation time	Times of NADPH Addition	Product formed 10^3 dpm/mg protein		
		6ξ-OH-E_1	16-OH-E_1	2-OH-E_1
7-1/2 min.	0	18.7±0.2	15.7±0.2	40.3±0.5
15	0	38.8±0.4	29.3±0.3	64.3±0.6
15	0, 7-1/2 min.	34.8±0.9	29.7±0.2	64.9±1.1

* 4 µmoles total added

** sp. act. 0.5 µc ^{14}C/100 µg

n = 2 animals with duplicate flasks for each time.

The possibility that NADH could possibly substitute for NADPH as the cofactor for the microsomal hydroxylase enzymes was tested next (see Table 14). As expected NADPH is a far better cofactor for all hydroxylase reactions; for example, compare 188,000 dpm of ^{14}C-2-OH-E$_1$, formed with 4 µmoles of NADPH vs. 22,000 dpm formed with the same amount of NADH. When NADH substitutes for NADPH the net formations of 2-hydroxy and 16α-hydroxyestrone are about one-tenth of that found when NADPH is the cofactor. In the case of 6ξ-hydroxylation, NADH is somewhat more effective. Here the net formation is about 30 percent of the control and furthermore, the amount of 6ξ-hydroxyestrone formed under these conditions exceeds that of 2-hydroxyestrone. Both protein-bound and ether-insoluble, water-soluble product formations are decreased when NADH acts as a cofactor. These rates are increased by increasing the cofactor concentration. The rates of hydroxylation are not significantly stimulated by doubling the NADH concentration. Looking at the reductive pathways of estrone metabolism, that is the conversion of estrone to estradiol-17β, we find almost a ten-fold increase in activity when NADH acts as a cofactor. This suggests a very active 17β A-hydroxysteroid dehydrogenase enzyme present in the microsomal preparation that requires NADH for optimal activity (37a).

2. Effect of Different Buffers and Phenobarbital Pretreatment.

Marks and Hecker have reported that 2-hydroxylation of estrone is stimulated by incubating in Tris instead of phosphate buffer.

Table No. 14. The comparison of NADH and NADPH as cofactors in estrone metabolism by rat liver microsomes.. Results expressed as dpm ($\bar{x} \pm 1$ S.E.) of product formed per mg of microsomal protein. Incubation time 10 minutes.

EFFECT OF NADH AND NADPH ON THE METABOLISM OF ESTRONE BY RAT LIVER MICROSOMES

Additions		10^3 dpm/mg* microsomal protein in estrone metabolites after incubation						
		E_1*	E_2	2-OH-E_1	16α-OH-E_1	6ξ-OH-E_1	H_2O sol products	Protein bound
NADPH	(4 µmoles)	348±4	32.3±0.3	188±1.9	98.3±0.7	101±0.9	28.3±0.2	26.7±0.2
NADH	(4 µmoles)	648±7	292 ±3.1	220±0.2	9.8±0.2	33.0±0.3	1.0±0.1	1.5±0.1
NADH	(8 µmoles)	616±6	338 ±3.0	26.7±0.3	10.6±0.2	35.8±0.3	2.7±0.1	3.0±0.2

* 1.1×10^6 dpm (50 µg) added to incubation flasks.

In order to test this finding the following experiments were carried out. Microsomes from both female and male rats were incubated in Tris and phosphate buffer. The results are given in Tables 15 and 16. With both male and female rats all hydroxylation reactions studied were depressed when Tris buffer was substituted for phosphate buffer. The significance of the buffer effect will be discussed in the following section. The effect of phenobarbital pretreatment (100 mg/kg daily for 5 days) upon estrone hydroxylase activities is presented in these tables. These incubations were carried out in Tris buffer. Phenobarbital had opposite effects on the hydroxylase activities in male and female rats. Male rats in most cases showed a decrease in 2- and 16α-hydroxylase activity; whereas, female rats showed a marked increase in both of these activities. 6ξ-Hydroxylation of estrone was slightly stimulated in both male and female rats after phenobarbital treatment.

The ether-insoluble, water-soluble and protein-bound product formation from these same animals is given in Table 16. It was observed that Tris buffer does not inhibit the formation of these products but either has no effect or stimulates their synthesis. Phenobarbital markedly increased the synthesis of water-soluble and protein-bound products in female rats which is in agreement with the increased 2-hydroxylation observed after phenobarbital treatment.

Since in the last series of experiments the Tris buffer was only 0.05 M and the phosphate buffer was 0.1 M, the same basic

Table No. 15. Effect of sex, phenobarbital pretreatment and incubation in different buffers on the metabolism of estrone-4-^{14}C by rat liver microsomes. Results expressed as dpm ($\bar{x} \pm 1$ S.E.) product formed per milligram microsomal protein. Livers from 3 animals, duplicate samples from each used.

EFFECT OF SEX, PHENOBARBITAL PRETREATMENT, AND DIFFERENT BUFFERS ON THE METABOLISM OF ESTRONE-4-^{14}C (2.2×10^6 dpm) BY RAT LIVER MICROSOMES

Sex	n*	Buffer	Pretreatment	10^3dpm/mg microsomal protein in estrone metabolites after incubation				
				E_2	2-OH-E_1	16α-OH-E_1	Y	6ξ-OH-E_1
♂	3	Tris	none	9.9±0.1	22±0.2	9.4±0.1	3.5±0.1**	48±0.6
♂	3	Tris	phenobarbital	12.6±0.1	13±0.3**	6.2±0.1**	18±0.1**	60±0.6
♂	3	phosphate	none	12.2±0.2	62±0.5**	19±0.2**	15±0.2	71±0.7
♀	3	Tris	none	38.0±0.3	2.5±0.1**	0.4±0.1**	- -	8.7±0.1
♀	3	Tris	phenobarbital	34.5±0.3	12±0.1**	2.7±0.1**	- -	15±0.2
♀	3	phosphate	none	52.0±0.4	24±0.2	2.4±0.1	- -	11±0.1

* Number of animals.
** Significant differance at $p < 0.05$.

Table No. 16. Effect of sex, phenobarbital pretreatment and incubation in different buffers on the metabolism of estrone-4-^{14}C by rat liver microsomes. The results are expressed as percent ($\bar{x}$ $\pm$ 1 S.E.) of the added radioactivity.

EFFECT OF SEX, PHENOBARBITAL PRETREATMENT, AND DIFFERENT BUFFERS ON THE METABOLISM OF ESTRONE-4-^{14}C IN RAT LIVER MICROSOMES

				Percent of Radioactivity		
Sex	n*	Buffer	Pretreatment	Mg protein	Protein binding	Water-soluble products
♂	3	Tris	none	3.1	14.3±0.2	10.4±0.2
♂	3	Tris	phenobarbital	3.1	11.7±0.1	13.3±0.1
♂	3	phosphate	none	3.1	9.9±0.1	10.6±0.1
♀	3	Tris	none	2.5	**3.2±0.1	**4.3±0.1
♀	3	Tris	phenobarbital	2.7	** 8.2±0.1	** 8.4±0.1
♀	3	phosphate	none	2.5	1.9±>0.1	2.9±>0.1

* Number of animals.
** Significant difference at $p < 0.01$

experiment was repeated using 0.1 M Tris and phosphate buffer (see Table 17). Here we find basically the same results as in previous experiments. This would tend to rule out the possibility that the differences observed are due to differences in molarity.

3. Physicochemical Alteration of Microsomal Enzyme Systems.

The following experiments were designed to clarify the question of whether one or more enzyme systems were involved in the several hydroxylase activities under consideration. The first experiment involved heat denaturation of crude microsomes at a constant temperature for variable time intervals. It is a definite possibility that if more than one enzyme system is involved in the hydroxylations, the thermal stabilities of the different systems may differ. To test this possibility crude microsomes at a concentration of 10 mg of microsomal protein per milliliter of Krebs-Ringer phosphate buffer, pH 7.4, were preheated at 47°C and samples taken for subsequent incubation at specified time intervals. The results are expressed as percent of control values (see Table 18 and Figure 10). The changes in the rates of 2- and 16α-hydroxylation of estrone are shown not to differ from each other but to differ from changes in the rate of 6-hydroxylation. The slight increases seen in 2- and 16α-hydroxylation rates may be due to a decrease in the rate of their breakdown. The most probable explanation of the rapid decline in 6-hydroxylation rates is a decrease in activity of the 6-hydroxylase enzyme rather than an activation of a breakdown enzyme. Next we tried

Table No. 17. Effect of sex and different buffers on the metabolism of estrone-4-^{14}C by rat liver microsomes. Results expressed as dpm of product formed per milligram microsomal protein. Incubation time 15 minutes. Livers pooled from 3 rats and triplicate samples assayed.

EFFECT OF SEX AND DIFFERENT BUFFERS ON THE METABOLISM OF ESTRONE-4-^{14}C BY RAT LIVER MICROSOMES

		10^3 dpm/mg microsomal protein in estrogen metabolites after incubation							
Sex	Buffer	E_1	E_2	2-OH-E_1	16α-OH-E_1	6ξ-OH-E_1	Y	H_2O Sol.	Protein bound
♂	Tris	142±1.5	9.1±0.1	23±0.2	11±0.1	31±0.4	7.1±0.1	12±0.1	18±0.2
♂	Tris	604** ±5.5							
♂	phosphate	96±1.0	27 ±0.3	29±0.3	15±0.2	61±1.0	9.9±0.1	10±0.1	14±0.2
♂	phosphate	523** ±5.0							
♀	Tris	570±5.7	7.5±0.1	1.3±0.1	0.8±0.1	2.3±0.1		2.5±0.1	+5.8±0.1
♀	Tris	595** ±6.0							
♀	phosphate	523 ±5.5	12 ±0.1	6.2±0.1	1.1±0.1	2.9±0.1		0.2±0.1	+1.1±0.1
♀	phosphate	546** ±5.1							

* Total amount of ^{14}C recovered, 6.5 x 10^5 dpm (50 µg) added.
** Incubated 0 minutes.
\+ Significant difference at $p < 0.05$

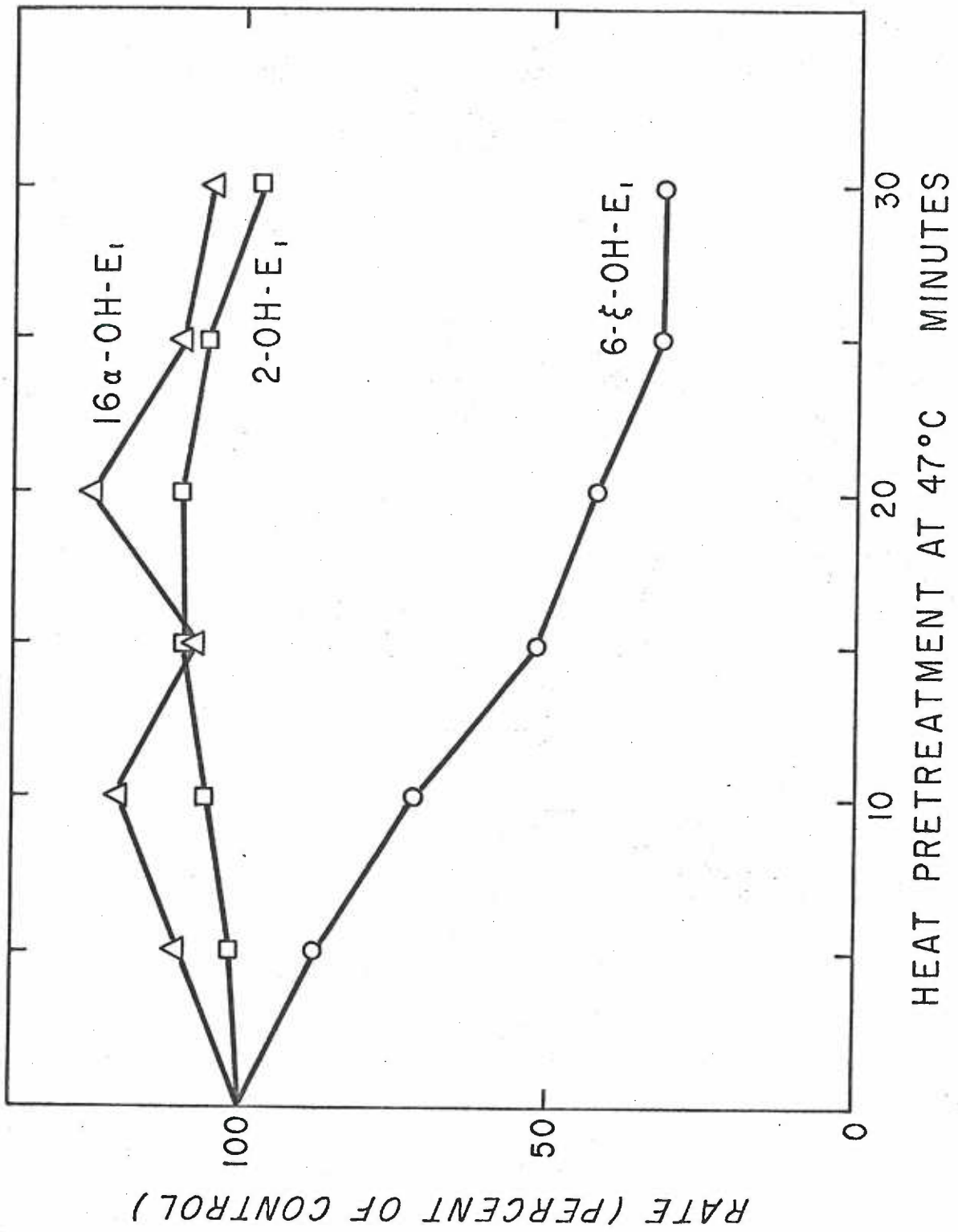
Table No. 18. Effect of heat pretreatment of liver microsomes at 47°C on estrone hydroxylase activities. Results expressed as dpm of product formed per milligram protein per minute. Livers from two animals were pooled and the values represent duplicate assays of two incubation flasks. The maximum deviation from the mean value was ± 10.0 % and it usually was ± 4%.

HEAT DENATURATION OF MICROSOMAL ESTRONE HYDROXYLASE ACTIVITY (10^3 dpm/mg protein/min)

Minutes of Pretreatment*	2-OH-E_1	6ξ-OH-E_1	16α-OH-E_1
0	4.98	3.15	3.28
5	5.11	2.81	3.65
10	5.37	2.30	4.07
15	5.48	1.68	3.66
20	5.44	1.36	4.22
25	5.33	1.06	3.59
30	4.83	1.02	3.50

* Crude microsomes heated at 47°C prior to incubation.
^{14}C-Estrone 7.7 x 10^5 dpm (50 µg); incubation time 15 minutes.

Figure No. 10. Effect of heat pretreatment of liver microsomes on their ability to hydroxylate estrone. Conditions: Microsomes at a concentration of 10 mg protein/ml phosphate buffer, pH 7.4, were heated at 47°C and samples taken at the specified intervals. One mg of protein was used. The incubation mixture included 4 µmoles of NADPH and 74 µmolar concentration of ^{14}C-estrone (100 µg) in total volume of 5 ml of Krebs-Ringer phosphate buffer, pH 7.4. The slope of the line for the decrease in 6ξ-hydroxylase activity is significantly different from that of 2- or 16α(t test, $p < 0.01$). The slopes for 2- and 16α-hydroxylase activity do not differ significantly.


16α-OH-E1
2-OH-E1
6-ξ-OH-E1
100
50
0
10
20
30
HEAT PRETREATMENT AT 47°C MINUTES
RATE (PERCENT OF CONTROL)

to repeat this experiment at a higher preincubation temperature in hopes of being able to differentiate between 2- and 16α-hydroxylation enzymes. I chose 53° C as the preincubation temperature. The results given in Table 19 and Figure 11 indicate that the enzymes are all inactivated at the same fast rate at this temperature. The rate of cytochrome P-450 inactivation differed from those of the hydroxylation enzymes. Its slower rate of inactivation points to the extreme lability of the hydroxylase enzymes since cytochrome P-450 is assumed to be a very labile microsomal protein.

Next I tried to dissociate 2-and 16α-hydroxylase activities by partial digestion of the microsomal enzymes with low concentrations of trypsin. This technique has proved useful in differentiating enzyme activities. Microsomes were prepared as usual and diluted to a concentration of 10 mg of protein per milliliter of Krebs-Ringer phosphate buffer (KRP) pH 7.4 for pretreatment with trypsin. At zero time trypsin was added to give a final concentration of 0.01 mg/ml of KRP and the mixture was then heated at 32°C. Trypsin was inactivated with soybean inhibitor as described in the Methods section. The results are given in Table 20 and Figure 12. Under the conditions used, the results indicate marked differences in the relative denaturability of cytochrome P-450 and the various hydroxylase enzymes. The slower rate of denaturation of P-450 supports the findings in the heat denaturation studies. Looking at Figure 12

Table No. 19. Effect of heat pretreatment at 53°C on rat liver microsomal estrone hydroxylase activities and the concentration of cytochrome P-450. Results expressed as dpm of product formed per milligram of protein. The incubation time was 15 minutes. Livers from two rats pooled and duplicate flasks with duplicate determinations done for each flask. The maximum deviation from the mean was 10%. The average deviation was 5%.

HEAT DENATURATION OF MICROSOMAL ESTRONE HYDROXYLASE ACTIVITY AND CYTOCHROME P-450

Minutes of Pretreatment*	Product (dpm x 10^3/mg protein)			mμmoles/mg protein
	6ξ-OH-E1	16α-OH-E1	2-OH-E1	Cytochrome P-450
0	33.9	59.5	103	0.86
5	26.4	44.8	87.3	0.63
7-1/2	16.2	32.3	52.7	-
10	9.4	18.9	33.9	0.53
15	0	1.7	7.2	0.36
20	0	0	0	0.28

* Crude microsomes heated at 53°C prior to incubation.

Figure No. 11. Effect of heat pretreatment on microsomal enzyme activities. Same data as used in Table 19 except expressed as percent of control.

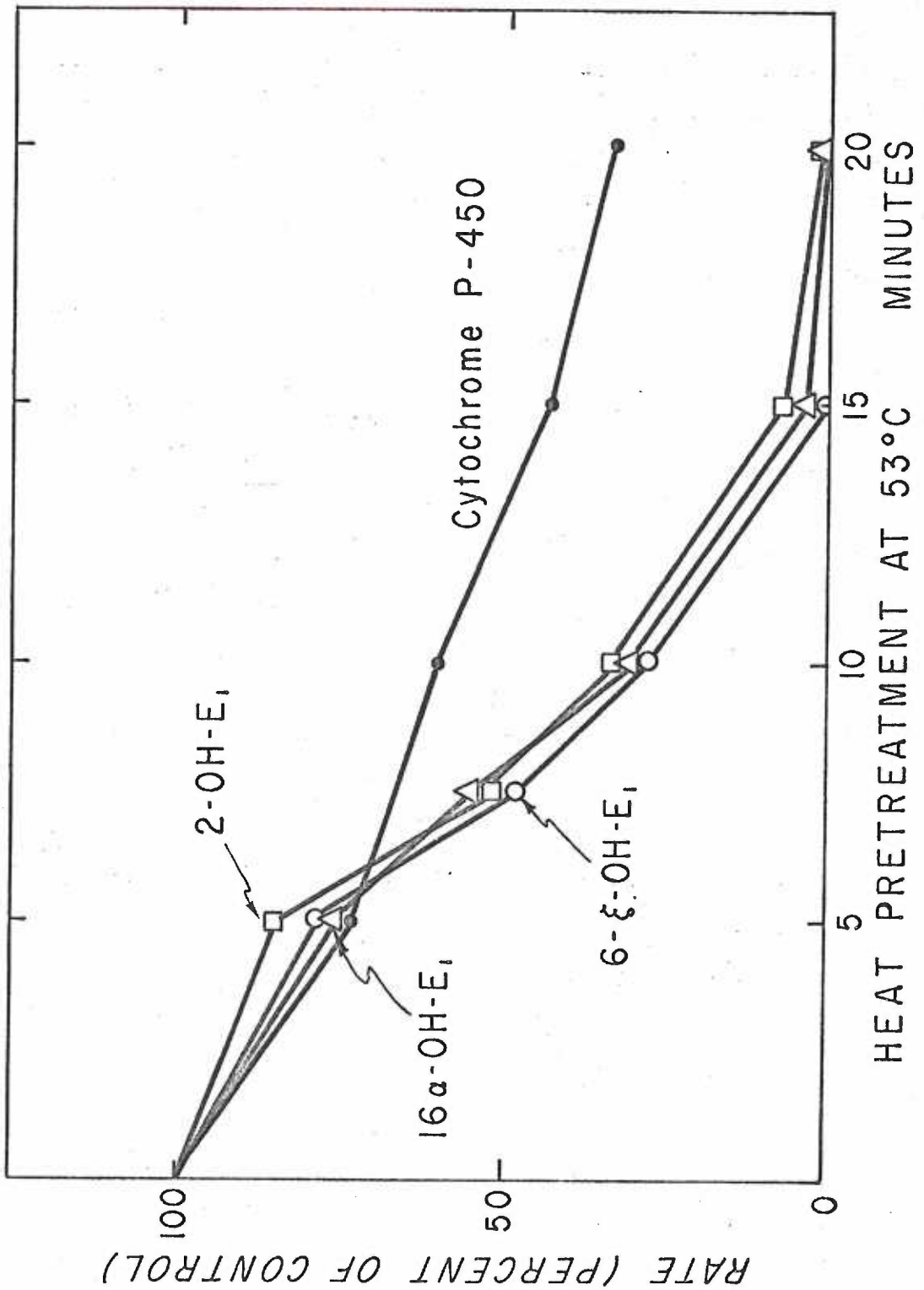

RATE (PERCENT OF CONTROL)
100
50
0
HEAT PRETREATMENT AT 53°C
5
10
15
20
MINUTES
Cytochrome P-450
2-OH-E1
16α-OH-E1
6-ξ-OH-E1

Table No. 20. Effect of pretreatment of rat liver microsomes with trypsin upon estrone hydroxylase activities and cytochrome P-450. Results expressed as dpm of product formed per milligram protein. Incubation time 7-1/2 minutes. Livers from 3 rats pooled incubation flasks run in quadruplicate.

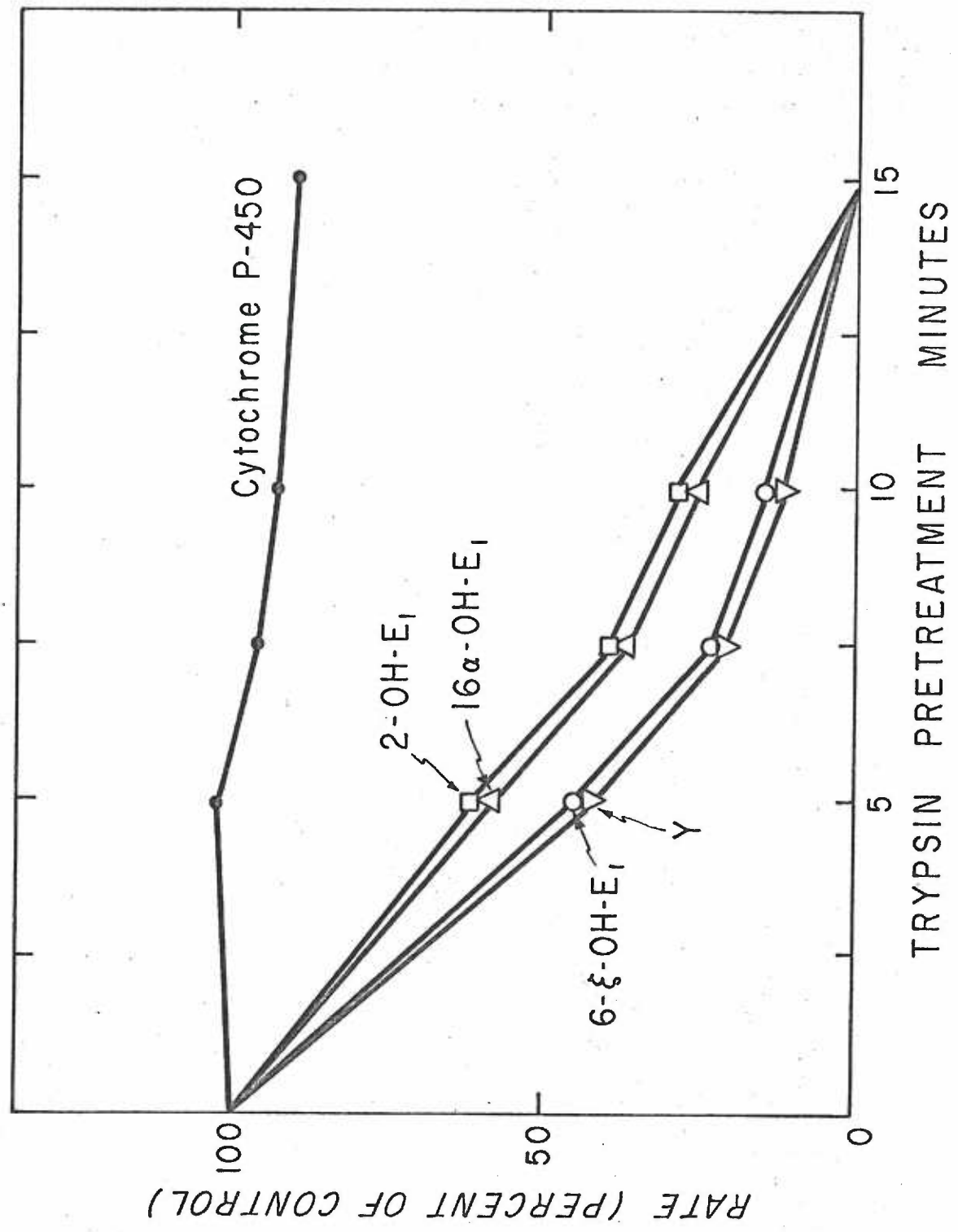
EFFECT OF PRETREATMENT OF MICROSOMES WITH TRYPSIN* UPON ESTRONE**
HYDROXYLASE ACTIVITY AND CYTOCHROME P-450

Minutes of Pretreatment	Product (10^3 dpm/mg protein)				mμmoles/mg protein
	6ξ-OH-E_1	Y	16α-OH-E_1	2-OH-E_1	Cytochrome P-450
0	25.0±0.3	13.1±0.2	13.0±0.3	24.7±0.5	0.82
5	11.3±0.3	5.6±0.1	7.5±0.2	14.9±0.6	0.84
7-1/2	5.8±0.1	2.7±0.0	4.8±0.0	9.6±0.1	0.79
10	4.2±0.1	2.0±0.0	3.5±0.1	6.9±0.3	0.76
15	0	0	0	0	0.73

* Approximately 0.1% by weight incubated at 32°C in phosphate buffer pH 7.4.

** 100 μg/0.5 μc.

Figure No. 12. Trypsin pretreatment of microsomes. The conditions are given in the Methods section. The results are expressed as percent of untreated controls. The percent of control rates for 6ξ-hydroxylation are significantly different (t.test $p<0.02$) from those for 2 - and 16α-hydroxylation at times 5, 7 1/2 and 10 minutes of treatment. The percent of control rates for 2- and 16α-hydroxylation do not differ significantly.


RATE (PERCENT OF CONTROL)
100
50
0
5
10
15
TRYPSIN PRETREATMENT MINUTES
Cytochrome P-450
2-OH-E1
16α-OH-E1
6-ξ-OH-E1
Y

one notes a difference in the rates of inactivation of 6-hydroxylase and 2- and 16α-hydroxylase enzymatic activities. A fourth activity whose product is denoted as Y closely resembles 6-hydroxylase activity in regard to its rate of inactivation. Again these inactivation studies point out the extreme lability of the hydroxylase enzymes.

4. Substrate Inhibition Studies.

Aminopyrine which is demethylated by rat liver microsomes (64) has been shown by Heinrichs and Colás to inhibit 7α- and 16α-hydroxylation of dehydroepiandrosterone by different mechanisms (64). In these studies two concentrations of estrone were used while the concentration of aminopyrine was varied. The results are expressed as 10^3 x dpm/mg microsomal protein in Table 21; these same data represented in Figures 13 and 14, where the results are expressed as percent of control values.

Aminopyrine is shown to be a very poor inhibitor of estrone 6- and 16α-hydroxylase enzymes (Figures 13 and 14) while it seems, paradoxically, to stimulate 2-hydroxylase activity. On closer examination, however, the increased net production of 2-OH-estrone can be largely accounted for by decreased protein-bound and ether-insoluble, water-soluble metabolites. The reason for the decrease in 16α-hydroxylase activity at 1.67×10^{-4} *M* aminopyrine concentration and 0.74×10^{-4} *M* estrone concentration is not clear and the result could not be reproduced. The inhibition of the breakdown of 2-OH-estrone (i.e., protein-bound and water-soluble product formation) clearly indicated

Table No. 21. The effect of aminopyrine on the metabolism of estrone by rat liver microsomes. The results are expressed as dpm of product formed per milligram of protein. Incubation time 7-1/2 minutes. Duplicate flasks for each determination. Livers pooled from two male rats. The maximum deviation from the mean value was ± 10%. The average deviation was ± 3.5%.

EFFECT OF AMINOPYRINE ON THE METABOLISM OF ESTRONE*

[Aminopyrine] x 10^{-4} *M*	10^3 dpm/mg microsomal protein				
	6ξ-OH-E_1	16α-OH-E_1	2-OH-E_1	Protein bound	Ether-insoluble Water-soluble
0.00	40.0	45.5	72.1	30.1	49.1
0.84	37.1	42.7	84.4	22.0	35.6
1.67	37.2	41.8	86.4	20.0	32.5
3.30	47.4	47.4	108.8	19.0	38.4
5.00	40.5	45.6	102.7	18.8	33.2
6.70	42.9	46.4	103.6	20.4	35.2

* Conc. 0.37 x 10^{-4} *M* sp. act. 0.5 μc/50 μg.

EFFECT OF AMINOPYRINE ON THE METABOLISM OF ESTRONE*

[Aminopyrine] x 10^{-4} *M*	10^3 dpm/mg microsomal protein				
	6ξ-OH-E_1	16α-OH-E_1	2-OH-E_1	Protein bound	Ether-insoluble Water-soluble
0.00	32.7	39.7	70.6	19.9	31.0
0.84	30.2	36.2	71.5	17.4	28.0
1.67	30.1	30.2	75.6	16.2	24.4
3.30	31.1	36.9	91.1	15.7	21.3
5.00	29.4	41.4	84.9	14.1	22.2
6.70	30.5	39.4	81.8	13.2	19.9

* Conc. 0.74 x 10^{-4} *M*

Figure No. 13. Effect of aminopyrine on estrone (3.4×10^{-5} *M*) metabolism by rat liver microsomes. Rates are expressed as percent of nontreated controls. Numerical data given in Table 21.

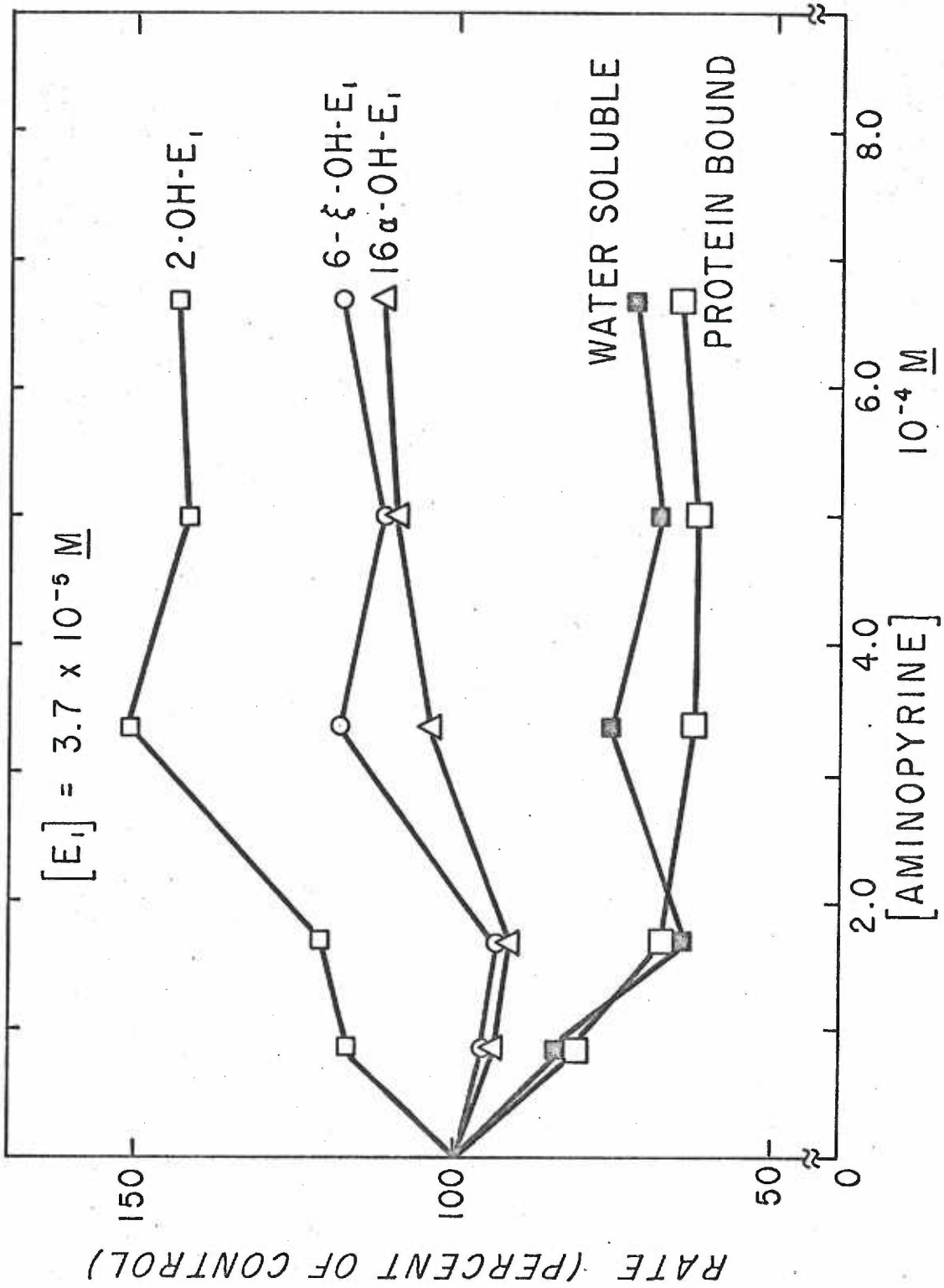
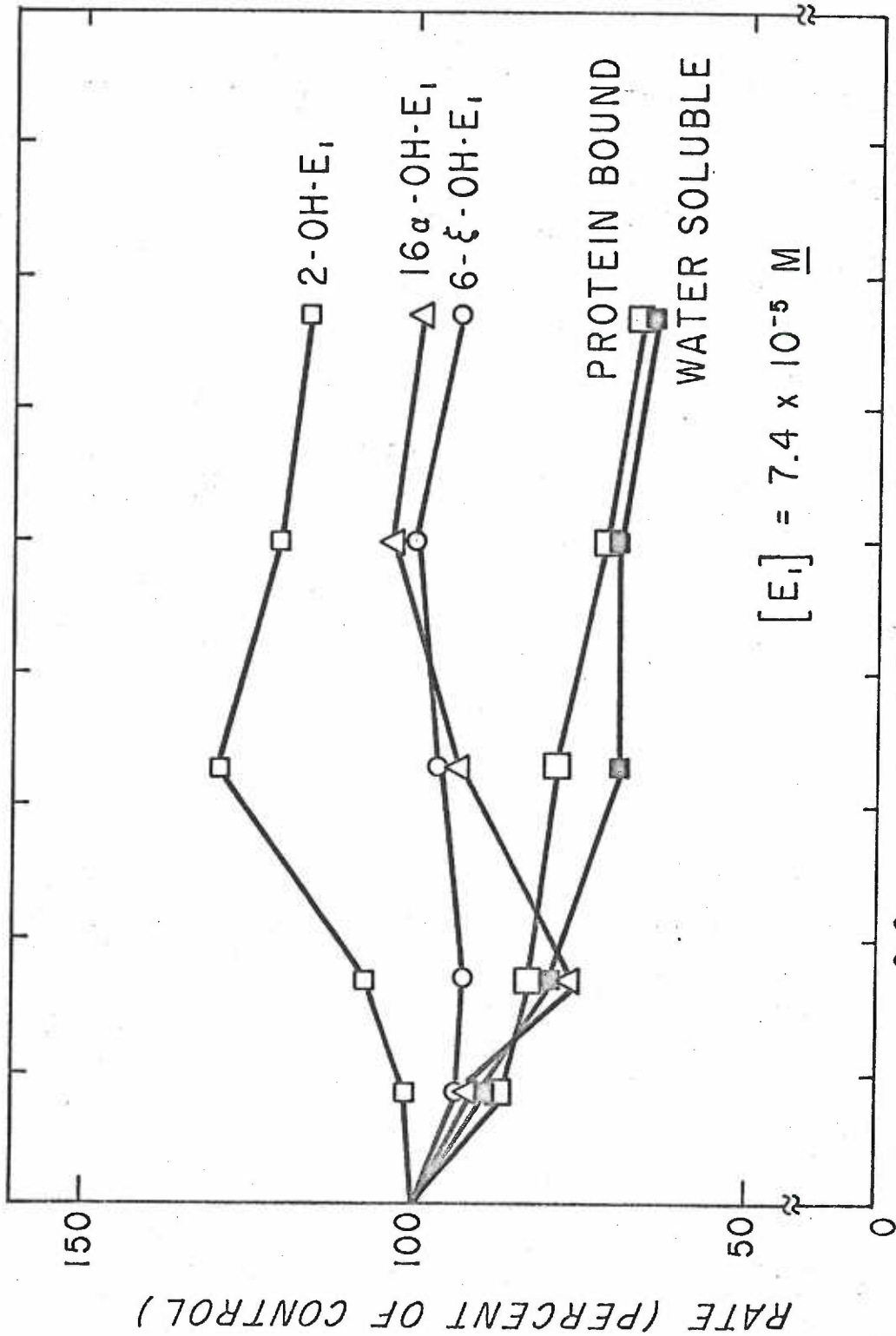

RATE (PERCENT OF CONTROL)
150
100
50
0
2.0
4.0
6.0
8.0
[AMINOPYRINE]
10⁻⁴ M
[E₁] = 3.7 x 10⁻⁵ M
2-OH-E₁
6-ξ-OH-E₁
16α-OH-E₁
WATER SOLUBLE
PROTEIN BOUND

Figure No. 14. Effect of aminopyrine on estrone (7.4×10^{-5} *M*) metabolism by rat liver microsomes. Numerical data given in Table 21.


RATE (PERCENT OF CONTROL)
150
100
50
0
2.0
4.0
6.0
8.0
[AMINOPYRINE]
10⁻⁴ M
[E₁] = 7.4 x 10⁻⁵ M
WATER SOLUBLE
PROTEIN BOUND
2-OH-E₁
16α-OH-E₁
6-β-OH-E₁

that different enzyme systems are responsible for the formation and breakdown of this steroid.

In a second set of experiments I used dehydroepiandrosterone (DHA) as an inhibitor. Under the usual experimental conditions dehydroepiandrosterone is primarily hydroxylated in two positions, C-7 and C-16 (26, 63, 123). Becuase of the similarities in the structures of estrone and DHA, I assumed that the enzyme responsible for 16α-hydroxylation of estrone also carries out 16α-hydroxylation of dehydroepiandrosterone. A second possibility is that the enzyme responsible for 6-hydroxylation of estrone would 7-hydroxylate dehydroepiandrosterone. Since dehydroepiandrosterone is not appreciably 2-hydroxylated, there should be little or no influence on 2-hydroxylated, there should be little or no influence on 2-hydroxylation of estrone in the presence of dehydroepiandrosterone while the 6-and 16α-hydroxylation reactions should be inhibited. Table 22 and Figures 15 and 16 clearly show that dehydroepiandrosterone inhibits all three hydroxylation reactions of estrone.

The net rates of formation at an estrone concentration of 3.7×10^{-5} *M* are 1.38, 0.68 and 0.44 mμmoles/mg. protein/min. for 2-, 6-and 16α-estrone, respectively. At 7.4×10^{-5} *M* estrone concentrations the rates are 0.77, 0.55 and 0.32 mμmoles/mg min. for 2-,6- and 16α-hydroxyestrone, respectively. At the lower estrone concentration (3.7×10^{-5} *M*) 6-hydroxylation is inhibited to a greater extent than either 2- or 16α-hydroxylation. At the higher estrone concentration, there was observed more inhibition of all hydroxylation reactions

Table No. 22. The effect of dehydroepiandrosterone on estrone metabolism by rat liver microsomes. The results are expressed as dpm ($\bar{x} \pm 1$ S.E.) of product formed per milligram of protein. Livers from 2 rats pooled quadtriplicate samples taken for incubation.

EFFECT OF DHA ON THE METABOLISM OF ESTRONE*

10^3 dpm/mg protein

[DHA] x 10^{-5} *M*	2-OH-E_1	6ξ-OH-E_1	16α-OH-E_1
0	31.8 ± 1.0	23.0 ± 0.3	13.3 ± 0.2
1.4	32.0 ± 0.6	19.5 ± 0.2	12.5 ± 0.2
4.7	29.6 ± 0.5	18.2 ± 0.3	12.5 ± 0.2
7.8	28.5 ± 0.4	14.7 ± 0.3	9.8 ± 0.1
11.2	26.6 ± 0.5	12.4 ± 0.3	9.1 ± 0.1
14.4	19.9 ± 0.4	10.1 ± 0.2	7.9 ± 0.2

* Conc. 3.7 x 10^{-5} *M*(50 µg/0.5 µc)

EFFECT OF DHA ON THE METABOLISM OF ESTRONE*

10^3 dpm/mg microsomal protein

[DHA] x 10^{-5} *M*	2-OH-E_1	6ξ-OH-E_1	16α-OH-E_1
0	29.5 ± 1.4	14.4 ± 0.3	9.4 ± 0.2
1.4	24.2 ± 0.5	10.1 ± 0.2	6.6 ± 0.3
4.7	15.6 ± 0.4	5.9 ± 0.1	4.3 ± 0.2
7.8	12.0 ± 0.2	4.6 ± 0.1	3.8 ± 0.2
11.2	9.8 ± 0.5	3.9 ± 0.2	3.9 ± 0.1
14.4	10.8 ± 0.4	4.3 ± 0.1	3.8 ± 0.1

* Conc. 7.4 x 10^{-5} *M* (100 µg/0.5 µc)

Figure No. 15. Effect of dehydroepiandrosterone on estrone hydroxylase activities. Numerical date are given in Table 22. Estrone concentration 3.7×10^{-5} *M*.

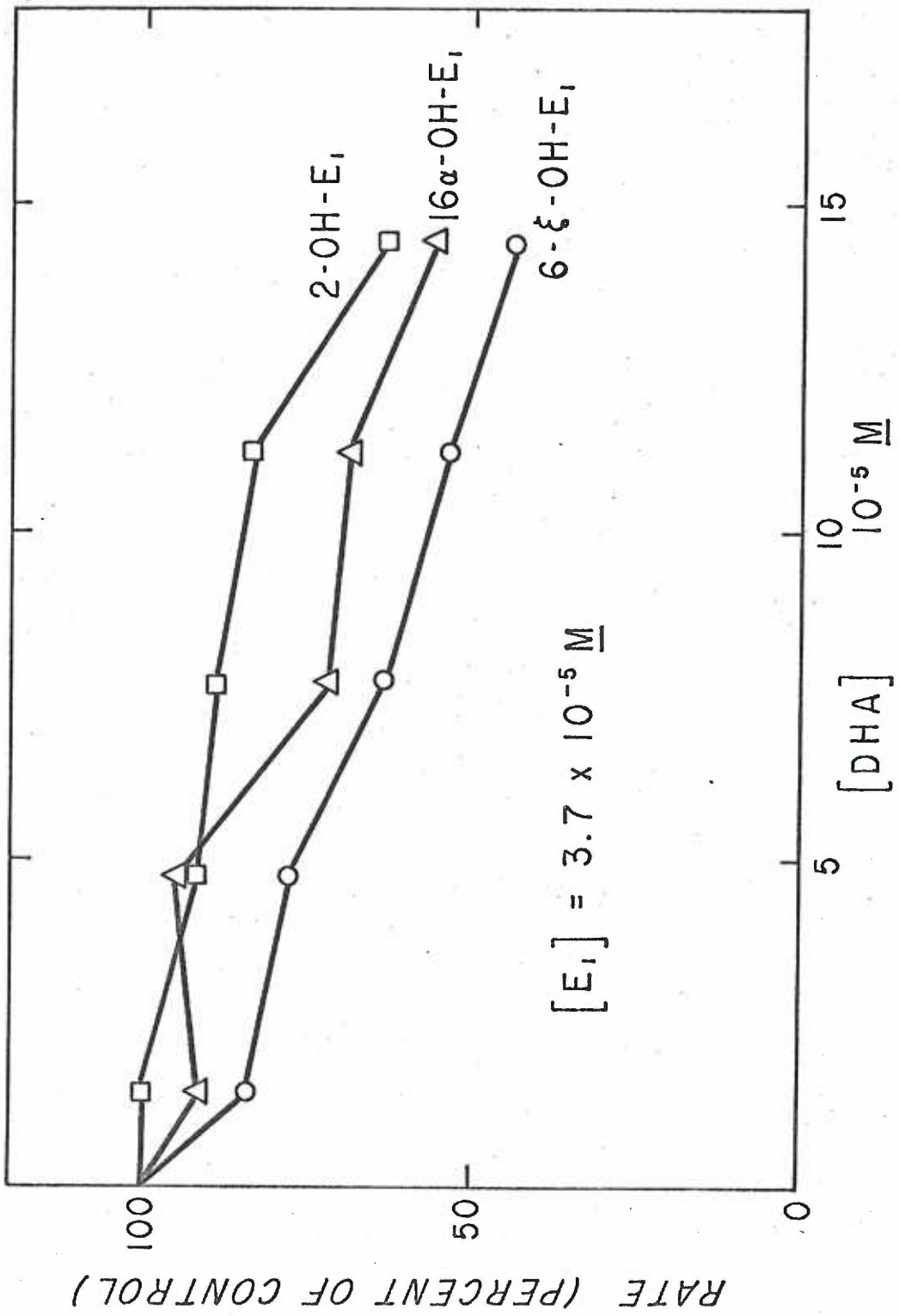
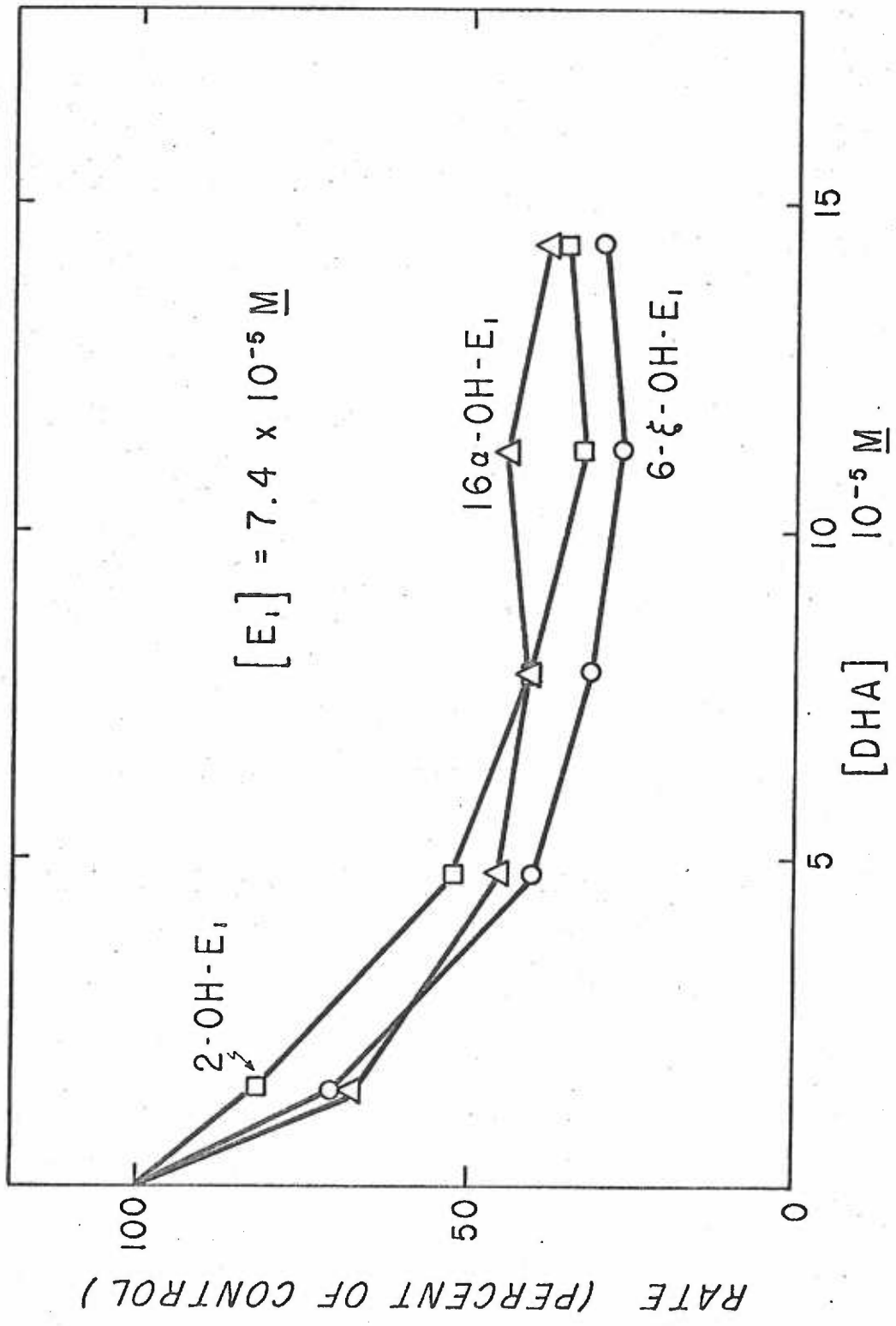

RATE (PERCENT OF CONTROL)
100
50
0
5
10
15
[DHA] 10⁻⁵ M
[E₁] = 3.7 x 10⁻⁵ M
2-OH-E₁
16α-OH-E₁
6-ξ-OH-E₁

Figure No. 16. Effect of dehydroepiandrosterone on estrone hydroxylase activities. The estrone concentration was 7.4×10^{-5} *M*. Numerical data are given in Table 22.


RATE (PERCENT OF CONTROL)
100
50
0
5
10
15
[DHA] 10^{-5} M
$[E_1] = 7.4 \times 10^{-5}$ M
2-OH-E_1
16α-OH-E_1
6-ξ-OH-E_1

than at the lower substrate concentration. One possible interpretation of these results is a mixed substrate inhibition which would account for the much greater inhibitory effect of high estrone concentrations.

5. Carbon Monoxide Inhibition Studies.

Carbon monoxide has been reported to compete with oxygen in certain steroid hydroxylation reactions (30). Conney et al. (30, 89) have shown that the various hydroxylation reactions of testosterone are inhibited at different rates by carbon monoxide. As one possible way to differentiate 2- and 16α-hydroxylating systems, we incubated microsomes in the usual manner. Instead of using air as the gas phase, we used gas mixtures containing various ratios of carbon monoxide and oxygen diluted in nitrogen. The results of such a study are given in Table 23 and Figure 16. All three reactions were inhibited by the presence of carbon monoxide, but 6-hydroxylation was more sensitive to carbon monoxide than either 2- or 16α-hydroxylation reactions. At a lower ratio (CO/O_2 = 1) of carbon monoxide to oxygen, 16α-hydroxylation seems to be inhibited more than 2-hydroxylation, but at higher ratios the degree of inhibition seems to be the same. The apparently higher inhibition of 16α-hydroxylation at a low CO/O_2 when compared to that of 2-hydroxylation may be due to a greater inhibition of the breakdown of 2-hydroxyestrone, which seemed to be the case as water-soluble and protein-bound product formation was almost completely inhibited at the lowest CO/O_2 ratio. Conney et al. have expressed the

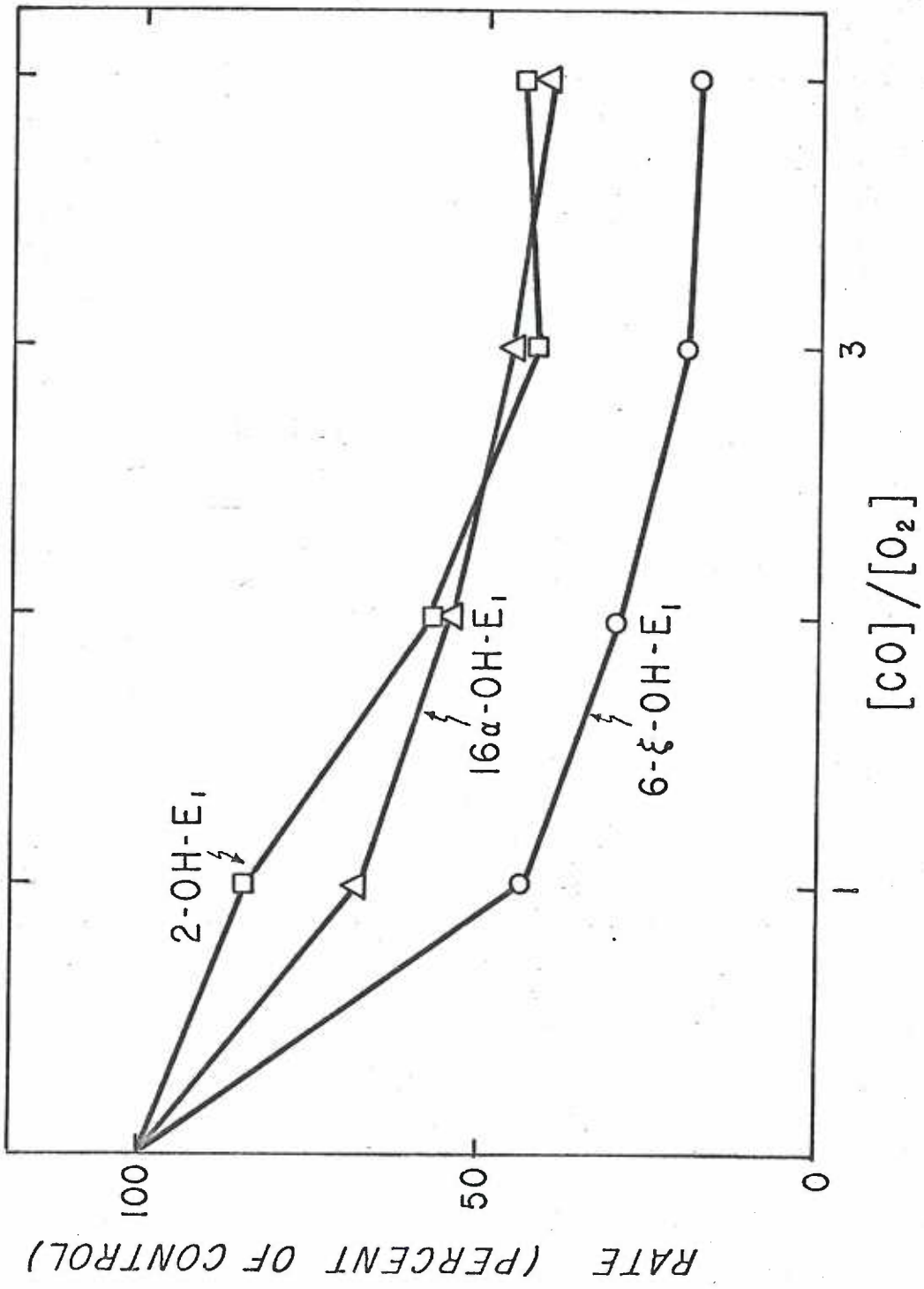
Table No. 23. Carbon monoxide inhibition of rat liver microsomal estrone hydroxylase activity. The results are expressed as dpm of product formed per milligram of protein per minute. Livers from 3 rats, duplicate flasks done for each point. The maximum deviation from the mean was ± 9.0 %. The average deviation was 4%.

CARBON MONOXIDE INHIBITION OF ESTRONE* HYDROXYLASE ACTIVITY

CO/O_2 Ratio	[CO]**[O_2]**		10^2 dpm/mg protein/min.		
			2-OH-E_1	6ξ-OH-E_1	16α-OH-E_1
Air (control)	-	21	32.3	16.0	15.4
O_2 - N_2		10	33.0	15.8	16.4
1	16.7	16.7	27.3	7.0	10.5
2	28.6	14.3	18.4	4.8	8.4
3	37.5	12.5	13.6	3.9	7.0
4	44.5	11.1	14.1	3.8	6.3

* 100 µg/o.3 µc-^{14}C
** percent

Figure No. 17. Effect of carbon monoxide on estrone hydroxylase activities.


RATE (PERCENT OF CONTROL)
100
50
0
1
3
[CO]/[O2]
2-OH-E1
16α-OH-E1
6-ξ-OH-E1

CO/O_2 ratios that give 50% inhibition of specified hydroxylation reactions of testosterone. For a subsequent comparison we have estimated that the CO/O_2 ratios for 50% inhibition of 2-, 16α- and 6-hydroxylation of estrone are 2.5, 2.5 and 0.83, respectively.

6. Approach to the Determination of the Rate of 2-Hydroxyestrone Formation.

Since 2-hydroxyestrone is thought to be metabolized to form protein-bound and ether-insoluble, water-soluble products, it was of interest to investigate the rate of its breakdown. Burstein has described a procedure to obtain a better approximation of the true initial rates of product formation by measuring the net rates of substrate and product metabolism (21, 22). The main assumption is that the reactions are first order. In the reaction sequence shown below:

$$\xleftarrow{k_3} \text{Estrone } (c_1) \xrightarrow{k_1} \text{2-OH-estrone } (c_2) \xrightarrow{k_2}$$

k_3, k_1 and k_2 are the first order constants [time^{-1}] of 2-hydroxyestrone formation, and the overall estrone and 2-hydroxyestrone metabolism, respectively; c_1 and c_2 are the concentrations of substrate and product at time t. The solution of the set of differential equations (A)

$$\frac{dc_2}{dt} = k_1c_1 - k_2c_2; \quad \frac{dc_1}{dt} = k_3c_1 \qquad (A)$$

is given by equation B:

$$c_2 = \frac{k_1 C}{k_2 - k_3} (e^{-k_3 t} - k_2 t) \qquad (B)$$

where C is the estrone concentration at time t = o. k_3 and k_2 can be calculated from the general equation:

$$k = \frac{\ln}{t} \left(\frac{a}{a - x} \right)$$

where a is the concentration of radioactivity present at the start of the incubation and a - x is the concentration remaining at time t. Knowing the values for C, c_2, k_3 and k_2, it is possible to solve for k_1 by rearranging equation B.

$$k_1 = \frac{c_2 (k_2 - k_1)}{C (e^{-k_3 t} e^{-k_2 t})}$$

The results of such an experiment are given in Tables 24 and 25. In Table 24 the calculations of k_3 and k_2 are given. Tracer ^{3}H-2-OH-estrone, prepared as previously described, was incubated with substrate amounts of ^{14}C-estrone. The ratio of ^{3}H dpm/^{14}C dpm was always greater than 1 and less than 10 to insure greater accuracy (106). As seen in Figure 7 the rate of estrone hydroxylation falls off for time intervals greater than a few minutes. The mean values of k_3 and k_2 are 0.020 min^{-1} and 0.0085 min^{-1}, respectively. Table 25 compares the values obtained by equation B and those obtained by single point analysis, that is, the rate at time t by directly reading the result

Table No. 24. The determination of rate constants for estrone and 2-hydroxyestrone breakdown. For a more complete description see the text.

DETERMINATION OF k_2 AND k_3

t	a*	(a - x)*	log a/a - x	k_2
minutes	dpm	dpm		min^{-1}
0	7.18×10^4			
5	7.18×10^4	7.03×10^4	0.0086	0.0040
7-1/2	7.18×10^4	6.68×10^4	0.0296	0.0091
10	7.18×10^4	6.50×10^4	0.0414	0.0094
15	7.18×10^4	6.01×10^4	0.0756	0.0113

* Tracer amount of ^{3}H-2OH-E_1 added to each incubation flask.

t	a*	a - x*	log a/a - x	k_3
minutes	dpm	dpm		min^{-1}
0	2.07×10^5			
5	2.07×10^5	1.90×10^5	0.0374	0.0172
7-1/2	2.07×10^5	1.78×10^5	0.0645	0.0200
10	2.07×10^5	1.71×10^5	0.0828	0.0190
15	2.07×10^5	1.44×10^5	0.1584	0.0238

* ξ_1 specific activity 2.07×10^5 dpm/100 µg (7.4×10^{-5} M)

Table No. 25. A comparison of the rates of formation 2-hydroxyestrone as determined by two approaches. For a more complete description see text page 124-125. Duplicate determinations done on triplicate flasks. Results are expressed as the mean ± 1 S.E.

COMPARISON OF RATES OF FORMATION OF 2-HYDROXYESTRONE*

time	Formation 10^{-9} moles x min^{-1} x mg $protein^{-1}$	
	k_1C**	observed
0		-
5	1.08±0.05	0.93±0.04
7.5	0.90±0.04	0.86±0.03
10	0.92±0.04	0.82±0.03
15	0.71±0.03	0.74±0.02

* Male rat liver microsomes at a concentration of 1 mg/glask (5ml) were used in these experiments. Four micromoles of NADPH was added as cofactor to each flask.

** Calculated.

with no corrections. It is seen that the calculated values of k_1C are generally greater than those observed by single point analysis. The decline in the k_1 values point out that the assumption of first order reaction kinetics is probably not justified under the current experimental conditions. The reason for the decrease in the rate constant k_1 at 15 minutes is not apparent. These results suggest, however, that the net rates of 2-hydroxylation of estrone at the time intervals studied in this work are similar in magnitude to the true rates of hydroxylation.

IV. DISCUSSION

A. Effect of thyroid function on estrone metabolism

The object of the first portion of this thesis was to replicate the *in vivo* observations of Fishman et al. (46,47) on the effect of thyroid function on estrogen metabolism. We had hoped to observe an inverse relationship between 2- and 16α-hydroxylation under control by thyroid hormone levels. If we had made these observations we might have studied the mechanism of action of thyroid hormone on the regulation of these two hydroxylase activities, but we failed to find evidence for a competitive relationship under the conditions we chose.

We originally tried to note a thyroid effect on estrogen metabolism in liver slices from thyroidectomized and thyroxine-treated rats. When rats were treated daily with 100 μg of thyroxine for seven days there was a significant decrease in the ability of these slices to hydroxylate estrone. For example, the fractions of ^{14}C-estrone incorporated into 2-hydroxyestrone in thyroxine-treated and normal rats were 2.9 ± 0.7% and 6.5 ± 1.1%, ($p < 0.01$) respectively; the values for 16α-hydroxyestrone were also decreased; 4.6 ± 2.5% for normal vs. 2.3 ± 0.5% for treated rats.

The amount of estradiol-17β synthesized was also decreased by about 50 percent (10.4 ± 1.4 percent vs. 5.4 ± 1.0 percent).

Livers from animals that had been thyroidectomized eight

days prior to the experiments had slightly decreased hydroxylase activities. The incorporation into 2-OH-estrone was decreased from 6.5 ± 1.1 percent to 4.7 ± 1.4 percent and into 16α-hydroxyestrone from 4.6 ± 2.5 to 2.8 ± 1.0 percent. Estradiol-17β formation was also slightly depressed by thyroidectomy (10.0 ± 1.4 vs. 8.3 ± 2.3 percent).

We next attempted to find out whether a thyroid effect on estrogen metabolism could be shown by using a subcellular fraction rather than slices. It was observed that pretreatment of rats with thyroxine had no significant effect on the ability of the 10,000 x *g* supernatant fraction from rat liver to hydroxylate estrone in either the 2- or 16α-positions. For example, normal and treated values for 2-hydroxylation are 12.2 ± 4.7 vs. 12.8 ± 0.8 percent, and for 16α-hydroxylation 8.7 ± 0.8 vs. 7.0 ± 0.9 percent incorporation. There was, however, a slight increase in the net formation of estradiol-17β (4.0 ± 1.0 vs. 6.1 ± 0.7 percent incorporation). Since changes in metabolism might be noted in the formation of ether-insoluble, water-soluble products and protein-bound products these fractions were examined. It is apparent that there is no significant effect of thyroxine on their formation (see Tables 10 and 11). In contrast to the lack of effect of thyroxine on net hydroxylation rates, thyroidectomy resulted in a decreased incorporation of estrone into both 2- and 16α-hydroxyestrone. For example, the percent incorporation into 2-hydroxyestrone by normal rats decreased from 12.2

± 4.7 to 6.2 ± 1.0 percent by thyroidectomized rats. For 16α-hydroxyestrone formation thyroidectomy resulted in a decrease from 8.7 ± 0.8 to 3.4 ± 1.0 percent incorporation. The incorporations into both the ether-insoluble, water-soluble and the protein-bound products were decreased about 50 percent by the treatment. There was no apparent effect on the 17β-hydroxysteroid dehydrogenase activity.

Since Lazier et al. (91) and Marks and Hecker (99) have provided strong indirect evidence that 2-hydroxylation is an obligatory step towards the formation of ether-insoluble, water-soluble, and protein-bound metabolites, we attempted to measure the overall kinetics of the formation of these two products. The estimated maximal velocity of formation of water-soluble, ether-insoluble products is decreased in thyroidectomized and thyroxine-treated rats from a normal of 0.13 to 0.04 and 0.03 μmoles x liter^{-1} x min^{-1}, respectively, while the overall apparent Km values are not changed (Km ≈ 0.5 x 10^{-5} *M*) and compare favorably to those values obtained by Lazier et al. (91) for female rats (1 x 10^{-5} *M*). In the case of the rate of formation of protein-bound products, thyroidectomy resulted in decreased rates of formation from 0.18 to 0.04 μmoles x liter^{-1} x min^{-1}, whereas, treatment with thyroxine slightly increased the rate of formation of these products from 0.18 to 0.30 μmoles x liter^{-1} x min^{-1}. As in the case of ether-insoluble, water-soluble product formation there was no significant change in the apparent Km

value (0.5×10^{-5} *M*). It is also interesting to note that this value is the same as for the formation of ether-insoluble, water-soluble products. The significance of the decreased rate of ether-insoluble, water-soluble product formation and increased rate of protein-bound product formation by thyroxine-treated rats may then be simply a decreased amount of low molecular wt.-SH group containing proteins (99).

Finally, we tried to change the pattern of estrone metabolism produced by liver microsomes from thyroidectomized rats by treatment with thyroxine in both male and female rats.

In the case of female rats there was no change in the incorporation of ^{14}C-estrone into 2- and 16α-hydroxyestrone upon thyroxine treatment. For 2-hydroxyestrone it was 3.5% vs. 3.2% per mg protein for the treated animals, and for 16α-hydroxyestrone 0.93% vs. 0.94% per mg protein. It was noted, however, that the concentration of microsomal protein increased from 7.2 mg/gram to 11.2 mg/gram. There was also an increase in the formation of protein-bound and ether-insoluble, water-soluble products upon thyroxine treatment (0.50% vs. 0.77% incorporation per mg protein for protein-bound products and 0.23% vs. 0.51% per mg protein for ether-insoluble, water-soluble products for thyroidectomized and treated rats, respectively).

The same results were obtained for microsomal preparations from male rats, namely, no apparent changes in hydroxylase activity and increases in concentration of microsomal protein and the amount

of protein-bound and ether-insoluble, water-soluble product formed. The sex differences in regard to estrogen hydroxylation rates were apparent for both 2- and 16α-hydroxylation as well as in the amount of protein-bound and ether-insoluble, water-soluble product formation. It is also noted that more estradiol-17β was formed by microsomes isolated from female rats than from males (5.44% vs. 3.38% per mg protein). The lesser amount obtained in the case of male rats may be due to an increased rate of breakdown of the estradiol formed or to a decreased availability of substrate due to the more rapid utilization of estrone by male microsomes.

The inability to demonstrate in rat liver preparation a competition between hydroxylations of estrone at C-2 and C-16 under the control by the level of thyroid hormone may be due to species differences. It is also possible that in man there are two specific enzyme systems involved and in the case of the rat a non-specific enzyme is responsible for both hydroxylations. Another plausible hypothesis is that there are two enzyme systems involved but sharing a common rate-limiting component.

The decreased 2- and 16α-hydroxylation of estrone by rat liver slices from thyroxine-treated rats is in agreement with the findings of Sulcova et al. (119) who noted that 7α-hydroxylation of dehydroepiandrosterone by rat liver is suppressed by thyroxine pretreatment.

B. Comparison of estrone hydroxylase activities

The lack of a specific thyroid hormone effect on 2- and 16α-hydroxylation of estrone by our rat liver preparations, and the obvious complexity in the pattern of products, directed our efforts toward an analysis of the systems responsible for steroid hydroxylation. According to one of the current hypotheses, a steroid reacts with a [P-450 Fe $^{++} \cdot O_2$] complex giving hydroxylated steroid plus oxidized cytochrome P-450 which is then reduced through a flavoprotein involving a non-heme protein in the case of adrenal mitochondria. Once reduced, the cytochrome is again free to react with oxygen (104, 108). In the case of steroid C-21 hydroxylation by adrenal cortex microsomes (31), and for C-11β hydroxylation of deoxycorticosterone by adrenal cortex mitochondria (24), the stoichiometry of the reaction has been measured and found to be in good agreement with the above mentioned mechanisms. If the substrate only reacts with the activated P-450 complex, it is difficult to visualize substrate or position specificity. Conney et al. (29) have suggested that steroid hormones are the normally-occurring substrates for the so-called drug-metabolizing enzymes, thus implying non-specificity. Later this group (87) compared Km values for oxidative metabolism of steroid hormones and several drugs and found that the Km values for drugs were about 10 times greater than for steroids, thus reinforcing the previous argument. Thus, at the point when I had started working on this project it was far from clear whether drugs and steroids were metabolized by the same systems or

whether there were specific liver microsomal enzymes for each steroid hydroxylation. We felt it unlikely, however, that one enzyme system hydroxylated estrone in all of the positions thus far reported and began to approach our problem with this in mind.

After the experimental portion of this thesis was completed Kuntzman et al. (89) reversed their position on the specificity of the steroid metabolizing enzymes and published a paper indicating these enzymes were specific and proposed that more than one enzyme system was involved in the 6β-, 7α- and 16α-hydroxylations of testosterone. They further suggested that more than one rate-limiting component participates in the various hydroxylations of testosterone and that more than one CO-binding pigment can exist in liver microsomes.

In order to study individual reactions we first wanted to establish conditions where the rates were as close to first order as possible. In early experiments we determined that approximately 1 mg of microsomal protein incubated for less than 10 minutes with 50 to 100 µg of estrone as substrate and 4 µmoles of NADPH as co-factor constituted optimal conditions. This mixture was contained in a total volume of Krebs-Ringer phosphate buffer of 5 ml. Using this system we were able to measure 2- and 16α-hydroxyestrone formation as well as 6ξ-hydroxyestrone formation. An unknown metabolite designated Y was also observed and measured on numerous occasions. It was not always possible to quantitate this metabolite because the

amount formed varied from experiment to experiment. It is likely that the hydroxylated products, except perhaps 2-hydroxyestrone, are metabolized at a slow rate and the decreased rate of formation is due to a decrease in the amount of substrate remaining (52, 64).

We were able to examine the rate of 2-hydroxyestrone metabolism by carrying out experiments in which substrate amounts of ^{14}C-estrone and tracer amounts of ^{3}H-2-hydroxyestrone were simultaneously incubated in the same flasks. Under these conditions, similar to those prevailing in all our experiments, we noted that the observed rate of 2-hydroxyestrone formation did not decrease as much as we had expected; however, the observed rate of formation at five minutes, the earliest time interval examined, was greater than at 15 minutes (0.93 vs. 0.74 x 10^{-9} moles x min^{-1} x mg $protein^{-1}$). The calculated values were not much greater than the observed values at each time interval and were found to decrease with time, pointing out that the assumption of first-order kinetics was unjustified. The rate constant for the metabolism of 2-hydroxyestrone increased from 0.0040 to 0.0113 min^{-1} during the time interval from 5 to 15 minutes. This could possibly be due to a substrate inhibition of the breakdown of this metabolite (88). Thus, as the level of estrone was decreased by its utilization, it would exhibit less of an effect on the breakdown of 2-hydroxyestrone. When NADH was substituted for NADPH as cofactor for estrogen metabolism studies several things became immediately apparent. First the rates of 2- and 16α-hydroxylation

decreased to about one-tenth of the control values. The incorporation into 2-hydroxyestrone decreased from 188,000 dpm/mg to 22,000 and 16α-hydroxyestrone went from 98,300 to 9,800 dpm/mg protein. Estradiol-17β formation was markedly increased when NADH was added as the sole cofactor, which was the expected result (32,300 dpm/mg to 292,000 dpm/mg). It was interesting to note that the rate of 6ξ-hydroxylation was still 33 percent of the control value when NADH was the cofactor, whereas those of 2- and 16α-hydroxylation had been reduced to only 10 percent of the control value. This was one of the first clues that 6ξ-hydroxylation is probably carried out by a different system than 2- or 16α-hydroxylation. The rate of incorporation of radioactivity to water-soluble and protein-bound products was almost completely inhibited when NADH acted as cofactor. This finding supports the reports of Lazier et al. (91) that NADPH is better cofactor for ether-insoluble, water-soluble product formation than NADH, and of Hecker and Betz (57) concerning protein-bound product formation.

The effect of phenobarbital treatment was examined next. Here we found that phenobarbital treatment slightly depressed both 2- and 16α-hydroxylation of estrone in liver microsomes, while greatly stimulating the formation of Y and slightly stimulating 6ξ-hydroxylation. The decreased formation of 16α-hydroxyestrone after phenobarbital treatment is in agreement with the findings of Heinrichs and Colás (64) for dehydroepiandrosterone 16α-hydroxylase. In female

animals, phenobarbital treatment resulted in increased rates of 2-, 16α- and 6ξ-hydroxylation of estrone and had no effect on the net formation of Y. Heinrichs and Colás also report increased rates of hydroxylation for dehydroepiandrosterone after phenobarbital treatment of female rats. Phenobarbital treatment had little effect on the rate of protein-bound or ether-insoluble, water-soluble product formation in livers from male rats while greatly stimulating their formation in female rats.

We next examined the effect of buffer on the rates of estrone hydroxylation. Marks and Hecker (99) have reported that incubation in Tris buffer instead of phosphate buffer greatly stimulates the net formation of 2-hydroxyestrone while slightly inhibiting the rate of formation of 16α-hydroxyestrone. For both male and female animals we found, in contrast to these results, that all hydroxylations studied were more active in an incubation media containing 0.1 *M* phosphate buffer rather than 0.05 *M* Tris buffer. For example, the incorporation into 2-hydroxyestrone increased from 22,000 to 62,000 dpm/mg of protein when the incubation system was suspended in Krebs-Ringer phosphate buffer instead of Tris. The magnitude of the increase in 6ξ-hydroxylation was less than for either 2- or 16α-hydroxylation; for example, in female rats 16α-hydroxylation increased from 400 to 2,700 dpm/mg, while 6-hydroxylation increased only from 8,700 ng to 11,000 dpm/mg. Marks and Hecker proposed that there was an increased formation of 2-hydroxyestrone in Tris buffer because phosphate ions stimulated the

breakdown of 2-hydroxyestrone to protein-bound and water-soluble metabolites. In contrast to their findings, we found little or no differences in the amount of radioactivity incorporated into either of these fractions when the incubations were carried out in 0.05 *M* Tris instead of 0.1 *M* phosphate buffer.

Since there was a difference in the concentration of the two buffers tested we decided to increase the concentration of the Tris buffer to 0.1 molar. When the incubations were repeated using a higher concentration of Tris buffer there was only a slight difference between the various hydroxylase activities, except for 6ξ-hydroxylase activity which was doubled in the case of microsomes from male animals (31,000 in Tris buffer vs. 61,000 dpm/mg in phosphate buffer). There was a decrease in the rate of formation of protein-bound and ether-insoluble product formation when microsome incubations were carried out in phosphate buffer (for protein-bound products, for example, 5,800 in 0.1 *M* Tris buffer vs. 1,100 dpm/mg in 0.1 *M* phosphate buffer). In view of this decrease, the increase in 2-hydroxylation (1,300 in Tris vs. 6,200 dpm/mg in phosphate buffer) may be more apparent than real. The differences between our results and those of Marks and Hecker are not easy to reconcile and must be due either to differences in the preparation of microsomes or in the fact that we used a Krebs-Ringer phosphate buffer containing, in addition to phosphate, calcium, magnesium, potassium and chloride ions, whereas they used a simple phosphate buffer according to Sorensen.

As two techniques which might be able to differentiate the various hydroxylase activities, we then tried heat inactivation and partial tryptic hydrolysis. Hernandez et al. (65) have used heat inactivation to demonstrate the similarities between NADPH-cytochrome *c* reductase and azo-reductase activities and Stärka and Kůtova (123) were able to increase the yield of 7-substituted C-19 steroids during incubation of rat liver homogenate by preheating. When crude microsomes were heated at 47°C we observed a slight increase in 2- and 16α-hydroxylase activities, whereas 6ξ-hydroxylase activity decreased to 30 percent of control values after 25 minutes of heat pretreatment. After this partial success we tried to distinguish between 2- and 16α-hydroxylase activities by heating at a higher temperature, 53°C. In this experiment we measured not only 2-, 6ξ- and 16α-hydroxylase activities but also cytochrome P-450. At 53°C the rate of inactivation of the microsomal activities are not only much greater than at 47°C but there were no differences in the rate of their inactivation. However, the rate of denaturation of cytochrome P-450 was slower than the rate of inactivation of the hydroxylase systems. For example, after 10 minutes of heat pretreatment all hydroxylase activities are decreased to about 25 percent of control values, whereas about 70 percent of the cytochrome P-450 is still present.

When crude microsomes were pretreated with dilute concentrations of trypsin all hydroxylase activities fell off quite rapidly

while the amount of cytochrome P-450 remained fairly constant. It was also apparent that 2- and 16α-hydroxylase activities were inactivated at a slower rate than those for 6ξ- or Y, which is in agreement with the greater heat lability of the 6ξ-hydroxylase system. Preliminary experiments in this laboratory have also pointed out that microsomal dehydroepiandrosterone 7α-hydroxylase activity is more labile than 16α-hydroxylase activity (Colás, unpublished results). These facts would certainly imply that not all liver microsomal hydroxylase reactions are carried out by the same enzyme systems. That the rate of denaturation of cytochrome P-450 was less than the rate of inactivation of the hydroxylase activities was somewhat unexpected in view of the prominent role that has been assigned to this cytochrome as the terminal oxidase in most schemes explaining steroid hydroxylation. However, it is possible that for all hydroxylations studied the P-450 reductase(s) systems are more labile than P-450 itself.

If Kuntzman et al. (86) are correct in assuming certain steroids and drugs are alternative substrates for a common enzyme system, these steroids should inhibit the metabolism of appropriate drugs in a competitive manner or, vice versa, certain drugs should competitively inhibit specific steroid hydroxylation reactions. The findings of Tephly and Mannering (125) support this view in that they reported that estradiol-17β, testosterone, androsterone, progesterone, and hydrocortisone competitively inhibit the hepatic oxidation of

ethylmorphine and hexobarbital. Also, Colla et al. (27) have reported that the experimental drug SU-9055 inhibits the 16α-, 17α-, and 18-hydroxylations of steroids *in vitro*. Similarly Heinrichs et al. (64) have reported that SU-9055 and aminopyrine inhibits hepatic 16α-hydroxylation of dehydroepiandrosterone. Heinrichs has also reported that, under the same conditions, low concentrations of both of these drugs stimulate 7α-hydroxylation of dehydroepiandrosterone. We decided to use aminopyrine as an example of a chemical inhibitor and chose conditions similar to those of Heinrichs et al. (64). In our hands we found that aminopyrine had very little effect on either 6ξ- or 16α-hydroxylation of estrone at either of the two concentrations of estrone studied, whereas there was 40 to 50 percent stimulation of 2-hydroxylation. However, upon closer inspection the increased net production of this metabolite could almost be exclusively accounted for by decreased formation of protein-bound and water-soluble products. Thus we can say that, unlike the 16α-hydroxylation of dehydroepiandrosterone, the 16α- as well as 2- and 6ξ-hydroxylations of estrone are not significantly inhibited by aminopyrine. In addition to this we can postulate that the enzyme system responsible for the formation of 2-hydroxyestrone is different from the one which converts it to protein-bound or water-soluble products.

To my knowledge, no one has yet described the effect of the addition of a steroid on the hydroxylation of a second steroid in

liver microsomal systems. However, Sharma and Dorfman (120) have reported that testosterone, androst-4-ene-3,17-dione and dehydroepiandrosterone inhibit C-21 hydroxylation of pregnenolone by bovine adrenal microsomes. For other examples, see the review by Dorfman (37). In order to test whether a C-19 steroid could inhibit any or all of the estrone hydroxylase activities, we included different amounts of dehydroepiandrosterone in our standard estrone incubation system. Under the experimental conditions used in our laboratory dehydroepiandrosterone is principally hydroxylated at the 7α- and 16α-positions. Since estrone and DHA are both 16α-hydroxylated, and in both steroids the ring conformations near the 16 position are the same, it seems reasonable to assume that the same enzyme might hydroxylate this site in both molecules. A less likely possibility is that 6α-hydroxylation of estrone and 7α-hydroxylation of DHA are carried out by the same enzyme. This possibility is suggested by several lines of reasoning; namely that C-6 in estrone and C-7 in DHA are both adjacent to centers of unsaturation, although in estrone it is not a true double bond, and that both positions are in the same ring. However, when we consider 2-hydroxylation of estrone we find that DHA is either not hydroxylated at this position or that the rate of its formation is too slow to measure. Thus, if there is only relative site specificity DHA should inhibit estrone hydroxylation at positions 6- and 16α- and have little or no effect on 2-hydroxylation. The results obtained from these experiments suggest our hypothesis to be wrong because

all hydroxylase activities were inhibited. At the lower estrone concentration (3.7×10^{-5} *M*) 6-hydroxylation appears, however, to be inhibited more than 2-hydroxylation; for example, at a DHA concentration of 7.5×10^{-5} *M* 2-hydroxylation is inhibited only 10 percent while 6ξ-hydroxylation is inhibited approximately 35 percent. The differences in the degree of inhibition between 2- and 16α-hydroxylations are smaller and, because of the variability in the data in this experiment, are less likely to be significant. At the higher estrone concentration we were surprised to get an even greater DHA inhibition of all hydroxylase activities and the degree of inhibition was very similar for all positions. This latter finding can be possibly explained on the basis of a mixed substrate inhibition, although to the author's knowledge this type of inhibition has not been described before for liver microsomal systems. The general inhibition of all of the hydroxylase activities can perhaps be explained in one of three ways: (1) that there are different hydroxylase enzyme systems, all sharing a common component (125); for example, cytochrome P-450 or some other factor coupled in some way to a number of enzymes and acting as a cofactor; (2) a general mixed function oxidase might be responsible for all the various hydroxylase activities and that the position specificity found is a property of the steroid molecule; or (3) that a steroid may interact with some carrier protein which then aligns the steroid to the enzyme in such a manner that a certain site is favored. The last possibility mentioned is purely speculative in

nature.

Carbon monoxide inhibition studies have been useful in testing the involvement of cytochrome P-450 in various microsomal and mitochondrial oxidative reactions (30, 43, 50, 65, 89, 121, 127). Conney et al. (30, 89), for example, have shown that not all hydroxylation reactions are inhibited at the same rate by carbon monoxide; they have found that the ratios of the concentrations of carbon monoxide and oxygen required for 50 percent inhibition of 16α, 6β and 7α-hydroxylations of testosterone were, respectively, 0.88, 1.59 and 2.50. Using the same method the [CO]/[O_2] ratios required for 50 percent inhibition of 2-, 6ξ- and 16α-hydroxylation of estrone are 2.5, 0.8 and 2.5, respectively. Conney et al. suggested that his results indicated the presence of one or more cytochrome P-450s in liver microsomes involved in steroid hydroxylation. Heinrichs and Colas (64) have also reported that 7α-hydroxylation of DHA is less sensitive to carbon monoxide inhibition than 16α-hydroxylation. I have also confirmed this result in another experiment. Recently the involvement of cytochrome P-450 in several mixed function oxidase reactions has been more clearly demonstrated by use of a photochemical action spectrum technique whereby workers have been able to partially relieve the carbon monoxide inhibition by irradiating the sample with light (30). The maximum reversal of inhibition was shown in each case to occur at 450 mμ. Recently a number of workers have suggested the presence of more

than one form of cytochrome P-450 largely on the basis of spectral studies (32, 66, 67, 116, 122). An added complication is that in at least one case of hydroxylation reaction (adrenal cortex C-21 hydroxylase activity) that was thought to be P-450 dependent on the basis of light reversal carbon monoxide inhibition (43) it has later been shown that partial purification of steroid 21-hydroxylase from the same source removes all detectable cytochrome P-450 (100). This would serve to emphasize that carbon monoxide inhibition studies do not necessarily prove the involvement of cytochrome P-450.

The involvement of cytochrome P-450 in estrogen hydroxylation reactions cannot be decided upon from the results contained in this thesis. The results clearly point out that there are at least two or more enzyme systems involved in estrone hydroxylation reactions and that the enzyme system responsible for the breakdown of 2-hydroxyestrone is not the same one required for its synthesis from estrone.

V. SUMMARY AND CONCLUSIONS

1. No inverse relationship between estrone 2- and 16α-hydroxylase activities was demonstrated which could be controlled by the level of thyroid activity.
2. Thyroxine treatment resulted in decreased 2- and 16α-hydroxyestrone formation by rat liver slices but no significant changes in activity were demonstrated using the 10,000 x *g* supernatant fraction. Thyroidectomy depressed both hydroxylase activities regardless of the preparation used. Thyroxine treatment of thyroidectomized animals had no effect on the hydroxylase activities studied.
3. Kinetic measurements of the rate of ether-insoluble, water-soluble product formation revealed that thyroxine treatment decreased the V_{max} for the overall series of reactions while not affecting the apparent Km.
4. Thyroxine treatment slightly increased the V_{max} for protein-bound metabolite formation while thyroidectomy decreased it. There was no significant effect on the apparent Km, however, with either treatment.
5. Phenobarbital pretreatment was shown to increase the rates of microsomal 2- and 16α-hydroxylation of estrone in female rats, while slightly inhibiting both reactions in male rats. In both

male and female rats 6-hydroxylation was slightly stimulated by phenobarbital pretreatment.

6. Incubation in Tris buffer instead of phosphate buffer had little effect on the hydroxylase activities in contrast to a report in the literature. The rate of formation of ether-insoluble, water-soluble and protein-bound metabolites was stimulated by incubating in Tris buffer instead of phosphate buffer in female rats.
7. A sex difference was demonstrated for all hydroxylase activities, liver tissue from male rats being more active than from female rats.
8. There was no decrease in the rates of 2- and 16α-hydroxylations of estrone when microsomes were heat-treated at 47°C prior to incubation, whereas the rate of 6ξ-hydroxylation rapidly declined. With preheating at 53°C all three activities decreased at similar rates, whereas cytochrome P-450 was inactivated at a slower rate.
9. Pretreatment of microsomes with trypsin resulted in parallel decreases in 2- and 16α-hydroxylation and more rapid declines in 6ξ-hydroxylation and the formation of Y under conditions where cytochrome P-450 was not appreciably inactivated.
10. (CO/O_2) ratios required for 50 percent inhibition of 2- and 16α-hydroxylation were the same (2.5), whereas the ratio for 50 percent inhibition of 6ξ- was only 0.8. These results are compared in the discussion to those reported for testosterone hydroxylations.
11. Aminopyrine was shown to have little or no effect on 6ξ- or 16α-hydroxylation rates, whereas the net rate for 2-hydroxylation was

increased. This increase could be largely accounted for by decreased rates of protein-bound and ether-insoluble, water-soluble product formation.

12. Dehydroepiandrosterone was shown to be a nonspecific inhibitor for all of the hydroxylase activities studied. At the higher estrone concentration (7.4×10^{-5} *M*) DHA was shown to be a more effective inhibitor than at the lower estrone concentration (3.7×10^{-5} *M*).

13. These data emphasize a number of differences between 2- and 16α- and 6ξ-hydroxylase activities and between all of these hydroxylase activities and the mixed function oxidase activity responsible for the conversion of estrone to protein-bound and ether-insoluble, water-soluble products.

REFERENCES

1. Alvares, A. P., and Mannering, G. T. Effects of thyroxine on phenobarbital and methylcholanthrene induction of hepatic microsomal enzyme systems. Fed. Proceedings, 1967, 26, 1146. (Abstract)

2. Balant, C. P., und Ehrenstein, M. Investigations on steroids. XX. 6β- and 6α-acetoxy and hydroxy derivatives of progesterone and androstenedione. J. Org. Chem., 1952, 17, 1587-1596.

3. Beer, C. T., and Gallagher, T. F. Excretion of estrogen metabolites by humans. I. The fate of small doses of estrone and estradiol-17β. J. Biol. Chem., 1955, 214, 335-349.

4. Beer, C. T., and Gallagher, T. F. Excretion of estrogen metabolites by humans. II. Fate of large doses of estradiol-17β after intramuscular and oral administration. J. Biol. Chem., 1955, 214, 351-364.

5. Biggerstaff, W. R., and Gallagher, T. F. 3,16β-Dihydroxy-$\Delta^{1,3,5}$-estratrien-17-one and related compounds. J. Org. Chem., 1957, 22, 1220-1222.

6. Bradlow, H. L., Hellman, L., Zumoff, B., and Gallagher, T. F. Interaction of hormonal effects: Influence of triiodothyronine on androgen metabolism. Science, 1956, 124, 1206-1207.

7. Bradlow, H. L., Fukushima, D. K., Zumoff, B., Hellman, L., and Gallagher, T. F. Influence of thyroid hormone on progesterone transformation in man. J. Clin. Endocrinol., 1966, 26, 831-834.

8. Breuer, H., and Nocke, L. Formation of oestriol-13,16β,17α by liver tissue *in vitro*. Biochim. Biophys. Acta, 1959, 36, 271-272.

9. Breuer, H., Nocke, L., and Knuppen, R. Stoffwechsel der Östrogene in Leberschnitten der Ratte. Zeit. Physiol. Chem., 1959, 315, 72-79.

10. Breuer, H., and Knuppen, R. Isolation of 6-hydroxyestradiol-17β and oestriol after incubation of oestradiol 17β with rat liver slices. Biochim. Biophys. Acta, 1960, 39, 408-411.

11. Breuer, H., Knuppen, R., Ortlepp, R., Pangels, G., and Puck, A. Biogenesis of 6-hydroxylated oestrogens in human tissues. Biochim. Biophys. Acta, 1960, 40, 560.

12. Breuer, H., Knuppen, R., and Schriefers, H. Biogenese von 6-hydroxy-östriol und 2-methoxy-östriol in leberschnitten der ratte. Zeit. Physiol. Chem., 1960, 319, 136-142.

13. Breuer, H., Knuppen, R., and Pangels, G. Biogenese und Zwischenstoffwechsel 6-substituierter östrogene beim Menschen. Zeit. Physiol. Chem., 1960, 321, 57-64.

14. Breuer, H. The metabolism of natural estrogens. Vitamins and Hormones, 1962, 20, 285-335.

15. Breuer, H., Knuppen, R., und Pangels, G. Konfiguration, biogenese und stoffwechsel von 6α- und 6β-hydroxylierten phenolischen steroiden. Biochim. Biophys. Acta, 1962, 65, 1-12.

16. Breuer, H., Knuppen, R., and Haupt, M. Metabolism of oestrone and oestradiol-17β in human liver in vitro. Nature, 1966, 212, 76.

17. Breuer, J., Breuer, F., Breuer, H., und Knuppen, R. Isolierung von 6-hydroxyöstriol aus dem Urin schwangerer Frauen. Zeits. für Physiol. Chem., 1966, 346, 279-289.

18. Brown, J. B. The relationship between urinary oestrogens and oestrogens produced in the body. J. Endocrinol., 1957, 16, 202-213.

19. Brown, H., Englert, E., and Wallach, S. Metabolism of free and conjugated 17-hydroxy-corticosteroids in subjects with thyroid disease. J. Clin. Endocrinol. Metab., 1965, 28, 167-179.

20. Burstein, S., and Fajer, A. B. Effect of thyroxine on urinary corticosteroid patterns in guinea pigs. Endocrinol., 1965, 77, 361-365.

21. Burstein, S. Genetic aspects of cortisol metabolism. Proceedings of the Second International Congress on Hormonal Steroids, Excerpta Medica Foundation, Amsterdam: 1967, 437-446.

22. Burstein, S., and Bhavnani, B. R. Effect of phenobarbital administration on the in vitro hydroxylation of cortisol and on overall substrate and product metabolism in the guinea pig and rat. Endocrinol., 1967, 80, 351-356.

23. Bush, I. E. The chromatography of steroids. New York: The Macmillan Company, 1961.

24. Cammer, W., and Estabrook, R. W. Energy linked reactions for steroid hydroxylations by adrenal mitochondria. Fed. Proceedings, 1966, 25, 517. (Abstract)

25. Cedard, L., and Knuppen, R. 6α-Hydroxylation in human placenta perfusion. Steroids, 1965, 6, 307-312.

26. Colás, A. The 16α-hydroxylation of dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) by rat liver slices. Biochem. J., 1962, 82, 390-394.

27. Colla, J. C., Liberti, J. P., and Ungar, F. Inhibition of 16α-hydroxylation in human testis tissue by SU-9055. Steroids, 1966, 8, 25-32.

28. Conney, A. H., and Schneidman, K. Decreased hypnotic action of progesterone and other steroids in rats pretreated with drugs that stimulate steroid metabolism. Fed. Proc., 1965, 24, 152. (Abstract)

29. Conney, A. H., Schneidman, K., Jacobson, M., and Kuntzman, R. Drug-induced changes in steroid metabolism. Ann. N. Y. Acad. Sci., 1965, 123, 98-109.

30. Conney, A. H., Ikeda, M., Levin, W., Cooper, D., Rosenthal, O., and Estabrook, R. Carbon monoxide inhibition of steroid hydroxylation in rat liver microsomes. Fed. Proceedings, 1967, 26, 1148. (Abstract)

31. Cooper, D. Y., Estabrook, R. W., and Rosenthal, O. The stoichiometry of C_{21} hydroxylation of steroids by adrenocortical microsomes. J. Biol. Chem., 1963, 238, 1320-1323.

32. Cooper, D. Y., Narasimhulu, S., Rosenthal, O., and Estabrook, R. W. Spectral and kinetic studies of microsomal pigments. In T. King, H. S. Mason, and M. Morrison (Ed.), Oxidases and Related Redox Systems, New York, N. Y.: John Wiley and Sons, 1965, pp. 838-860.

33. Cooper, D. Y., Levin, S., Narasimhulu, S., Rosenthal, O., and Estabrook, R. W. Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. Science, 1965, 147, 400-402.

34. Corker, C. S., Norymberski, J. K., and Thow, R. Some aspects of the zimmerman reaction. Biochem. J., 1962, 83, 583-587.

35. Dallner, G. Studies on the structural and enzyme organization of the membranous elements of liver microsomes. Acta Path. et Microbiol. Scand., 1963, Suppl. No. 166, 1-94.

36. Diczfalusy, E., and Lauritzen, C. Oestrogene Beim Menschen. Berlin: Springer-Verlag, 1961, pp. 203-210.

37. Dorfman, R. I. Control of steroid hormone biosynthesis. Proceedings of the VIth Pan-American Congress of Endocrinology 1965, 23-31.

37a Dorfman, R. I., and Ungar, F. Metabolism of Steroid Hormones. New York: Academic Press, 1965.

38. Engel, L. L. The biosynthesis of estrogens. Cancer, 1957, 10, 711-715.

39. Engel, L. L., Baggett, B., and Carter, P. In vivo metabolism of estradiol 17β-16-^{14}C in the human being: Isolation of 2-methoxyestrone-^{14}C. Endocrinol., 1957, 61, 113-114.

40. Engel, L. L., Baggett, B., and Halla, M. The formation of ^{14}C-labeled estriol from 16-^{14}C-estradiol-17β by human fetal liver slices. Biochim. Biophys. Acta, 1958, 30, 435-436.

41. Engel, L. L., Cameron, C. B., Stoffyn, A., Alexander, J. A., Klein, O., and Trofimaw, N. D. The estimation of urinary metabolites of administered estradiol-17β-16-C^{14}. Anal. Biochem., 1961, 2, 114-125.

42. Engel, L. L., Baggett, B., and Halla, M. In vitro metabolism of estradiol-17β by human fetal liver: Formation of estriol, 16-epiestriol, estrone and estriol glucosiduronic acid. Endocrinol., 1962, 70, 907-914.

43. Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. The light reversible carbon monoxide inhibition of the steroid C^{21}-hydroxylase system of the adrenal cortex. Biochem. Zeit., 1963, 338, 741-755.

44. Fishman, J., Cox, R. I., and Gallagher, T. F. 2-Hydroxyestrone: A new metabolite of estradiol in man. Arch. Biochem. Biophys., 1960, 90, 318-319.

45. Fishman, J., Bradlow, H. L., and Gallagher, T. F. Oxidative metabolism of estradiol. J. Biol. Chem., 1960, 235, 3104-3107.

46. Fishman, J., Hellman, L., Zumoff, B., and Gallagher, T. F. Influence of thyroid hormone on estrogen metabolism in man. J. Endocrinol. Metab., 1962, 22, 389-392.

47. Fishman, J., Hellman, L., Zumoff, B., and Gallagher, T. F. Effect of thyroid on hydroxylation of estrogen in man. J. Clin. Endocrinol., 1965, 25, 365-368.

48. Gornall, A. G., Bardawill, C. J., and David, M. M. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., 149, 177, 751-766.

49. Graubard, M., and Pincus, G. The oxidation of estrogens by phenolases. Proc. Natl. Acad. Sci., 1941, 27, 149-152.

50. Greengard, P., Psychoyos, S., Tallan, H. H., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W. Aldosterone synthesis by adrenal mitochondria. III. Participation of cytochrome P-450. Arch. Biochem. Biophys., 1967, 121, 298-303.

51. Hagopian, M., and Levy, L. K. The conversion of 16-^{14}C-17β-estradiol to estriol by isolated rat livers. Biochim. Biophys. Acta, 1958, 30, 641.

52. Hall, P. E., and Fotherby, K. Metabolism *in vitro* of 6-hydroxyprogesterone. Biochem. J., 1967, 104, 23P.

53. Hecker, E., and Zayed, S. M. A. D. 17β-Hydroxyestra-p-quinol-(10β) as a metabolite of 17β-estradiol in vitro. Biochem. Biophys. Acta, 1961, 50, 607-608.

54. Hecker, E., and Zayed, S. M. A. D. Bildung von 17β-Hydroxy-östra-p-chinol-(10β) und ihre Beziehung zur Proteinbindung von östradiol-(17β)-[16-^{14}C] in Rattenlebermikrosomen. Zeit. Physiol. Chem., 1961, 325, 207-223.

55. Hecker, E., and Zayed, S. M. A. D. Metabolism and action of estrogen. II. Formation of 17β-hydroxyestra-p-quinol-(10β) and its relation to protein formation from 17β-estradiol-16-C^{14} in rat liver microsomes. Hoppe-Seyler's Zeit. Physiol. Chem., 1961, 325, 209-223.

56. Hecker, E., and Marks, F. 17-Oxo-oestra-p-chinol-(10β), ein neuer Metabolit von Oestron-(16-^{14}C) in vitro. Naturwissenschaften, 1963, 50, 1-3.

57. Hecker, E., and Betz, D. Zum Stoffwechsel und Wirkungsmechanismus der östrogene V. Activität des mikrosomalen, Ostradiol-(17β) inaktivierenden Enzymsystems aus Rattenleber unter verschiedeneu Versuchsbedingungen. Zeit. Physiol. Chem., 1964, 338, 260-271.

58. Hecker, E., and Marks, F. Zum Stoffwechsel und Wirkungsmechanismus der Oestrogene VII. Die o-Hydroxylierung von Oestron in Rattenleber und ihre Beziehung zur Proteinbindung sowie zur p-Hydroxylierung und zu Hydroxylierung in aliphatischen Positionen. Biochem. Zeit., 1965, 343, 211-226.

59. Hecker, E., Walter G., and Marks, F. Zur Bildung wasserlöslicher Metaboliten aus Oestron in Rattenlebermikrosomen. Biochim. Biophys. Acta, 1965, 111, 546-548.

60. Hecker, E., and Marks, F. Zum Stoffwechsel und Wirkungsmechanismus der östrogene. VI. 10β-Hydroxylierung und Proteinbindung von Oestron in Rattenlebermikrosomen. Zeit. Physiol. Chem., 1965, 340, 229-242.

61. Heinrichs, W. L., Feder, H. H., and Colás, A. The steroid 16α-hydroxylase system in mammalian liver. Steroids, 1966, 7, 91-98.

62. Heinrichs, W. L. Hydroxylations of dehydroepiandrosterone by microsomal fractions from mammalian liver. Doctoral thesis, 1967, University of Oregon Medical School.

63. Heinrichs, W. L., Mushen, R. L., and Colás, A. The 17β-hydroxylation of 3β-hydroxyandrost-5-en-17-one by hepatic microsomes. Steroids, 1967, 9, 23-40.

64. Heinrichs, W. L., and Colás, A. The selective stimulation, inhibition and physiochemical alteration of the 7-, and 16α-hydroxylases of 3β-hydroxyandrost-5-en-17-one and drug metabolizing enzymes in hepatic microsomal fractions. Biochem. "In Press".

65. Hernandez, P. H., Gillette, J. R., and Mazel, P. Studies on the mechanism of action of mammalian hepatic azoreductase. I. Azoreductase activity of reduced nicotinamide adenine dinucleotide phosphate cytochrome c reductase.

66. Imai, Y., and Sato, R. Evidence for two forms of P-450 hemoprotein in microsomal membranes. Biochem. Biophys. Res. Comm., 1966, 23, 5-11.

67. Imai, Y., and Sato, R. Studies on the substrate interactions with P-450 in drug hydroxylation by liver microsomes. J. Biochem., 1967, 62, 239-249.

68. Imai, Y., and Sato, R. Anomalous spectral interactions of reduced P-450 with ethyl isocyanide and some other ligands. J. Biochem., 1967, 62, 464-473.

69. Jellinck, P. H. The enzymatic oxidation of [16-^{14}C] oestrone *in vitro*. Biochim. Biophys. Acta, 1960, 41, 37-45.

70. Jellinck, P. H., Lazier, C., and Copp, M. L. Nature of the water-soluble estrogen metabolites formed by rat liver in vitro. Can. J. Biochem., 1965, 43, 1774-1776.

71. Jellinck, P. H., Lewis, J., and Boston, F. Further evidence for the formation of an estrogen-peptide conjugate by rat liver in vitro. Steroids, 1967, 10, 329-345.

72. Jirku, H., Hogsander, U., Levitz, M. 15α-Hydroxyestrone "sulfate". A biliary metabolite of estrone sulfate in the non-pregnant female. Biochim. Biophys. Acta, 1967, 137, 588-591.

73. Kato, R., and Takahashi, A. Thyroid hormone and activities of drug-metabolizing enzymes and electron transport systems of rat liver microsomes. Mol. Pharmacol., 1968, 4, 109-120.

74. King, R. J. B. Biosynthesis of 2-methoxyestriol by rat liver preparations. Biochem. J., 1960, 74, 22P.

75. King, R. J. B. Oestriol metabolism by rat- and rabbit-liver slices. Isolation of 2-methoxyoestriol and 2-hydroxyoestriol. Biochem. J., 1961, 79, 355-361.

76. King, R. J. B. Metabolism of oestriol *in vitro*: cofactor requirements for the formation of 2-hydroxyoestriol and 2-methoxyestriol. Biochem. J., 1961, 79, 361-369.

77. Klingenberg, M. Pigments of rat liver microsomes. Arch. Biochem. Biophys., 1958, 75, 376-386.

78. Knuppen, R., and Breuer, H. Preparation of 6-hydroxyestrone. Liebigs Ann., 1961, 639, 194-19[illegible].

79. Knuppen, R. Papierchromatographie phenolischen steroide. Z. Vitamin Hormon. Fermentforsch., 1963, 12, 355-401.

80. Knuppen, R., Behm, M., and Breuer, H. Biogenese von 6α-Hydroxyöstron in der Nebenniere. Zeit. für Physiol. Chem., 1964, 337, 145-149.

81. Knuppen, R., Haupt, O., and Breuer, H. The isolation of 6α-hydroxyoestrone from urine of pregnant women. Biochem. J., 1966, 101, 397-401.

82. Koerner, D. R., and Hellman, L. Effect of thyroxine administration on the 11β-hydroxysteroid dehydrogenases in rat liver and kidney. Endocrinol., 1964, 75, 592-601.

83. Kornel, L. A new method of elution of conjugated steroids from paper strips. Anal. Chem., 1964, 36, 443-444.

84. Kraychy, S., and Gallagher, T. F. 2-Methoxyestrone, a metabolite of estradiol-17β in the human. J. Am. Chem. Soc., 1957, 79, 754.

85. Kraychy, S., and Gallagher, T. F. 2-Methoxyestrone, a new metabolite of estradiol-17β in man. J. Biol. Chem., 1957, 229, 519-526.

86. Kuntzman, R., Jacobson, M., Schneidman, K., and Conney, A. H. Similarities between oxidative drug-metabolizing enzymes and steroid hydroxylases in liver microsomes. J. Pharm. and Exp. Therap., 1964, 146, 280-285.

87. Kuntzman, R., Lawrence, D., and Conney, A. H. Michaelis constants for the hydroxylation of steroid hormones and drugs by rat liver microsomes. Mol. Pharmacol., 1965, 1, 163-167.

88. Kuntzman, R., and Jacobson, M. Testosterone inhibition of 6β-, 7α- and 16α-hydroxytestosterone metabolism. Fed. Proceedings, 1967, 26, 1147. (Abstract)

89. Kuntzman, R., Levin, W., Jacobson, M., Conney, A. H. Studies on microsomal hydroxylation and the demonstration of a new carbon monoxide binding pigment in liver microsomes. Life Sciences, 1968, 7, 215-224.

90. Langer, L. J., Alexander, J. A., and Engel, L. L. Human placental estradiol-17β dehydrogenase. J. Biol. Chem., 1959, 234, 2609-2614.

91. Lazier, C., and Jellinck, P. H. Inhibition of ^{14}C-estrone metabolism in rat liver microsomes by 2-hydroxyestrogens and related compounds. Can. J. Biochem., 1965, 43, 281-290.

92. Leeds, N. S., Fushima, D. K., and Gallagher, T. F. Studies of steroid ring D epoxides of enol acetates; a new synthesis of estriol and of androstane-3β,16α,17β-triol. J. Am. Chem. Soc., 1954, 76, 2943-2948.

93. Levin, M. E., and Daughaday, W. H. The influence of the thyroid on adrenocortical function. J. Clin. Endocrinol. and Met., 1955, 15, 1499-1511.

94. Levitz, M., Spitzer, J. R., and Twombly, G. H. The conversion of estradiol 17β-16-^{14}C to radioactive 16-ketoestradiol-17β in man. J. Biol. Chem., 1956, 222, 981-990.

95. Lisboa, B. P., and Diczfalusy, E. Separation and characterization of steroid oestrogens by means of thin layer chromatography. Acta Endocrinol., 1962, 40, 60-81.

96. Longwell, B., and Wintersteiner, O. Estrogens with oxygen in Ring B III. 6-Keto-α-estradiol. J. Biol. Chem., 1940, 133, 219-229.

97. Lucis, O. J., and Hobkirk, R. Formation of labelled 2-methoxy-estrone by rat liver subcellular fractions. Steroids, 1963, 2, 669-686.

98. McGuire, J. S., and Tomkins, G. M. Effect of thyroxine administration on the rate and steric course of enzymatic reduction of steroids. Nature, 1958, 182, 261-262.

99. Marks, Friedrich, and Hecker, E. Zum Stoffwechsel und Wirkungsmechanismus der Östrogene, VIII. Biogenese und Stoffwechsel von 2-Hydroxy-Östron in Beziehung zur Bildung Proteingebundener und wasserlöslicher Östronmetaboliten und zur NADPH-Oxydation in Rattenlebermikrosomen. Zeit. fur Physiol. Chem., 1966, 345, 22-40.

100. Matthijssen, C., and Mandel, J. E. Preparation of an adrenal steroid 21-hydroxylating system exhibiting activity in the absence of the carbon monoxide-binding pigment, cytochrome P-450. Biochim. Biophys. Acta, 1967, 146, 613-614.

101. Mitropoulos, K. A., and Myant, N. B. The metabolism of cholesterol in the presence of liver mitochondria from normal and thyroxine-treated rats. Biochem. J., 1965, 94, 594-603.

102. Moury, D. N., and Crane, F. L. Quantitative study of the effects of thyroxine on components of the electron-transfer system. Biochem., 1964, 3, 1068-1072.

103. Mueller, G. C., and Rumney, G. Formation of 6β-hydroxy and 6-keto derivatives of estradiol-16-^{14}C by mouse liver microsomes. J. Am. Chem. Soc., 1957, 79, 1004-1005.

104. Nakamura, Y., Otsuka, H., and Tamaoki, B. Requirement of a new flavoprotein and a non-heme iron containing protein in the steroid 11β- and 18-hydroxylase system. Biochim. Biophys. Acta, 1966, 122, 34-42.

105. Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. Spectrophotometric properties of a triton-clarified steroid 21-hydroxylase system of adrenocortical microsomes. Life Sciences, 1965, 4, 2101-2107.

106. Okita, G. T., Kabhra, J. J., Richardson, F., and LeRoy, G. V. Assaying compounds containing ^{3}H and ^{14}C. Nucleonics, 1957, 15, 111-114.

107. Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., 1964, 239, 2370-2378.

108. Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. J. Biol. Chem., 1964, 2379-2385.

109. Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. Isolation from adrenal cortex of a nonheme iron protein and a flavoprotein functional as a reduced triphosphopyridine nucleotide-cytochrome P-450 reductase. Arch. Biochem. Biophys., 1966, 117, 660-673.

110. Pangels, G., and Breuer, H. Cellular location of 17β-estradiol-16α-hydroxylase. Naturwissenschaften, 1962, 49, 106-107.

111. Pearlman, W. H., and Pincus, G. Conversion of estrone to estriol in vivo. J. Biol. Chem., 1942, 144, 569-570.

112. Peterson, R. E. The influence of the thyroid on adrenal cortical function. J. Clin. Invest., 1958, 37, 736-743.

113. Peterson, R. E. The miscible pool and turnover rate of adrenocortical steroids in man. Recent Progress in Hormone Research. G. Pincus (Ed.), Academic Press, New York: 1959, Vol. 15, 231-261.

114. Raw, I., and DaSilva, A. A. Effect of thyroxine on the level of cytochrome b5 and P-450. Exper. Cell Res., 1965, 40, 677-678.

115. Remmer, H., and Merker, H. J. Effect of drugs on the formation of smooth endoplasmic reticulum and drug-metabolizing enzymes. Ann. N. Y. Acad. Sci., 1965, 123, 79-97.

116. Remmer, H., Schenkman, J., Estabrook, R., Sasame, H., Gillette, J., Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. Drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol., 1966, 2, 187-190.

117. Riegel, I. L., and Mueller, G. C. Formation of protein bound metabolite of estradiol-16-C^{14} by rat liver homogenates. J. Biol. Chem., 1954, 210, 249-257.

118. Ryan, K., and Engel, L. Hydroxylation of steroids at carbon 21. J. Biol. Chem., 1957, 225, 103-114.

119. Schwers, J., Eriksson, G., Wiqvist, N., and Diczfalusy, E. 15α-Hydroxylation: A new pathway of estrogen metabolism in the human fetus and newborn. Biochim. Biophys. Acta, 1965, 100, 313-316.

120. Sharma, D. C., and Dorfman, R. I. Effects of androgens on steroid C-21 hydroxylation. Biochem., 1964, 3, 1093-1097.

121. Silverman, D. A., and Talalay, P. Studies on the enzymatic hydroxylation of 3,4-benzpyrene. Mol. Pharmacol., 1967, 3, 90-101.

122. Sladek, N. E., and Mannering, G. J. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Comm., 1966, 24, 668-673.

123. Stârka, L., and Kutovâ, J. 7-Hydroxylation of dehydroepiandrosterone by rat-liver homogenates. Biochim. Biophys. Acta, 1962, 56, 76-82.

124. Suzuki, M., Imai, K., Ito, A., Omura, T., and Sato, R. Effects of thyroidectomy and triiodothyronine administration on oxidative enzymes in rat liver microsomes. J. Biochem., 1967, 62, 447-455.

125. Tephly, T. R., and Mannering, G. J. Inhibition of drug metabolism. V. Inhibition of drug metabolism by steroids. Mol. Pharmacol., 1968, 4, 10-14.

126. Valcourt, A. J., Thayer, S. A., Doisy, E. A., Jr., Elliott, W. H., and Doisy, E. A. The metabolism of radioestrone in the rat. Endocrinol., 1955, 57, 692-696.

127. Wada, F., Hirata, K., Shibata, H., Higashi, K., and Sakamoto, Y. Involvement of P-450 in several reactions of lipid metabolism in liver microsomes. J. Biochem., 1967, 62, 134-135.

128. Westerfeld, W. W. The inactivation of oestrone. Biochem. J., 1940, 34, 51-58.

129. Williams, G. R. Unpublished results.

130. Wilson, H., and Fairbanks, R. A rapid micromethod for the determination of 17-hydroxy- and 17-ketosteroids. Arch. Biochem. Biophys., 1955, 54, 440-456.

131. Wintersteiner, O., and Moore, M. The epimeric 6-hydroxy-3,17 - estradiols. J. Amer. Chem. Soc., 1959, 81, 442-443.

132. Zondek, B. Über das Schicksal des Follikelhormons (Follikulin) im Organismus. Skand. Arch. Physiol., 1934, 70, 133-167.

Appendix

Figure No. 18. Infrared spectra of A: 16α-hydroxyestrone synthesized by the author. B: 16α-hydroxyestrone purchased from IKAPHARM, Ramat-Gan, Israel. The steroids were mixed with KI for the preparation of the pellets.

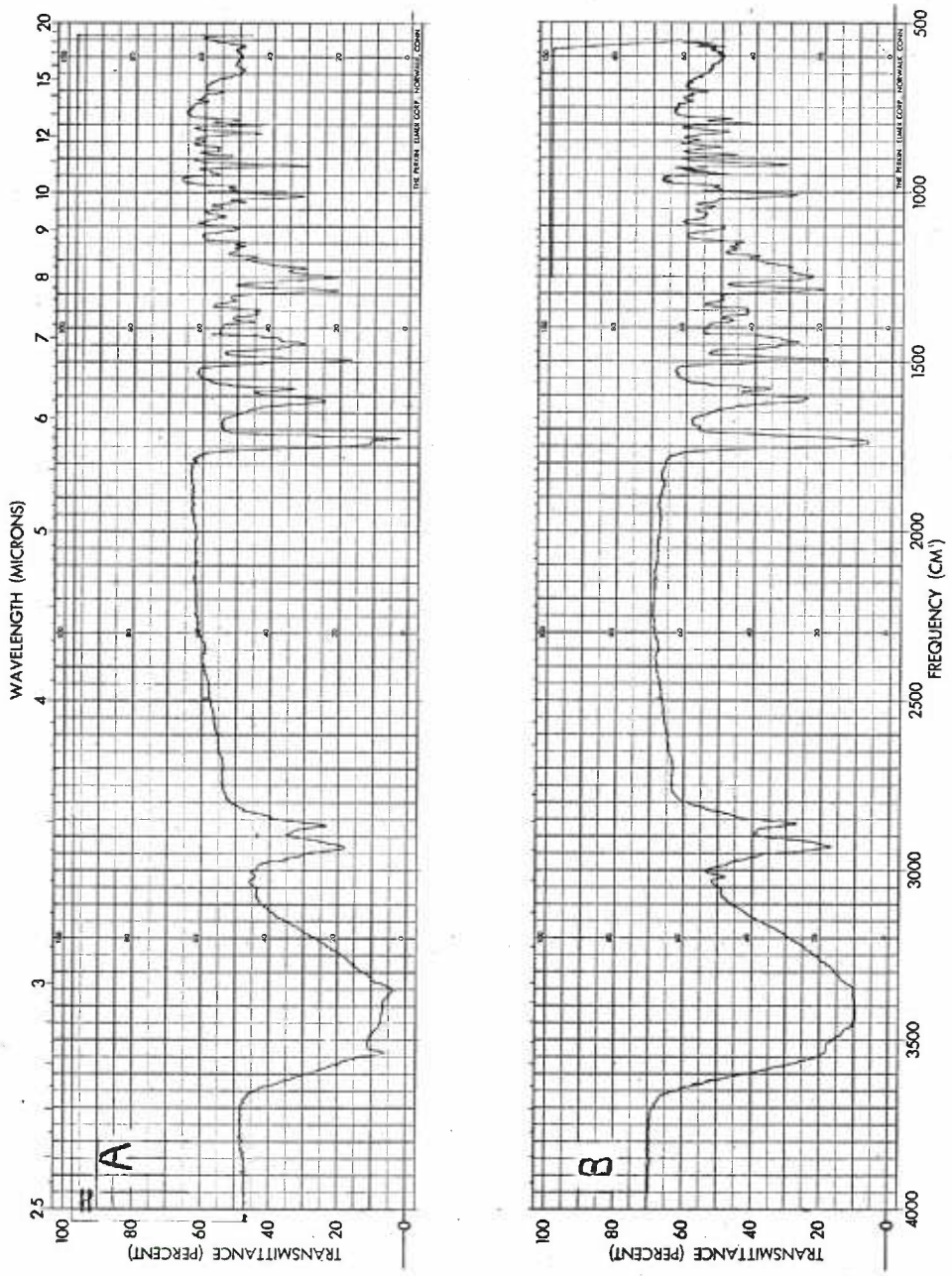
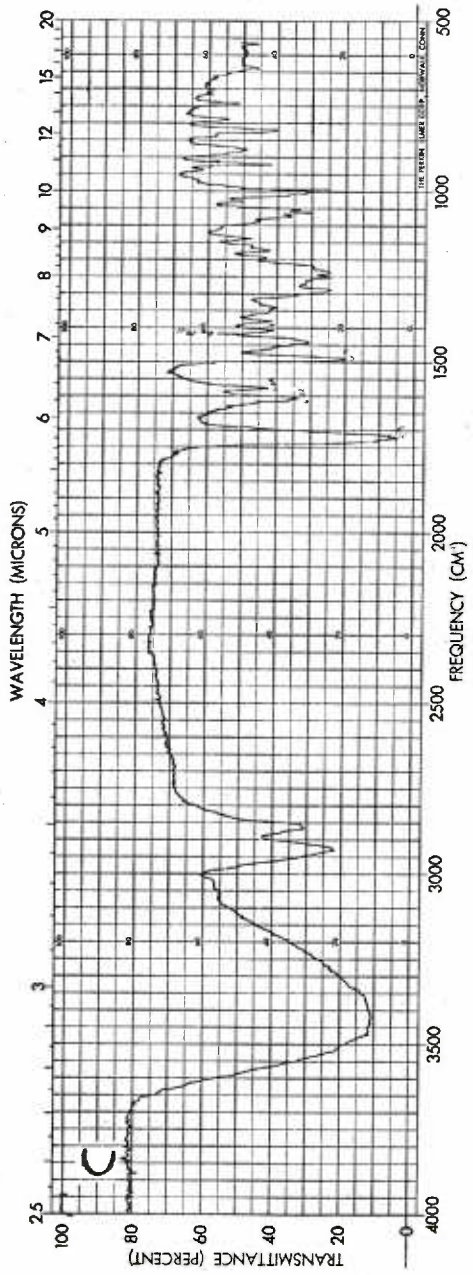

A
WAVELENGTH (MICRONS)
TRANSMITTANCE (PERCENT)
B
FREQUENCY (CM⁻¹)
TRANSMITTANCE (PERCENT)

Figure No. 19. C: Infrared spectra of 6α-hydroxyestrone in KI pellet.


WAVELENGTH (MICRONS)
FREQUENCY (CM⁻¹)
TRANSMITTANCE (PERCENT)
C