HYDROXYLATIONS OF DEHYDROEPIANDROSTERONE BY MICROSOMAL FRACTIONS FROM MAMMALIAN LIVER

by

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To Phyllis, Stephen, and Lynn

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TRIVIAL AND SYSTEMATIC NAMES

3β-OH-Δ^5-steroids - 3β-hydroxy-Δ^5-steroids

cholesterol - cholest-5-en-3β-ol

pregnenolone - 3β-hydroxypregn-5-en-20-one
16α-OH-pregnenolone - 3β,16α-dihydroxypregn-5-en-20-one

progesterone - pregn-4-ene-3,17-dione
16α-OH-progesterone - 16α-hydroxypregn-4-ene-3,17-dione

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

DHA, dehydroepiandrosterone - 3β-hydroxyandrost-5-en-17-one
7α-OH-DHA - 3β,7α-dihydroxyandrost-5-en-17-one
7β-OH-DHA - 3β,7β-dihydroxyandrost-5-en-17-one
7-keto-DHA - 3β-hydroxyandrost-5-ene-7,17-dione
16α-OH-DHA - 3β,16α-dihydroxyandrost-5-en-17-one
androstenetriol - androst-5-ene-3β,16α,17β-triol

androstenedione - androst-4-ene-3,17-dione
2β-OH-androstenedione - 2β-hydroxyandrost-4-ene-3,17-dione
6β-OH-androstenedione - 6β-hydroxyandrost-4-ene-3,17-dione
7α-OH-androstenedione - 7α-hydroxyandrost-4-ene-3,17-dione
11β-OH-androstenedione - 11β-hydroxyandrost-4-ene-3,17-dione
16α-OH-androstenedione - 16α-hydroxyandrost-4-ene-3,17-dione

testosterone - 17β-hydroxyandrost-4-en-3-one
2β-OH-testosterone - 2β,17β-dihydroxyandrost-4-en-3-one
6β-OH-testosterone - 6β,17β-dihydroxyandrost-4-en-3-one
7α-OH-testosterone - 7α,17β-dihydroxyandrost-4-en-3-one
16α-OH-testosterone - 16α,17β-dihydroxyandrost-4-en-3-one

androsterone - 3α-hydroxy-5α-androstan-17-one
etiocholanolone - 3α-hydroxy-5β-androstan-17-one

estrone - 3-hydroxyestra-1,3,5(10)-trien-17-one
2-OH-estrone - 2,3-dihydroxyestra-1,3,5(10)-trien-17-one
16α-OH-estrone - 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one

estradiol - estra-1,3,5(10)-triene-3,17β-diol
2-OH-estradiol - estra-1,3,5(10)-triene-2,3,17β-triol
16α-OH-estradiol, estriol - estra-1,3,5(10)-triene-3,16α,17β-triol

TABLE OF CONTENTS

LIST OF TABLES AND ILLUSTRATIONS

DISCUSSION

I. INTRODUCTION

A. Statement of the problem.

The enzymatic hydroxylation of steroid hormones is an important transformation in the regulation of a variety of metabolic processes. The biological activities of hydroxylated steroids are dependent upon their stereospecific configurations even as the characteristic hydroxylations in biological systems are likewise stereospecific and often occur in a stepwise fashion. To define the mechanism(s) of hydroxylation or the physiologic controls of such systems, one would ideally resort to *in vitro* preparations of purified enzymes characterized by a high specific activity. Most attempts to solubilize and purify microsomal steroid hydroxylase systems, and other enzymes which are tightly-bound to membranes, have resulted in a failure to preserve their enzymatic activities. Alternatively, a concentration of the intact membranes containing a large number of oxidative enzyme systems, including steroid hydroxylases, has been achieved by mechanical means (selective separations by ultracentrifugation) or by physiological means (enzyme induction by drug treatments). As a result of the limited concentration that has been possible, one can ascribe to the hepatic endoplasmic reticulum, and especially to the agranular subfraction, the properties and functions of performing oxido-reductions on a wide variety of drug and steroid substrates. Because of the stimulation of so many enzyme systems by some of the drugs which had been administered, a low enzyme specificity for drug and steroid substrates has been postulated.

This study was directed toward the mechanism and physiologic controls of the steroid hydroxylase system(s) from mammalian liver. Biological and biochemical aspects of 7-, and 16α-hydroxylation of dehydroepiandrosterone (DHA) were compared with those of two representative oxidative enzymatic activities, *p*-hydroxylation of acetanilide and *N*-demethylation of aminopyrine, in an effort to explore the interrelationships which might exist between steroid hydroxylase systems and drug-metabolizing enzymes. The isolation and identification of a new metabolite of DHA in microsomal systems was fortunate in that it permitted a wider comparison than was contemplated at the beginning of this work.

B. Biosynthesis of estriol in the human fetoplacental unit.

The search for the metabolic precursors of estriol (the most abundant 16α-hydroxylated estrogen known as a urinary product of the human fetoplacental unit) which lasted for 3 decades, was successful in the past five years when certain neutral 16α-hydroxylated C_{19}-steroids were found to fulfill a major part of that role (32,88). Quantitation of DHA and 16α-OH-DHA, mostly as the sulfate-esters, in the fetal circulation at term gestation demonstrated over 150 µg./ml. of plasma of both compounds. In addition to their large concentrations, a significant arterial-venous difference existed signifying the production of DHA and 16α-hydroxylation by the fetus (18,38,101).

The steroid 16α-hydroxylase system was known from *in vitro* studies to be active in the human fetal steroidogenic organs which include adrenal gland, ovary and testis, and also in the fetal liver (142).

Placental preparations failed to exhibit a significant 16α-hydroxylation activity (101); however, high rates of *in vitro* placental conversion of 16α-OH-DHA to 16α-OH-androstenedione (17,101) and to estriol (101) substantiated a placental role in the Δ^5-3β-hydroxysteroid dehydrogenation, and aromatization of such precursors.

The abundance of several 3β-hydroxy-Δ^5-steroids in umbilical cord blood (41) and the poor ability of fetuses to aromatize DHA attests to a relative inactivity of the DHA-3β-hydroxysteroid dehydrogenase system in the human fetus (102). The microsomal fraction from the steroidogenic organs and the placenta is a rich source of this enzyme which is NAD dependent. The reaction is postulated to comprise two steps: the first involves the oxidation of the 3β-hydroxyl function to the 3-ketone, and the second the shift of the Δ^{5-6} double bond to the Δ^{4-5} position resulting in the 3-keto-Δ^4 grouping. Crystalline isomerase has only been isolated from microorganisms (10,37,147).

The aromatization of neutral steroids occurs in a microsomal enzyme system which requires NADPH and O_2 and comprises 19-hydroxylation and elimination of the C-19 angular methyl group as formaldehyde or formic acid along with elimination of the C-1β proton followed by aromatization of ring A. Even though three separate enzymes, a 19-hydroxylase, and 19-oxidase, and an aldehydelyase have been implicated in the system (10), none have been isolated and details of the mechanism are partially lacking (147). In spite of that, it is known that the activity of the aromatase system is high in placental and even in fetal liver preparations with Δ^4-3 keto-steroid substrates (80,140). Because of the inactive 3β-hydroxysteroid dehydrogenase system in the fetus, it

appears that the Δ^4-3 keto-configuration of precursors obtained elsewhere in the fetal circulation, probably in the placenta, is a prerequisite for the fetal aromatization of certain C_{19} steroids (102).

The 17β-hydroxysteroid dehydrogenase system in the microsomal fraction has been studied extensively (37,67). NAD is the preferred coenzyme and the optimal pH for the reduction is 6.2 (10). It is present in the liver, placenta, ovaries and testes and because of a specificity for aromatic steroids, it has been designated as the 17β-*A*-hydroxysteroid dehydrogenase in contrast with the 17β-*N*-hydroxysteroid dehydrogenase which is active for neutral steroids (37). The enzymatic reduction of 16α-OH-estrone to estriol was shown in term placentas perfused *in vitro* (14). The recent analyses of $^3H/^{14}C$ ratios in urinary estrone, estradiol and estriol after simultaneous administration of DHA-4-^{14}C sulfate and DHA-7-^{3}H-sulfate into the human fetal and maternal circulations respectively, confirmed that the pathways in the fetus favor the formation of 16α-hydroxylated estrogens in comparison to the pathways in the maternal organism which govern the metabolism of DHA sulfate primarily to estrone and estradiol (7).

Therefore, at least two metabolic pathways for the formation of urinary estriol are operative in human pregnancies: 16α-hydroxylation of DHA within the fetus followed by placental dehydrogenation at C-3, Δ^5-Δ^4-isomerization, aromatization of ring A, and finally, 17β-reduction to estriol; or dehydrogenation, isomerization and aromatization of circulating DHA to estrone by the placenta followed by 16α-hydroxylation of the circulating estrone by the fetus and finally, placental reduction at C-17.

C. Steroid 16α-hydroxylation *in vitro*.

Of the four enzymatic transformations necessary for the biosynthesis of estriol from DHA, the least is known about the 16α-hydroxylase system. Although there has been a wide interest in recent months, only a few reports of hepatic 16α-hydroxylation *in vitro* antedated 1965. The first report in 1948 dealt with incubations of slices from rabbit liver with DHA hemisuccinate and a product was androst-5-en-3β, 16α, 17β-triol (132). In 1956, the perfusate from testosterone circulated through a dogs liver yielded 16α-OH-testosterone, confirming that neutral steroids could be substrates (2). In 1958, two reports described the conversion of estradiol-17β to estriol in incubations of a coarse brei of male rat liver and slices of human fetal liver (42,43,65). The localization, in 1962, of the estradiol 16α-hydroxylase in the microsomal fraction of rat liver was also confirmed for the estrone 16α-hydroxylase (68,123). The microsomal localization has also been confirmed very recently with porcine ovaries with nine different steroid substrates (81). Previously, in 1962, the 16α-hydroxylation of the neutral steroid, DHA, was demonstrated in rat liver slices and was reported to be greatly stimulated by the addition of NADPH, to require oxygen and to be virtually zero in tissue from female rats (16). Furthermore, castration of the males greatly decreased their hepatic hydroxylation activity and treatment of the females with testosterone increased the 16α-hydroxylation. Some, but not all of these findings, were later confirmed in similar systems when estrone-^{3}H and estradiol-^{14}C were the substrates; however, no cofactor was added to the incubation flasks (3). At the present time, hepatic 16α-hydroxylation is also known

to occur with the following substrates: pregnenolone, progesterone, and androstenedione (127,140,142,155). In addition to liver and ovary, the transformation has been observed in preparations from adrenal gland, testis and kidney (142).

D. Stimulation of "androgen hydroxylases" by drug treatment.

Four years ago, the first of several reports appeared in which the activity of "androgen hydroxylase systems", which required NADPH and oxygen, was localized in the microsomal fraction of male rat liver and was stimulated three to seven fold by the treatment of the immature animals with the drugs phenobarbital or chlorcyclizine[1] (see Table No. 1). Among the "polar products" measured, metabolites of testosterone-4-^{14}C were identified as 2β-, 6β-, 7α-, and 16α-OH-testosterone-4-^{14}C. The predominant products of androstenedione-4-^{14}C metabolism were 6β- and 7α-OH-androstenedione-4-^{14}C, and two other compounds with the chromatographic properties of 11β- and 16α-OH-androstenedione were also observed (21). The hydroxylase activities increased less than 50% of the control values after treatment with 3-methylcholanthrene. When phenylbutazone[2] was used for similar treatments, reported in 1964, the quantity of polar metabolites formed, based upon a fraction corresponding chromatographically to 6β-hydroxylated products of testosterone and androstenedione was stimulated by about 150 and 300%, respectively (22).

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[1] 1-(4-chlorobenzhydryl)-4-methylpiperazine

[2] 4-butyl-1,2-diphenyl-3,5-pyrazolidinedione

Table No. 1. Steroid hydroxylations by liver microsomes from normal immature and drug-treated male rats (From Conney, et al). Semiquantitative data has been taken from chromatographic profiles and the enzymatic rates were calculated into mμmoles/mg./min. assuming 20 mg. microsomal protein/g. liver.

STEROID HYDROXYLATIONS[1] BY LIVER MICROSOMES FROM NORMAL IMMATURE AND DRUG-TREATED RATS (From Conney, et al.).

^{14}C-labelled Substrate	Condition	2-	6β-	7α-	16α-	Polar Products mμmoles/ g. liver/5 min.	mμmoles/ mg./min. **
Progesterone	control		+		0	235 ± 84 *	2.35
	phenobarbital		+++		+	1055 ± 288	10.55
	phenylbutazone					425 ± 24	4.25
Androstenedione	control		+	++	0	228 ± 18 *	2.28
	phenobarbital		++	+++	+	1710 ± 103	17.10
	3-methylcholanthrene					291	2.91
	phenylbutazone		++	+++	+	800 ± 82	8.00
Testosterone	control	±[2]	+	++	0	185 ± 12 *	1.85
	phenobarbital	++[2]	++	+++	+	1042 ± 72	10.42
	3-methylcholanthrene					258 ± 15	2.58
	phenylbutazone		++	+++	+	451 ± 39	4.51
Estradiol	control	±	+		0	15 *	0.05
	phenobarbital	+++	++		+	342	1.14

1. The rate of each transformation is graded 0 to +++ on the basis of the area under the chromatographic peaks which correspond to tentatively identified products.

2. 2β-configuration.

* mμmoles/g. liver/15 min.

** Calculation assumes 20 mg. microsomal prot./g. liver.

Similar responses were observed after treatment of immature dogs with phenylbutazone. In the latter experiments, the 7α- and 16α-hydroxylated products of androstenedione and testosterone were determined separately: hydroxylation at C-7 was unchanged by phenylbutazone but at C-16, the hydroxylating activity was 300 to 500% greater. In the experiments with rat liver microsomes data on each steroid transformation were not reported separately but the peaks corresponding to the 7α-, and 16α-hydroxy derivatives, and to a third unknown product appeared to be larger (in the published chromatographic profiles) after phenylbutazone treatment. Microsomes from the immature control rats formed polar products corresponding on the chromatograms to 6β- and 7α-OH-androstenedione or testosterone but no 16α-hydroxy compounds.

Later in 1964, in another report from the same laboratory (91), the authors suggested, on the basis of the similar metabolic properties of the hepatic enzymes which oxidatively metabolized testosterone, estradiol and hexobarbital, that steroid hormones are normally occurring substrates for oxidative drug-metabolizing enzymes in rat liver microsomes. Accordingly, they had observed a several-fold greater production by tissue from adult male rats of "polar products" which included 6β-, 7α-, and 16α-OH-testosterone and 2-, 6β-, and 16α-OH-estradiol. Also, the amount of hexobarbital metabolized was several-fold greater than that observed with tissue from adult female rats. No sex difference was apparent in similar preparations from mice. Furthermore, the same transformations were several-fold greater in adult (300 g.) male rats than in immature (50 g.) male rats. Treatment of immature male rats

and mature females with the insecticide, chlordane[1], or with phenobarbital similarly stimulated the hydroxylation of steroids and the oxidation of hexobarbital. The *in vitro* addition of a known inhibitor of oxidative drug-metabolism (SKF 525A)[2], affected all of the activities. The polycyclic hydrocarbon, 3-methylcholanthrene which is known to stimulate the oxidative drug metabolism of certain drugs including the *p*-hydroxylation of acetanilide and *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene, did not alter significantly the metabolism of testosterone, estrone or hexobarbital (24).

The same research team extended their observations of hepatic steroid metabolism to show that phenobarbital or phenylbutazone treatment increased by 175 to 450% the microsomal metabolism of progesterone-4-^{14}C to "polar products" and that the hypnosis normally resulting in rats from progesterone administration *in vivo* was abolished in animals stimulated similarly with phenobarbital, chlorcyclizine, phenylbutazone or chlorinated insecticides (25).

E. Steroid 7-hydroxylation *in vitro*.

The increased 7α-hydroxylation of androstenedione and testosterone in rat liver microsomes after drug treatments was interesting since the same transformation had been previously reported to occur with DHA in homogenates of liver from normal rats (143). The first report (36) of DHA 7α-hydroxylation, and 7β-hydroxylation too, had described the

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[1] 1,2,4,5,6,7,8,8-octachloro-4,7-methane-3α,4,7,7-α-tetrahydroindane

[2] β-diethylaminoethyldiphenylpropylacetate, an inhibitor of drug-metabolizing enzymes

transformation by cultures of Rhizopus (M 2045). Subsequently, it was shown that liver homogenates of rats of either sex hydroxylated DHA at apparently equal rates at the 7α-position, and that the rates were even higher in spleen, kidney and lung homogenates. Furthermore, liver and lung tissue from calves had an activity parallel to that of female rats and in both species the activity was twice as large as in rabbits (146). The 7α-hydroxylation of deoxycorticosterone proceeded rapidly in liver slices from a number of rodents but not from some other mammals (133) and the enzyme system responsible for the 7α-hydroxylation of both DHA and pregnenolone was also localized in the microsomal fraction of rat liver (144).

In contrast, the *in vitro* formation of 7β-hydroxy products of DHA or of any other steroid hormone had not been shown with mammalian systems as it had been with microbial ones. The 7β-hydroxylation *in vitro* of cholesterol-26-^{14}C by a 20,000 *g* supernatant was first reported in 1965 with a yield of 0.09% in comparison to a 1.1% yield of the 7α-hydroxy product (107). A preliminary report in 1960 of the 7-hydroxylation of 3β-hydroxy-Δ^5-cholenic acid by the 44,000 *g* supernatant from rabbit liver (150) has been amplified in a recent symposium to show that the yields of the 7β-epimer far exceed those of the 7α-epimer in the rabbit (156) and that reduced NADP was a required cofactor. A 7β-hydroxylated compound isolated from bovine testes was tentatively identified as 3β, 7β-dihydroxycholesterol (114).

In bile, of course, 7α-hydroxy C_{24}-steroids comprise important metabolic products (31 is a review) and 7β-substituted cholanic acids have also been isolated from the bile from rats (106,116,156), the bear

and coypu (31), and humans (8,71,138). The 7β-hydroxyl function appears to influence the further catabolism of bile acids by rat liver preparations (149).

The 7β-hydroxylation of C_{19} steroids *in vivo* was demonstrated when such compounds were identified as urinary products of administered 3α-hydroxy-5β-androstan-17-one or testosterone-4-^{14}C (54).

F. Biological differences in steroid metabolism.

The 7α-hydroxylation of androstenedione-4-^{14}C had been noted in liver microsomes of normal or diabetic female rats (60) or normal ones of either sex (1). In the latter study, the mature males incorporated 1.3% of the radioactivity into the 7α-OH-androstenedione fraction while the females incorporated 0.8%. The corresponding values for 6β-OH-androstenedione were 4.1 and 1.2%, also suggesting a sex difference for the 7α- and 6β-hydroxylation of androstenedione. In addition to the sex-related 16α-hydroxylation of DHA, male rats metabolize a variety of other steroid substrates differently from females. The ten-fold greater 2- and '6ξ' hydroxylation of estradiol-16-^{14}C by hepatic microsomes (76) represents another example of the sex-dependent differences in steroid metabolism which could also be decreased by gonadectomy or treatment with heterologous sex hormones. The differences in metabolic activities which appear after 40 days of age were greatest in rats 59 to 70 days old. This is one to four weeks earlier than the age at which the oxidation rates are maximal for the hepatic microsomal metabolism of many other compounds, mainly drugs (58,82,85,124,128). The treatment of the animals with androgens (9,52),

progestogens (78), or alteration of their endocrine (83,59) or nutritional status (84) also influenced the sex-dependent microsomal oxidative metabolism of certain drugs as it had that of steroid substrates. The thyroid function in humans profoundly influenced the urinary excretion of 2- and 16α-hydroxylated estrogens (46).

G. Related metabolism of steroids and drugs.

There are extensive studies of the mechanism of steroid hydroxylations at the C-11 and C-21 positions of the molecule by adrenal mitochondrial and microsomal fractions, respectively. The stereospecific hydroxylation of secondary carbon atoms appears to proceed through the electrophilic substitution of the hydrogen atom by a hydroxyl function. Molecular oxygen, not that from water, is involved (37,147). The reactions are examples of external mixed function oxidations (103), many of which have been related in recent years to the membrane-bound hemoprotein, cytochrome P-450 (104). This component of the microsomal (and mitochondrial) electron transport chain is reversibly inactivated by carbon monoxide (26), a fact which was important in linking the CO inhibition of steroid 21-hydroxylation of C_{21} steroids (130) to cytochrome P-450. Recent spectral studies using steer adrenal microsomal fractions treated with Triton N-101[1] have shown that the addition of 17α-hydroxyprogesterone, but not of 17α, 21-dihydroxyprogesterone produced a trough at 420 mμ and a peak at 388 to 390 mμ (110). These low-amplitude spectral changes (absorbancy

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[1]Nonylphenol sidechain of oxyethylene having 9 to 10 units

of 10 μ*M* 17α-hydroxyprogesterone added = 0.03-0.04) were also given by 90 μ*M* androstenedione, a C_{19} steroid, and they disappeared upon the addition of NADPH in the case of the hydroxylatable steroid; with androstenedione, addition of NADPH to the aerobic preparation increased the absorbancy but the spectral response to NADPH was not observed under anaerobic conditions. At the higher concentrations, progesterone and 17α-hydroxy-5ξ-pregnane-3,20-dione also produced the same reactions as 17α-hydroxyprogesterone; however, reduction of the keto groups at either C-3 or C-20 abolished the reactions. These observations have been interpreted as spectral evidence for an enzyme-substrate complex of the 21-hydroxylase system.

A large number of hepatic microsomal enzymes active in the metabolism of steroids, lipids and xenobiotic compounds similarly require O_2 and NADPH for reducing equivalents (55,104,134,136), the latter being transferred by cytochrome P-450. The oxidative demethylations of codeine and monomethyl-4-aminopyrine, and the hydroxylation of acetanilide by hepatic microsomes were also inhibited by CO and the inhibition was reversed by mono-chromatic light of 450 mμ (26) as had been observed with the adrenal microsomal C-21 hydroxylation. Hepatic microsomal *N*-demethylation of aminopyrine was also inhibited approximately 90% by a mixture of N_2:CO:O_2 (gas-phase ratio of 56:40:4) (85,120). These similarities in the adrenal and hepatic microsomal metabolism were extended by the observation that the addition of several different drug substrates to the hepatic preparations produced the same type of low-amplitude changes in the Soret band (44,72,126). Aminopyrine,

phenobarbital, hexobarbital, amobarbital and SKF 525-A produced a 385 mμ peak absorbance and a 420 mμ trough, but aniline or DPEA[1] caused an approximately opposite spectrum: an absorption band at 430 mμ and a decreased absorption at about 390 mμ. The absorbancy changes were related to the concentration of the substrates. Barbital and benzene, substrates which are also metabolized by microsomes, did not produce the spectral change and neither did diethylamine or amino acids, neither of which are metabolized in these systems (72).

The relationship between the steroid and drug substrates, and the enzymes has been explored in still another way. In spite of the limitations imposed by studying structurally-complex, enzymatically-active particles, kinetic data has demonstrated the competitive inhibition of *N*-demethylation of ethylmorphine by hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone and acetanilide (129). Barbital and acetazoleamide, drugs not metabolized by hepatic microsomes of rats, were not inhibitory for the *N*-demethylation. The similarities of the inhibitions in normal and phenobarbital-induced microsomal systems were interpreted to indicate a quantitative, rather than a qualitative, change by the induction process which resulted in a different set of inhibition interactions when 3-methylcholanthrene was used as the inducing agent (139).

Michaelis constants for the oxidative metabolism of steroids and drugs have been offered as evidence for the interrelationship of the involved enzymes. The apparent K_m values for the metabolism of

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[1] 2,4-dichloro-6-phenylphenoxyethylamine hydrochloride, an inhibitor of drug oxidation

progesterone, testosterone, and estradiol-17β to "polar products", which ranged from 1.6×10^{-5} to $4.9 \times 10^{-5} M$, were ten or more times smaller than those for all of the drugs compared (92). Similarly, the apparent K_i values were obtained for the competitive inhibition of *N*-demethylation of ethylmorphine by estradiol, testosterone, androsterone, diethylstilbesterol and cortisol (148). These results indicate some interrelationship of steroids and drugs and enzymes active in their metabolism; however, they do not indicate the site of the competition.

H. Biological differences in drug metabolism.

The recent spectral and kinetic observations described above for a few drug or steroid substrates with microsomal fractions were preceded, years ago in many instances, by descriptions of the biological and biochemical aspects of their reactions. In rabbit liver microsomes for example, at least 8 different types of oxidative enzyme activities are recognizable (49): side chain oxidation of hexobarbital, *N*-demethylation of aminopyrine, deamination of amphetamine, *p*-hydroxylation of acetanilide, sulfoxidation of chlorpromazine, *O*-dealkylation of codeine, reduction of aromatic nitro groups and reduction of azo linkages. All of these enzymatic activities are active in the microsomal fractions from which two reproducible subfractions have been prepared by using a sucrose density-gradient and centrifugation at higher *g* forces for longer periods of time. Thus the smooth subfraction from rabbit liver, corresponding to the endoplasmic reticulum without attached ribosomes (agranular endoplasmic reticulum), has a greater enzymatic activity (smooth/rough ratios from 2:1 to 8:1) for all 8 types of drug oxidations than does the rough microsomal fraction

which corresponds to the endoplasmic reticulum with attached ribosomes (granular endoplasmic reticulum) (48,51,62,73). Not only were the enzymatic activities selectively localized in the less-dense membranes but a number of different redox components were similarly located in tissue from rabbit (73,105) and porcine (56) sources but the distribution was less clear with the microsomal subfractions from the mouse (112) or the rat (28). In the latter, the *N*-demethylase activity was also equal in both the smooth and rough subfractions (119); however, a recent study has attributed that response to the method of preparation rather than to the initial distribution of the enzymes (51,62).

Microsomes from rabbit and rat livers are different in still other respects. The rates of *p*-hydroxylation and the deamination of amphetamine are very low in rat liver microsomes in comparison to those from rabbit liver (51), and the sex-dependence observed in rats for steroid hydroxylase systems exists for several drug-metabolizing enzymes as well. Thus, in addition to the hexobarbital oxidase already mentioned, hepatic preparations from male rats have larger rates than females for the *N*-demethylation of aminopyrine (124), methylaminoantipyrine and pithidine (135), aromatic hydroxylation of 3,4-benzpyrine, and the aliphatic hydroxylation of pentobarbital (93). Similar sex-differences have not been found regularly in the microsomal metabolism of other laboratory animals. Also, different strains of rats are likely to exhibit different rates (125,135).

I. Induction of enzymatic activities.

All of the common laboratory animals which have been tested, on the other hand, respond similarly to treatment with a variety of

drugs which stimulates their microsomal oxidative metabolism. Although the stimulation of steroid hydroxylations has not been reported for rabbits as was described for rats, the 6β-hydroxylation of cortisol was increased after phenobarbital was administered to rats, guinea pigs, monkeys (Cebus albifrons) and humans (6,12,13,23) and cholesterol biosynthesis from acetate was increased by similar treatment to hamsters (77). These related findings suggest that the induction of enzymes by various drugs occurs generally in mammals. The progressive increase in the hepatic endoplasmic reticulum after drug-treatments, as observed by the electron microscope, has been limited to the agranular type corresponding to smooth microsomes and has been documented in tissues from the rabbit, rat, hamster, and the dog (77,125).

The increases in activity of drug-metabolizing enzymes, after the administration of the compounds, have not always corresponded to a proliferation of agranular endoplasmic reticulum evaluated morphologically (50), or by alterations of the protein and RNA contents (79). From the large number of agents that have been used to stimulate the activities, three major groupings have been discerned (51). Polycyclic hydrocarbons such as 3-methylcholanthrene or 3,4-benzpyrene stimulate a few drug-metabolizing enzymes but the effect of the anabolic steroids is more restricted. In contrast, a third group, comprising diverse compounds such as phenobarbital, cholanthrene, tolbutamide and others, seems to have little specificity and shows the best correlation between the enzymatic activities and the proliferation of the smooth endoplasmic reticulum. The maximum proliferation has been directly related to the amount and time of drug administration and, except for isolated

examples, reverts within 24 to 48 hours after cessation of the treatment to the equivalent morphology and activity of the pretreatment status (125).

The correspondence between the increased enzymatic activity and the agranular reticulum has confirmation from the simultaneous increases in the structural and functional elements of the microsomal vesicles. The content of protein, RNA, cytochromes b_5 and P-450 as well as other heme and non-heme redox components, phospholipids and pyridine nucleotide oxidoreductases and other elements are increased primarily in the smooth subfraction by drug treatment (28,86,104,119,121,125,135). This correspondence has provided indirect evidence for a functional relationship between the membrane components, such as the hemoproteins, and the enzymatic activities. A number of experiments, based upon the incorporation of ^{32}P or glycerol-^{14}C into the phospholipid components of the enzymes or of radioactively-labeled amino acids, supply conclusive evidence that the induction process involves the biosynthesis of new membranes (30,57,86).

Many efforts have been made to solubilize and purify microsomal oxidative enzymes without altering the active system. Invariably, although purification of some components has been possible, the state of others has been altered and the enzymatic activities have been greatly diminished or completely lost (104,108,118,134). There is good evidence that extraction of the lipid from biological membranes or the addition of bile salts causes disaggregation of membrane particles composed of electron transport complexes and that reaggregation occurs after some lipids are restored or the bile salts are removed (64). The enzymatic activities parallel the aggregation.

J. Objectives of this study.

The objective at the beginning of this work in mid-1964 was to study the biochemical properties of the hepatic DHA 16α-hydroxylase *in vitro*. I planned to carry out a descriptive study of some biological variations of the 16α-hydroxylase system in comparison with other hepatic steroid hydroxylase systems, in an effort to find some biochemical basis for the specificity of steroid hydroxylations. Of course, the comparison of the enzymatic products of DHA necessitated their identification in our laboratory.

Simultaneous developments in the related fields of biological oxidation and molecular pharmacology interested me in comparisons between the hepatic DHA hydroxylases and drug-metabolizing enzymes in the same tissue. Thereafter, the objectives focused upon attempts to establish some specific differences between the activities of the enzyme systems after pharmacologic or physicochemical manipulations and to correlate them with the redox component, cytochrome P-450.

II. MATERIALS AND METHODS

A. Animals.

1. Sprague-Dawley rats were purchased from Pacord Research, Inc., Beaverton, Oregon. Except for special studies, their ages were between 9 and 12 weeks. The diet was Ralston-Purina laboratory pellets and water *ad libitum*. Livers from animals which were fasted overnight (about 16 hours without pellets) were used in the experiments concerning lyophilization, age effects, and the effect of fasting on the metabolic activities. Those rats which received drug treatments were injected intraperitoneally and the control animals were given the same amount of the appropriate vehicle. The drugs and daily dosages per kg. of body weight were: sodium phenobarbital[1], 100 mg.; 3-methylcholanthrene[2], 20 mg.; and phenylbutazone[3], 134 mg. All animals were treated daily for five days and killed on the sixth day.

Progressively older rats (16 to 24 weeks) were used for the experiments testing the effect of sodium deoxycholate on the enzymatic activities and cytochrome P-450 content.

2. New Zealand white rabbits were bought from local food suppliers. They were mature (5 to 9 lbs.) and the females were nulligravid. Their diet was Triangle Special Rabbit Diet, Traingle Milling Co., Hillsboro, Oregon, except for one pair which received Alber's Rabbit Greens. The rabbits were fasted 24 hours before death.

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[1]Luminal, 160 mg./ml., Winthrop Laboratories, New York

[2]Sigma Chemical Corp., St. Louis, Mo., 25 mg./ml. sesame oil

[3]A gift from Geigy Pharmaceuticals, Ardsley, New York, 100 mg./ml. sesame oil

3. Livers from rhesus monkeys from the colony at the Oregon Regional Primate Research Center were quick-frozen and brought to our laboratory for processing. These tissues were used within 4 to 6 weeks. The daily diet of adult animals was Purina monkey chow and apples were offered at biweekly intervals. Gravid monkeys received a daily multi-vitamin supplement[1] and non-breeder adults ate cabbage each week instead of vitamins. For the first two days of life, newborn monkeys were maintained on 5% glucose in water with additions, for 2 to 3 days thereafter, of Similac[2] formula. After 30 days, apples, bananas and Purina chow were added, and after 90 days, no Similac was fed.

4. Tissue from three human anencephalic infants was excised and quick-frozen within 30 minutes after clinical death and prepared within 4 weeks. Appropriate consent had been obtained. The related pregnancies were of 37 to 45 weeks duration; Cases No. 3 and 4 in prior reports (19,69) and L.B., Case No. 5, was a 5 lb. 7 oz. female delivered after 43 weeks gestation; the former were males.

B. Preparation of tissue.

1. Total microsomes.

All of the livers from rats and rabbits were perfused with 0.25 *M* sucrose after they were blotted and weighed but the livers or organs from other species were only washed with sucrose before blotting and weighing them. Perfusion was incorporated to remove hemoglobin prior to homogenization.

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[1]Unicap, chewable[R] Upjohn Co., Kalamazoo, Michigan

[2]Similac[R], Ross Laboratories, Columbus, Ohio

All specimens, except rhesus livers, were minced with scissors and homogenized in 0.88 *M* sucrose (1/3;w/v) by means of three complete excursions of a teflon pestle driven at 400 rpm. inside a glass homogenizer (1.25×10^{-2} mm. of clearance). The rhesus liver was homogenized in 0.25 *M* sucrose (1/2;w/v) in a Virtis "45" apparatus for two minutes. The nuclei and mitochondria were sedimented simultaneously by centrifuging at 10,000 *g* for 35 minutes. The supernatant was recentrifuged after dilution to isotonic osmolarity with 5 volumes of 0.04 *M* KCl.[1] The combined pellets were suspended in 0.1 *M* sodium phosphate buffer, pH 7.4 (2/1;v/w fresh tissue) for mitochondrial enzymatic studies.

The 10,000 *g* supernatant was centrifuged at 78,000 *g* ($r_{ave.}$ in a Spinco #30 rotor at 30,000 rpm.) for 60 minutes. The resulting supernatant was retained for appropriate determinations. The sedimented total microsomal fraction was resuspended in 0.1 *M* phosphate buffer, pH 7.4 (1/1;v/w fresh tissue) for incubation experiments.

2. Microsomal subfractions.

The procedure to separate microsomes into smooth and rough subfractions was based on those of Mason *et al.* (105) and Fouts (48). A suspension of the total microsomal fraction in 3 to 4 volumes of a 0.88 *M* sucrose and 0.04 *M* KCL mixture (1/5;v/v) was carefully layered over 2 ml. of 1.42 *M* sucrose and centrifuged at 105,000 *g* ($r_{ave.}$ in a Spinco #40 rotor at 40,000 rpm.) for 8 hours. The smooth microsomal subfraction was aspirated along with the 1.42 *M* sucrose and the rough

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[1] A single 10,000 *g* centrifugation was used for livers from two humans and the rat preparations related to lyophilization, age effects, and the effect of delays after the preparation of microsomal fractions.

microsomal pellet dislodged by gentle washing from the transparent glycogen layer underneath. The smooth subfraction was resuspended in 3 to 5 volumes of 0.04 *M* KCl and the rough subfraction in 1 to 2 volumes of the 0.88 *M* sucrose-0.04 *M* KCl mixture. Both subfractions were resedimented by centrifuging at 78,000 *g* for 60 minutes. The final homogenates of the pellets in 0.1 *M* phosphate buffer pH 7.4 had a protein concentration of 5 to 15 mg./ml. Figures No. 1 and 2 are electron photomicrographs to substantiate the separation achieved.

For the experiments to test the effect of deoxycholate[1] on the enzymatic activities, total microsomal fractions representing 2 g. fresh liver/ml. were treated as follows:

1. DOC - Crystalline sodium deoxycholate, 0.5 mg./mg. protein, was dissolved in the microsomal solution (60 mg. prot.) at room temperature by gentle shaking. After 30 minutes, the clear protein solution was pipetted onto a Sephadex G-25-fine column[2], (12.5 g.; 2.2x15 cm.) and eluted with 0.1 *M* phosphate buffer, pH 7.4. The mid-portion of the eluate (10 to 12 ml.) was collected for the analyses.[3]

All of the samples were prepared as described except, of course, for initial procedures that are described now.

2. N_2 - A 15 ml. centrifuge tube containing the microsomal fraction was closed with a tight-fitting multiple-dose vial stopper penetrated by three stainless steel needles. After 5 minutes of a constant

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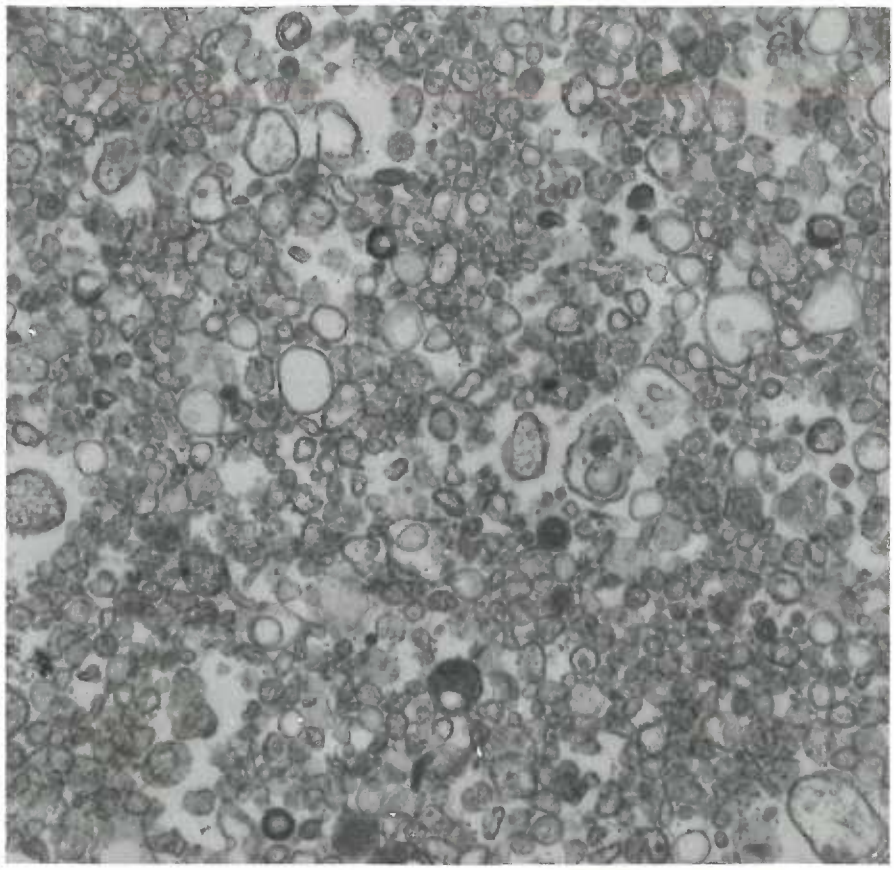
[1]Sigma Chemical Co., St. Louis, Mo.

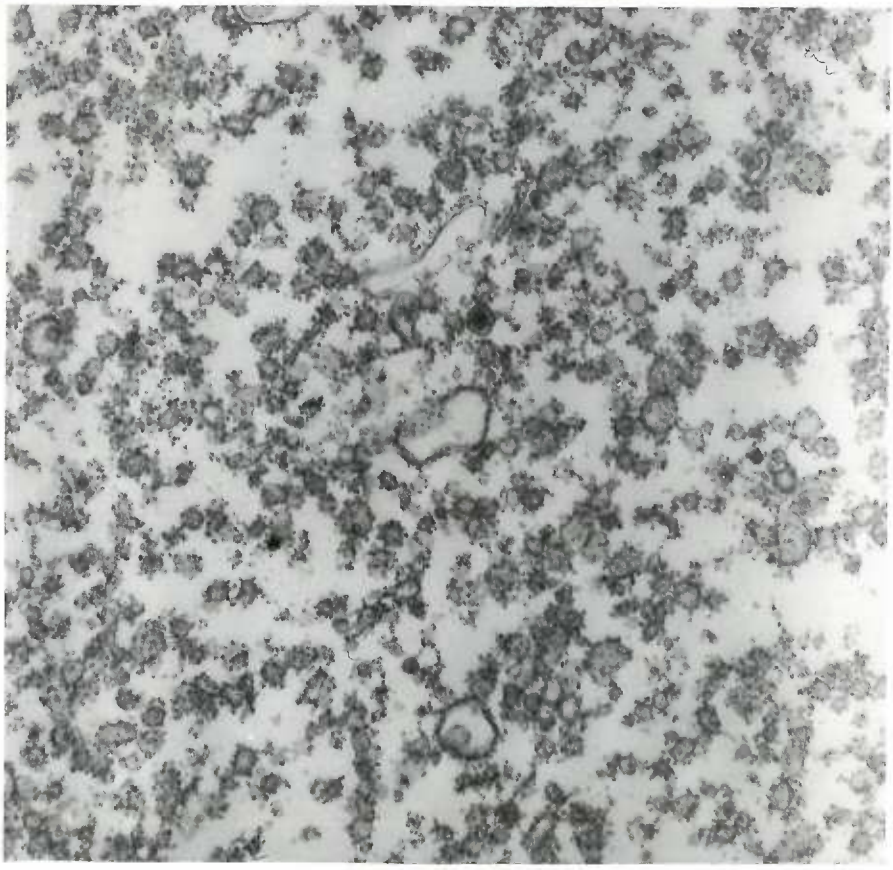
[2]Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

[3]Dr. J.L. Gaylor gave valuable advise about this system and granted permission to use the columns.

Figure No. 1. Micrograph of the subcellular fraction designated smooth microsomal fraction. Very few dense ribosomal particles are observed. Fractions were fixed in osmium tetraoxide, dehydrated rapidly in a series of graded ethanol solutions (4° C.), and embedded in Epon 812. Sections cut on a LKB Ultratome were stained with uranyl acetate and lead citrate and viewed in an RCA EMU-3F electron microscope. X48.000.

Figure No. 2. Micrograph of the subcellular fraction designated rough microsomal fraction. Nearly all the vesicles are characterized by attached ribosomal particles. X48.800.





flow of purified N_2 merely brisk enough to stir the solution, the deoxycholate crystals were introduced by means of a N_2 filled syringe into the solution; the N_2 exited through the remaining needle. The needles were withdrawn at the beginning of the 30 minute DOC treatment leaving the N_2 atmosphere during the gentle agitation which followed. Thereafter the cleared solution was chromatographed as described above.

3. Propylene glycol - Propylene glycol, 0.25 ml., corresponding to the vehicle for the steroid substrates, was pipetted and stirred into appropriate microsomal fractions. The DOC treatment and chromatography followed.

4. Steroid in propylene glycol - DHA or androstenedione, 6.25 mg. in 0.25 ml. of propylene glycol were either pipetted and treated with DOC in air as in preparation 3, or introduced into the microsomes in a N_2 atmosphere from a N_2-filled syringe. For the latter procedure, the DOC was then poured into the syringe which was reflushed with N_2 and the microsomal solution containing the steroid in propylene glycol was aspirated and mixed with the DOC in the syringe. The solution was left in the N_2 atmosphere of the centrifuge tube for 30 minutes as described above. Thereafter, the solution was chromatographed on the Sephadex column as described.

Lyophilized microsomes were prepared using a system of cylindrical flasks suitable for tissue and one for a moisture trap immersed in an ethanol-dry ice bath and coupled to a vacuum pump and pressure indicator.

Approximately 50 ml. of the microsomal solutions could be lyophilized in 3 to 4 hours. The powder was weighed and usually stored *in vacuo* at -14°C. The weight of lyophilized microsomal powder from 1 g. of fresh tissue was usually about 40 mg.

C. Chemical methods.

1. Protein content.

The protein content of all tissue fractions was determined by the biuret method described for serum protein determinations (61). The respective volumes of mitochondrial, microsomal and supernatant fractions pipetted for analysis were 0.025, 0.20, and 0.40 ml. After incubation in the presence of 0.2 ml. of 2% sodium deoxycholate for two hours at 37°C, enough 0.9% NaCl was added to make 1 ml. After addition of 4.0 ml. of 0.45 *M* NaOH to another sample, the tubes were left at room temperature for 30 minutes before reading the absorbancies at 540 mμ in a Zeiss PMQ II spectrophotometer. Simultaneous duplicate determinations of 4.0 mg. each of crystalline bovine serum albumin[1] provided the reference value. Each mixture of biuret reagent had been tested with the reference standard to establish its reliability. From 72 duplicate determinations of serum albumin at the 4.0 mg. level, the sensitivity was calculated as 0.07 mg. and the precision as ± 0.12 mg. (3.0%)[2] (11,141).

2. RNA content.

The RNA content of smooth and rough microsomal subfractions was determined by using the orcinol reaction for pentose (15,28). About

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

[2]See Table No. 2 (p. 27) for the formulas

Table No. 2. Reliability of the colorimetric procedures.

RELIABILITY OF THE COLORIMETRIC PROCEDURES

Determination	No. Duplicate Determinations	Sample Vol. (ml.)	Amount of Standard Compound	Precision[1] Value	Precision[1] %	Sensitivity[2] Value
Biuret	72	5.0	4 mg. bovine serum albumin	0.12 mg.	3.0	0.07 mg.
Orcinol	28	2.5	10 µg. D-ribose	0.03 µg.	0.3	0.02 µg.
Hantzsch	20	6.0	4 µg. formaldehyde	0.13 µg.	3.3	0.11 µg.
	20	6.0	8 µg. "	0.26 "	3.3	0.22 "
Folin-Ciocalteu	28	6.7	100 mµmoles p-OH-acetanilide	2.9 mµmoles	2.9	2.0 mµmoles
	8	6.7	200 "	1.9 "	1.0	3.7 "
	25	6.7	300 "	0.9 "	0.3	0.7 "
Blue Tetrazolium	74	2.3	15.2 µg. 16α-OH-DHA	0.28 µg.	1.9	0.11 µg.
Pettenkofer (modified)	13	1.0	2.5 µg. DHA	0.13 µg.	5.2	0.16 µg.
	26	"	5.0 "	0.34 "	6.8	0.25 "
	29	"	10.0 "	0.46 "	4.6	0.31 "
	25	"	20.0 "	0.47 "	2.4	0.35 "

1. Precision: $s = \sqrt{\frac{\Sigma (d^2)}{2N}}$, where d is the difference in absorbance in a duplicate determination and N is the number of duplicate determinations.

2. Sensitivity: $\frac{t \times s}{\sqrt{N}}$, where t is the t-value corresponding to $N-1$ degrees of freedom at the $P = .001$ level and s is the precision value.

2 mg. of microsomal protein were precipitated in 5 ml. of ethanol and centrifuged for ten minutes at 2,000 rpm. in an International Model V centrifuge. After decanting the supernatant, the pellet was washed with 5 ml. of deionized water to remove glycogen and the mixture was recentrifuged. The supernatant was aspirated and the pellet was treated with 2.5 ml. of 10% perchloric acid and heated for 20 minutes at 70°C. After cooling and recentrifugation, the supernatant was saved and the hydrolysis was repeated. The combined supernatants were diluted with deionized water up to 12.5 ml. To duplicate 2.5 ml. samples, 2.5 ml. of orcinol reagent was added and the mixtures were heated for 40 minutes in a boiling water bath. After cooling, the color complex was extracted using 2.5 ml. of isoamyl alcohol with repeated inversion of the stoppered tubes. After centrifugation, the absorbancies were determined at 675 mμ using appropriate blank samples and the values were compared to those given by 10 mg. samples of D-ribose.

From 28 duplicate determinations at the 10.0 μg. level, the sensitivity was calculated as 0.02 μg. and the precision as ± 0.03 μg. (0.3%). The RNA content was calculated by multiplying the ribose content by 3.76, the reciprocal of the average ratio of the optical densities of 1 μg. of RNA and 1 μg. of ribose.

D. Enzymatic methods.

1. Succinic-cytochrome *c* reductase.

Solutions of mitochondrial, microsomal and supernatant fractions equivalent to 0.0125, 0.10 and 0.025 g. of fresh tissue/ml. in 0.1 *M* phosphate buffer pH 7.4 were prepared. For the determinations of enzyme activity, 0.05, 0.1, and 0.1 ml., respectively, of the solutions

were pipetted into two 10x0.5x4.0 cm. quartz cuvettes (the volumes correspond to 6.25×10^{-4}, 1×10^{-2}, and 2.5×10^{-3} g. of fresh liver for the mitochondrial, microsomal, and supernatant fractions, respectively). Together with 1.0 µmole of KCN (0.10 ml.) and 1.0 mg. cytochrome c[1] (0.10 ml.), enough 0.02 *M* phosphate buffer pH 7.4 was added to make a total volume of 0.4 ml. To the reference cuvette, 0.50 ml. of phosphate buffer was added and in the sample cuvette 5.0 µmoles of succinate in phosphate buffer (0.50 ml.) was placed and mixed with a transfer pipette at time zero. Spectrophotometric readings at 550 mµ corresponding to the reduction of cytochrome *c* at room temperature were made at 30 second intervals and the mµmoles reduced were calculated taking the difference between the molar extinction coefficients of the oxidized and reduced cytochrome *c* as 1.97×10^4 $cm^{-1}M^{-1}$(63). Activity is expressed as mµmoles/mg./minute.

2. Cytochrome P-450.

One ml. of a microsomal protein solution containing 2 to 3 mg./ml. was pipetted into two 0.5x1.0x4.0 cm. quartz cuvettes which were placed into a Cary model 14 recording spectrophotometer equipped with a scattered transmission attachment and a high-intensity quartz lamp operated at 70 volts. After the absorbancy difference had been adjusted to zero, CO was bubbled into the sample solution for 20 seconds before several crystals of sodium dithionite were dissolved in both cuvettes and mixed with a transfer pipettes. The difference spectrum between 500 to 400 mµ was recorded and the absorbancy at 450 mµ minus that at

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[1]Type III from horse heart, Sigma Chemical Co., St. Louis, Mo.

490 mμ and the molar extinction coefficient 91 $cm.^{-1}mM^{-1}$ were used to calculate the cytochrome P-450 content (28,118).

In the experiments testing the inhibition of P-450 in the presence of SU-9055, various amounts of the latter were added to the sample cuvette in 0.1 ml. of 10^{-3} *M* tartaric acid. The same volume of tartaric acid was added to the reference cuvette to prepare the difference-spectra system.

3. Acetanilide *p*-hydroxylase activity.

Between 1 to 3 mg. (0.20 ml.) microsomal protein was incubated in a 25 ml. Erlenmeyer flask with 4.0 mmoles (0.1 ml.) acetanilide[1] in 0.04 *M* Tris buffer, pH 8.2, 2.0 μmoles (0.10 ml.) NADPH[2] in deionized water, and 0.60 ml. Tris buffer for 30 minutes with shaking in an air atmosphere in a water bath at 37°C. Duplicate samples and a flask without acetanilide were incubated and 4.0 mmoles of acetanilide was added to the blank after the reactions were stopped by adding 0.10 ml. of 20% trichloracetic acid. The solubility coefficient of the product was decreased by the addition of 0.5 g. of crystalline NaCl and the *p*-hydroxyacetanilide was extracted into 10 ml. of a solution of amyl alcohol-ether (1.5/100). Each flask was stoppered immediately to retard evaporation and swirled several times. After five minutes, 8 ml. of the 9.7 ml. ether phase was pipetted into 4.5 ml. of 0.10 *M* NaOH in a 25 ml. separatory funnel. The NaOH phase (4.7 ml.) was drained into a test tube where 1.0 ml. of a mixture of 1:5 Folin-

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[1]Mallinckrodt Chemical Works, St. Louis, Mo.

[2]Triphosphopyridine nucleotide, reduced form, Disodium salt, Sigma Chemical Co., St. Louis, Mo., General Biochemicals, Chagrin Falls, Ohio

Ciocalteu reagent; deionized water was added and inverted immediately. The blue color developed during a 30 minute reaction time in a 37°C water bath. The absorbancy at 765 mμ (see Figure No. 3) was compared to that given by 0.10 and 0.30 μmoles of *p*-hydroxyacetanilide[1], although the wave length used in the reported method was 691. The average value of duplicate samples minus the value of the blank were used to calculate the rate which was expressed as *p*-hydroxyacetanilide formed in mμmoles/mg. protein/minute (90).

From 28 duplicate determinations at the 0.10 μmole level and 25 determinations at the 0.30 μmole level, the respective sensitivities were 2.0 mμmoles and 0.70 mμmoles and the precision values were ± 2.9 mμmoles (2.9%) and ± 0.90 mμmoles (0.3%).

4. Aminopyrine *N*-demethylase activity.

Between 1 to 3 mg. (0.20 ml.) of microsomal protein was incubated in a 25 ml. Erlenmeyer flask with 5.0 mmoles aminopyrine[2] (1.0 ml. in 0.05 Tris buffer, pH 7.5), 2.0 μmoles (0.10 ml.) NADPH, and 0.70 ml. Tris buffer. After 20 minutes incubation with shaking in air at 37°C, the reactions were stopped by adding 0.25 ml. of 25% zinc sulfate and 0.25 ml. of saturated barium hydroxide. Duplicate samples and a flask without aminopyrine were incubated and 5.0 mmoles of aminopyrine was added terminally to the blank and 1.0 ml. of Tris buffer to each sample. The mixtures were decanted into centrifuge tubes for sedimentation of the residues. The 3.0 ml. supernatants reacted in stoppered

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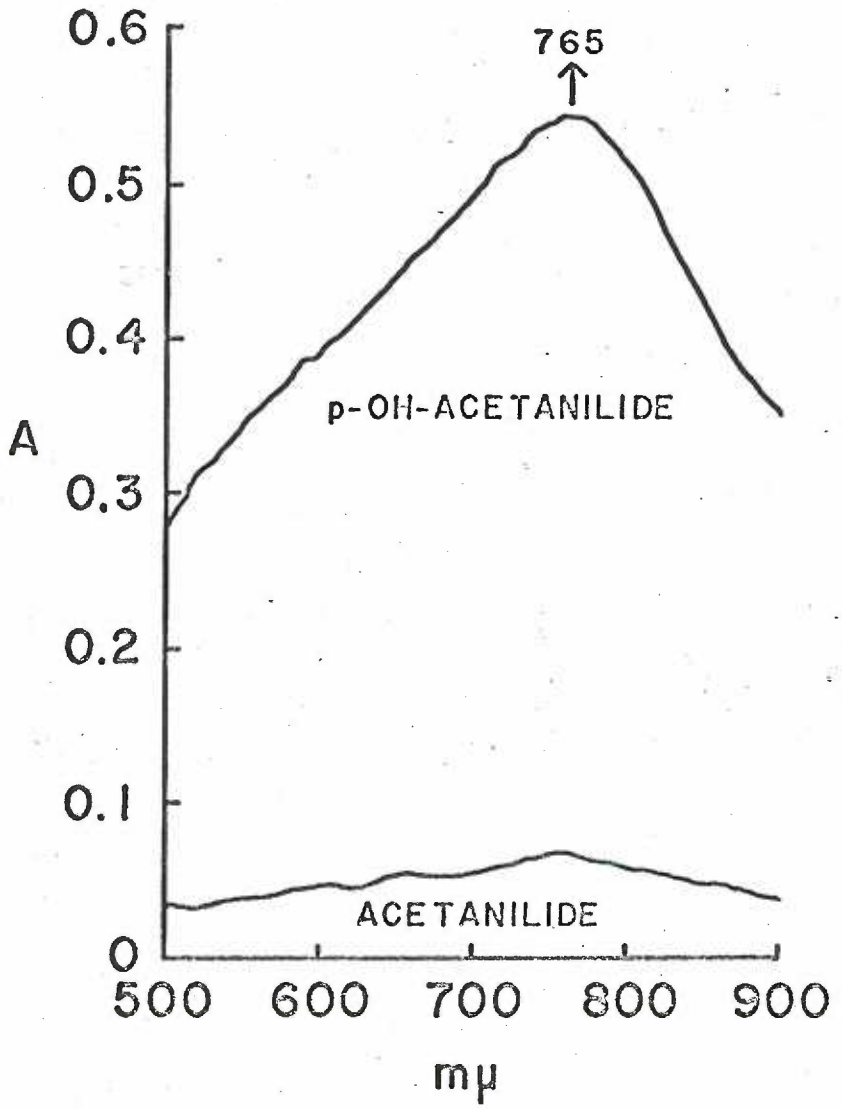
[1]Eastman Organic Chemicals, Rochester, N.Y.

[2]K & K Laboratories, Inc., Hollywood, California

Figure No. 3. Spectra of p-hydroxyacetanilide and acetanilide (100 μmoles/2.3 ml.) in Folin-Ciocalteu reagent.

SPECTRA OF p-OH-ACETANILIDE AND ACETANILIDE IN THE FOLIN-CIOCALTEAU REAGENT (100 mμMoles/2.3 ml)



tubes with 3.0 ml. of formaldehyde reagent for 40 minutes at 37°C. The absorbancies at 412 mμ were compared to those given by 4.0 and 8.0 μg. of formaldehyde treated simultaneously. The average value of duplicate samples minus the value of the blank were used to calculate the rate which was expressed as formaldehyde formed in mμmoles/mg. protein/minute (111,120). From 20 duplicate determinations at the 4.0 and 8.0 μg. levels, the respective sensitivities were 0.11 μg. and 0.22 μg., and the precisions were ± 0.13 μg. (3.3%) and ± 0.26 μg. (3.3%).

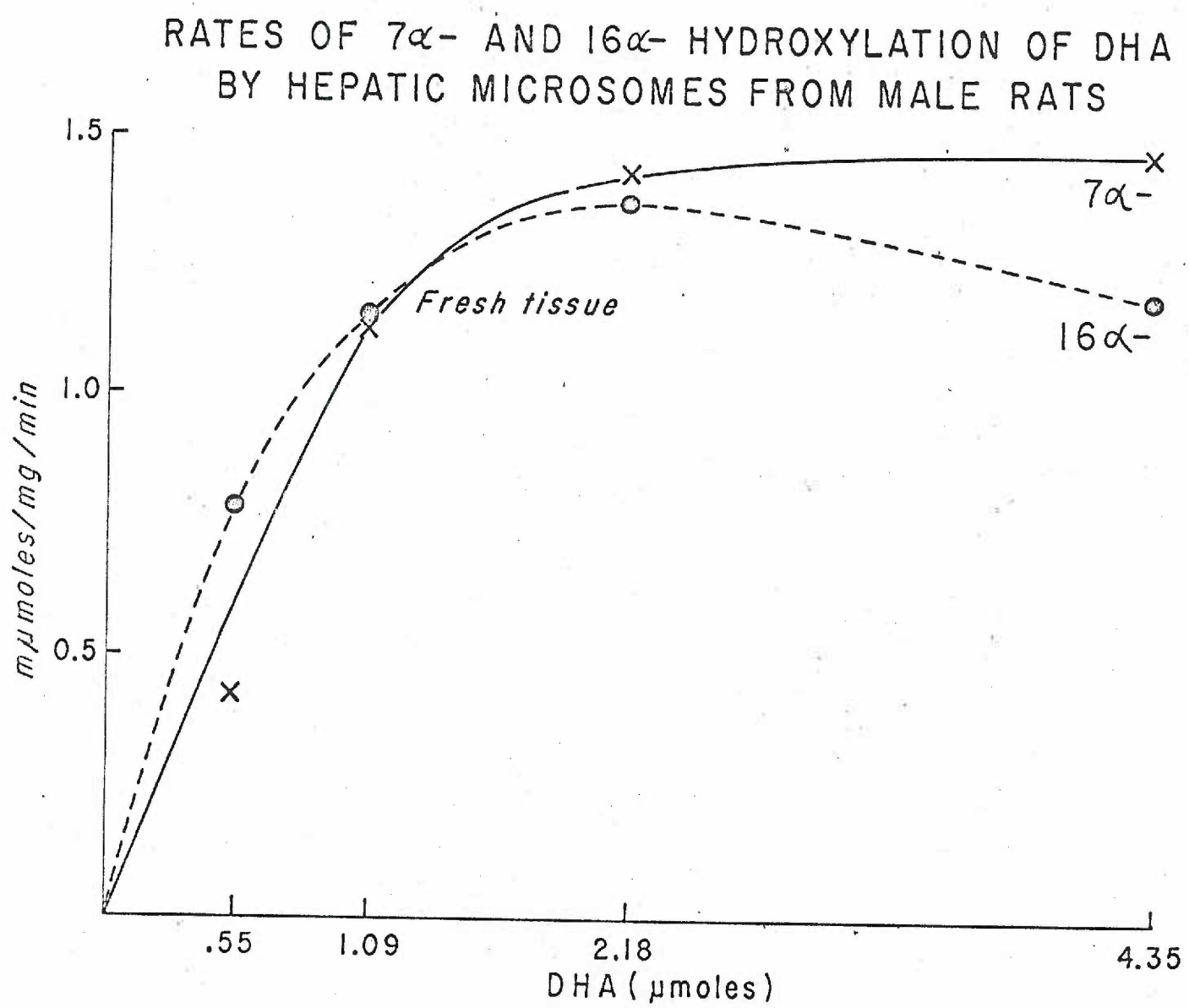
5. Steroid hydroxylase activity.

In preliminary experiments with the 16α-hydroxylase system, the incubation conditions for microsomal fractions were adopted from previous studies with liver slices (16). The basic incubation mixture contained 8.65 μmoles of DHA in 0.1 ml. propylene glycol, microsomes (fresh or lyophilized) equivalent to 0.3 to 1.0 g. fresh liver in 5 ml. of 0.25 *M* sucrose, 4.16 μmoles of NADPH and 15.0 ml. of Krebs-Ringer phosphate, and the reaction was carried out at 37°C with shaking in air for 2.5 hours. These conditions were used, with slight modifications as indicated in the text, for all studies with lyophilized microsomes and with those obtained from the rhesus and the rats in the study of age effects and substrate specificity. Incubation conditions were constant within any set of experiments.

Optimal incubation conditions for the steroid hydroxylase system were determined experimentally for hepatic microsomal systems and they were utilized for the remaining experiments. The final 6.0 ml. mixture in a 25 ml. Erlenmeyer flask consisted of 1 to 3 mg. of microsomal protein, 1.09 μmoles of DHA in 0.1 ml. of propylene

glycol (1.83×10^{-4} *M* in the incubation mixture), 12.2 µmoles of NADPH in 0.6 ml. of deionized water, and 5.1 ml. of Krebs-Ringer phosphate buffer, pH 7.4. The contents were incubated for 30 minutes with shaking in air at 37°C and the reaction was stopped by placing the flasks in crushed ice. Control incubation mixtures with substrate added terminally were always included. Results from control flasks thus prepared were the same as when the substrate was incubated with tissue that had been inactivated by boiling for three minutes. The substrate concentration of 1.83×10^{-4} *M* (1.09 µmoles) was chosen as the optimal one because the effect of doubling that concentration represented only about a 20% increase in activity in both the lyophilized and fresh microsomal fractions (see Figure No. 4). Recovery experiments of DHA or 16α-OH-DHA after the incubations showed that 70 to 90% of the added substrate remained unmetabolized and that most of the added product also could be recovered. The microsomal solutions prepared equivalent to 1 g. fresh tissue/ml. were used for the incubation studies immediately after their preparation, to prevent deterioration in the enzymatic activities. Therefore, it was not possible to use a constant amount of protein. Since the reaction was linear to 4.3 mg. of microsomal protein, the range 1 to 4 mg. was used as the optimum amount. An excess of NADPH was added to insure maximum rates. Although addition of 6.1 µmoles of NADPH gave maximal rates of 16α-hydroxylation in two experiments, that amount was doubled to 12.2 µmoles to insure an excess for all tissue preparations. No inhibition was observed in the presence of an excess of cofactor and the pH of the mixture did not change from 7.4 during the reaction period. A comparison of the rates reported

Figure No. 4. Rates (mμmoles/mg./min.) of 7α- and 16α-hydroxylation of different amounts of DHA by rat liver microsomes. Conditions: 30 min. incubations at 37° C. of a mixture of DHA, 12 μmoles NADPH, 1-4 mg. microsomal protein and 5.1 ml. of buffer - final volume 6 ml.


RATES OF 7α- AND 16α- HYDROXYLATION OF DHA
BY HEPATIC MICROSOMES FROM MALE RATS
1.5
1.0
0.5
mμmoles/mg/min
7α-
16α-
Fresh tissue
.55
1.09
2.18
4.35
DHA (μmoles)

for 16α-hydroxylation by slices with those by microsomes shows that the latter have a higher rate of transformation in short periods of incubation (see Figure No. 5). Intervals of 30 minutes were chosen because in that period the rate of substrate utilization was low; enough product had accumulated for accurate colorimetry and little product was metabolized in that period. Therefore, the rates reported as mμmoles of 16α-OH-DHA formed/mg. microsomal protein/minute represent net rates for the described conditions and not necessarily initial rates, especially in the experiments where the microsomal enzymatic activities were stimulated by drug treatments. The rates are uncorrected.

In the incubations that tested the effects of aminopyrine, SU-9055, KCN, and CO on the enzymatic activities, the former three compounds (amounts given in the Results section) were added as the last reagent. Experiments with CO were done in a ventilated hood using a bell jar to mix the CO and O_2 (4:1) which were quantitated by flow meters. The Warburg flasks were wrapped in aluminum foil to prevent light interference. The system containing microsomes, substrate and buffer in the flask and NADPH in the side-arm was flushed by bubbling the gas mixture into the solutions for one minute prior to closing and mixing them. Each flask was unstoppered and reflushed with the gas mixture at ten minute intervals during the incubation.

The extraction method used for rhesus liver (3 extractions with boiling acetone and 3 more with chloroform), was adopted from studies with slices. However, the rates of recovery of added 16α-OH-DHA (18 μg.) from incubation media was 87 to 103% when only two 40 ml. chloroform extractions were made (see Table No. 3). The

Figure No. 5. Rates (mμmoles/mg./min.) of 16α-hydroxylation of DHA for different durations of incubation by lyophilized liver microsomes from male rats. The incubation mixture contained 1.09 μmoles DHA, 6.24 μmoles NADPH and microsomal powder equivalent to 0.328 g. of fresh tissue in Krebs-Ringer phosphate pH 7.4 - total volume 5.4 ml.

RATES (mμMol/mg/min) OF 16α-HYDROXYLATION OF 3β-HYDROXYANDROST-5-EN-17-ONE FOR DIFFERENT DURATIONS OF INCUBATION BY LYOPHILIZED LIVER MICROSOMES FROM MALE RATS.

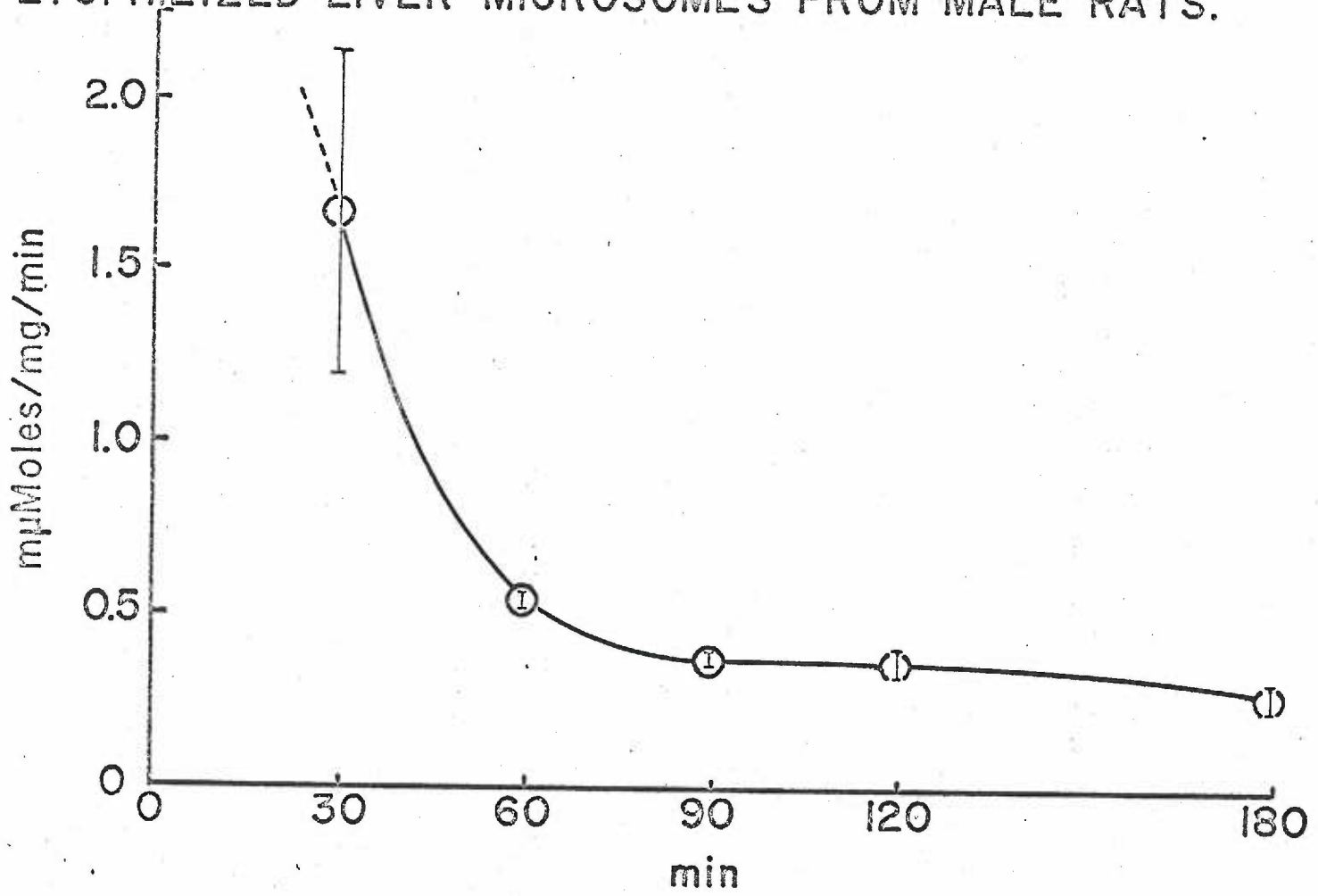


Table No. 3. Recovery of 16α-OH DHA added to different microsomal-tissue mixtures.

RECOVERY OF 16α-OH-DHA ADDED TO DIFFERENT MICROSOMAL-TISSUE MIXTURES

Mixture *	18 µg. added		313 µg. added	
	µg. recovered	%	µg. recovered	%
Unincubated tissue	18.4	103	252	81
Boiled tissue	16.5	92	—	—
Incubated tissue	15.6	87	157	50
Before incubation (30 min.)	10.2	66	69	22

*2.5 to 3.48 mg. protein, 12 µmoles NADPH, KRP buffer, pH 7.4, and the steroid in 6 ml.

latter extraction procedure was adopted for subsequent experiments. The combined chloroform extracts were washed with water, dried with 9.0 g. of anhydrous sodium sulfate, filtered and evaporated in an all-glass Büchi rotary evaporator.

For most of the experiments with rhesus liver and all of those using lyophilized preparations, including the age and substrate studies with rat liver, the product was quantitated as the crude residue. Subsequently, however, they were dissolved in 0.1 ml. of methanol and applied in 4 adjacent lanes onto 0.025 mm. layers of silica gel G[1] activated at 120°C for one hour. The 20x20 cm. plates were developed in system C [ethyl acetate-cyclohexane, 50:50 (98)], system B [ethyl acetate-*n*-hexane-ethanol, 80:15:5 (99)], or system M [ethyl acetate-*n*-hexane-acetic acid, 75:20:5 (98)] to a distance of 15 cm. Inspection with a Chromatovue scanner systematically revealed no products absorbing ultraviolet light. One lane from each residue and appropriate amounts of reference compounds were sprayed in a ventilated hood with a picric acid reagent for 3β-hydroxy-Δ^5-steroids [100 mg. of picric acid in 36 ml. of glacial acetic acid and 6 ml. of 70% perchloric acid (40)]. The colors developed maximally when the plate was warmed. Photographic records of all plates were obtained using Polaroid color film type 48 (see Figure No. 6). Areas of silica gel within the remaining lanes that represented 75% of the incubation products and a blank area were scraped off for elution and quantitation. The elution usually consisted

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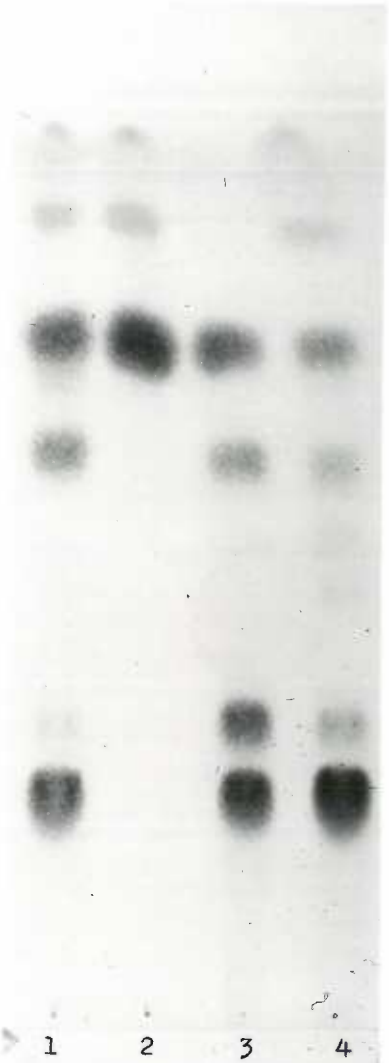
[1] E. Merck A.G., Darmstadt, Germany

Figure No. 6. Photograph of a thin-layer chromatogram containing one-fourth of the residue from representative incubations of a hepatic microsomal fraction with steroid. The spots, which develop after spraying with picric reagent, represent (in lane 3-reference steroids, from the bottom of the photograph, upward) about 5 μg. of 7α-OH-DHA, 7β-OH-DHA, 16α-OH-DHA, and DHA.



HEPATIC MICROSOMES
1-normal male rat
2-blank
3-reference steroids
4-phenobarbital-induced male rat

of two extractions with 2 ml. of 95% aq. methanol (45) followed each time by centrifugation. Recoveries of the 16α- and 7α-hydroxy products from the silica gel ranged from 87 to 97%. Duplicate aliquots were evaporated *in vacuo* for colorimetric analysis with the blue tetrazolium (BT) reaction for 16α-OH-DHA (47) and modifications of the Lifschütz reaction (94,95) for the 7-hydroxylated epimers of DHA.

a. DHA 16α-hydroxylase activity. The product (C), labeled 16α-OH-DHA, was identified in thin-layer chromatography (TLC) eluates by a correspondence with the reference compound in three TLC systems as the free alcohol (The R_F's in systems B, C, and M were 0.68, 0.23, and 0.53) and in paper system E-1 [2,2,4-trimethylpentane:methanol: water (10:9:1 by vol.)] (39) as the acetylated derivative (R_F 0.55) and by the spectral characteristics in concentrated sulfuric acid and in the Oertel-Eik-Nes reaction (117) at 15 minutes and two hours (see Table No. 4 and 5). The product gave a positive colorimetric response to the picric acid, Pettenkofer and BT reagents sprayed on the chromatograms.

For quantitation, 0.175 ml. of a solution of 0.07% blue tetrazolium reagent in 95% ethanol (w/v) and 0.125 ml. of a solution of 1% tetramethylammonium hydroxide in 90% ethanol (w/v) reacted with the residue for one hour at 25°C. Two ml. of 1% acetic acid (v/v) in 50% ethanol was added and the absorbancies at 480, 520 and 560 were read permitting application of Allen's correction [A_{520} corrected = $2(A_{520}) - (A_{480} + A_{560})$]. Comparison of the corrected absorbancies, minus the values for silica gel blanks, with those given by duplicate samples of 15.2 µg. of reference steroid permitted calculations of

Table No. 4. Spectral data obtained from the reaction of concentrated sulfuric acid (1.0 ml. mixture - 2 hrs. at room temperature) with pure 16α-OH-DHA and product C isolated from incubation mixtures of hepatic microsomal fractions.

SPECTRAL DATA* FROM 16α-OH-DHA AND PRODUCT C FROM INCUBATIONS OF HEPATIC MICROSOMAL FRACTIONS.

	16α-OH-DHA (≈ 6 μg.)		Product C (≈ 7 μg.)	
	mμ ;	Absorbancy	mμ ;	Absorbancy
Maxima	(570;	0.161	570;	0.114
	(490;	0.128	470;	0.131
	(376;	0.326	375;	0.301
	(270;	0.129	270;	0.128
Minima	(510;	0.131	520;	0.103
	(450;	0.113	450;	0.128
	(315;	0.172	315;	0.129
	(230;	0.068	260;	0.119

* Corrected for the silica gel blank.

Table No. 5. Spectral data in Oertel-Eik-Nes reaction (2 ml.) for 16α-OH-DHA and product C isolated from incubation mixtures of hepatic microsomal fractions and DHA.

SPECTRAL DATA IN THE OERTEL-EIK-NES REACTION (2 ML.) FOR 16α-OH-DHA AND PRODUCT C.

	16α-OH-DHA[1]		Product C[2]	
(15 min. period)	mμ	Absorbancy	mμ	Absorbancy
Maxima	615	0.087	610	0.032
	405	0.414	405	0.109
	380	0.330 (I)	380	0.100 (I)
Minima	450	0.021	440	0.029
(120 min. period)				
Maxima	615	0.351	615	0.068
	405	0.139 (I)	405	0.069 (I)
	380	0.279	380	0.087
Minima	450	0.039	440	0.040

1. 12 μg. of reference steroid.
2. Represents 1/26 of the yield of the following incubation: 4 mg. DHA, 192 mg. microsomal protein, 154 μmoles NADPH, and 97.6 ml. of buffer - total volume 121.6 ml; duration 2.5 hrs. The absorbancies of the product eluted from the 16α-OH-DHA zone (R_F = 0.23 - System C) in TLC were corrected by subtracting readings obtained from a comparable incubation without DHA.

hydroxylation rates which are expressed as mμmoles of 16α-OH-DHA formed/mg. protein/minute (47). The molar extinction coefficient of pure 16α-OH-DHA is 13,164 cm.$^{-1}$ M^{-1} in the BT reagent. From 74 duplicate determinations at the 15.2 μg. level, the sensitivity was 0.11 μg. and the precision was ± 0.28 μg. (1.9%).

For the detection of the 16α-hydroxylated products when different substrates were incubated, the BT reaction was used systematically and the results are expressed with reference to 16α-OH-DHA.

b. DHA 7-hydroxylase activity. The evidence for the identification of 7α-, and 7β-OH-DHA is given for both compounds in the Results section where the formation of the latter compound, as a new hydroxylation of a steroid hormone *in vitro*, is described.

For quantitation of each 7-hydroxy epimer of DHA, 1.0 ml. of freshly pre-mixed Pettenkofer reagent was added to duplicate evaporated aliquots. The reagent is composed of a mixture (1:5:15 v/v) of glacial acetic acid, 1% furaldehyde (in acetic acid:water/50:50) and 17 *N* sulfuric acid (75). The resulting color with its maximum absorbance at 580 mμ was stable between 10 and 20 minutes thereafter (see Figure No. 7). The absorbancies at 555, 580 and 605 were read in the ten minute interval and the corrected absorbancy was compared to values determined similarly with different amounts of pure 7α-, and 7β-OH-DHA which exhibited linear responses to 10 μg./ml., the maximum amount of each tested (see Figure No. 8). The respective molar extinction coefficients at 580 mμ were 17,580 cm.$^{-1}$ M^{-1} for the 7α-hydroxy compound and 19,800 cm.$^{-1}$ M^{-1} for the 7β-hydroxy epimer.

Figure No. 7. Effect of time on the modified Lifschütz reaction with the 7-hydroxy-epimers of dehydroepiandrosterone (5 μg./ml.). The absorbancies represent corrected values.

EFFECT OF TIME ON THE MODIFIED LIFSCHÜTZ REACTION WITH THE 7-HYDROXY-EPIMERS OF 3β-HYDROXYANDROST-5-EN-17-ONE (5 µg/ml).

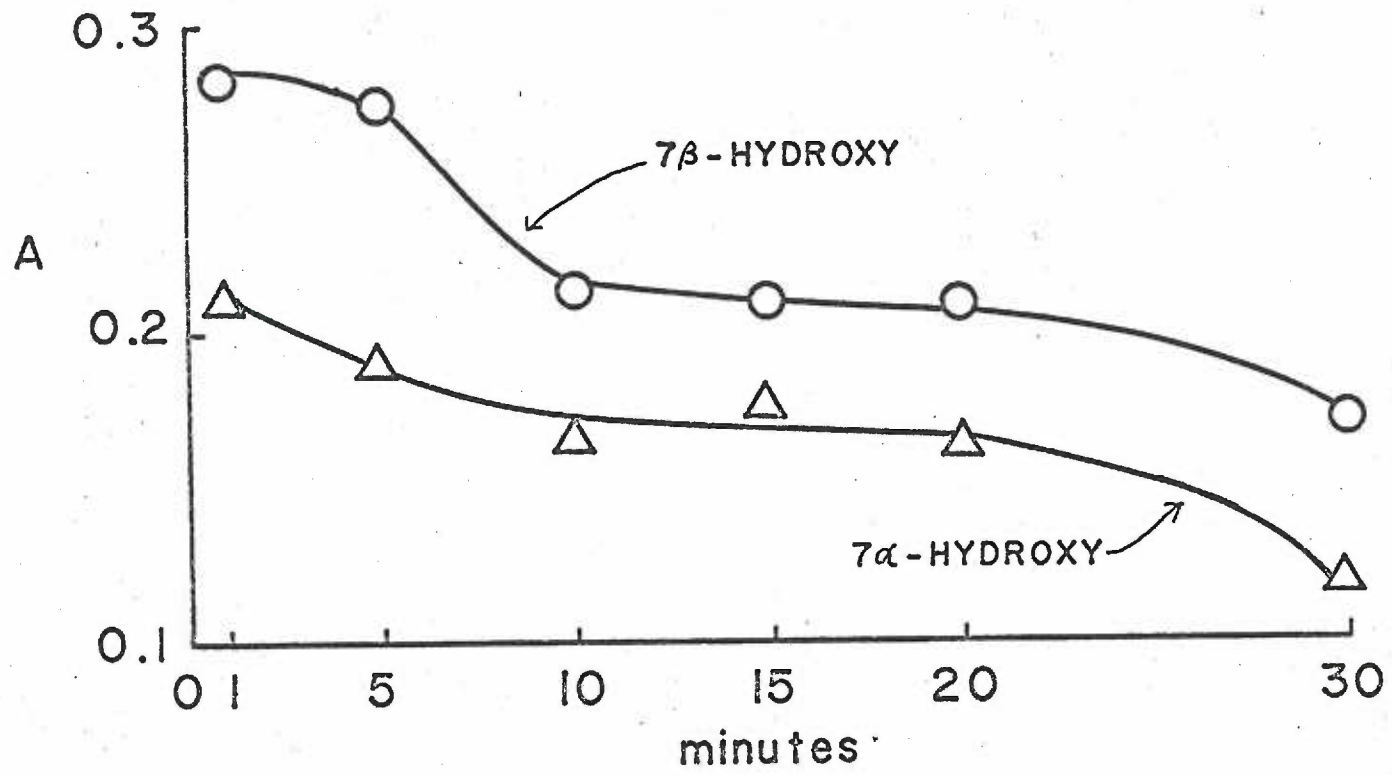
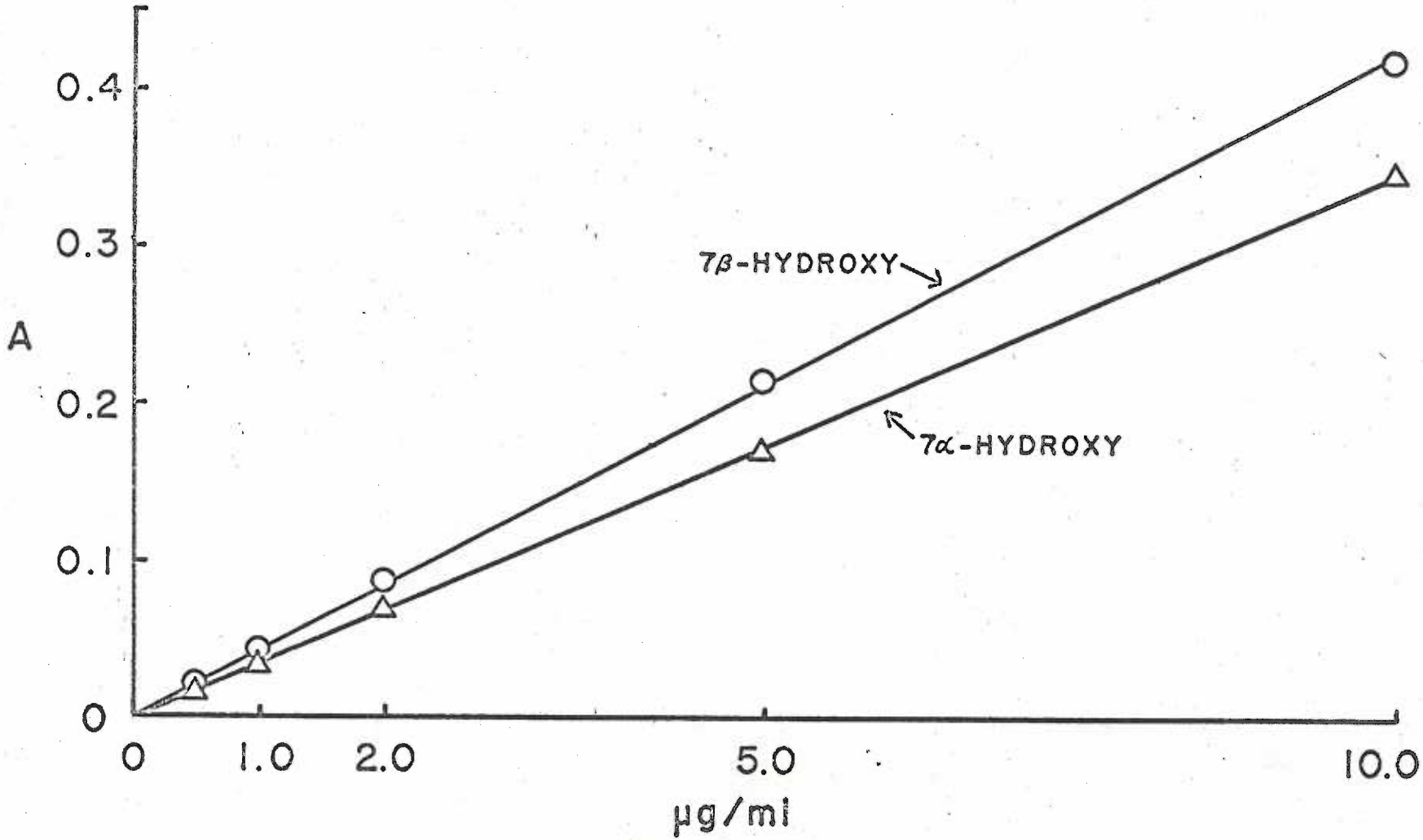


Figure No. 8. Linearity of the modified Lifschütz reaction with the 7-hydroxy-epimers of dehydroepiandrosterone. The absorbancies represent corrected values.

LINEARITY OF MODIFIED LIFSCHUTZ REACTION WITH 7-HYDROXY-EPIMERS OF 3β-HYDROXY-ANDROST-5-EN-17-ONE.



6. Materials for steroid hydroxylase studies.

Steroids: With the exception of 16α-OH-DHA, the compounds were gifts to Dr. Colás or purchased from commercial suppliers. These steroids were used without further purification after checking, in some instances, melting points and their spectral properties.

DHA, Schering Co., Berlin; M.P. 151.5 to 152.5°C was dissolved in propylene glycol (1.09 μmoles/0.1 ml.) as the usual substrate solution.

16α-OH-DHA, supplied by Dr. Colás from a lot prepared from synthetic 3β, 16α, 17α-trihydroxypregn-5-en-20-one by periodate oxidation. M.P. 183 to 188°C.

7α-OH-DHA was given by Dr. David K. Fukushima, M.P. 174 to 180°C.

7α-diacetoxydehydroepiandrosterone was given by Dr. R.M. Dodson, M.P. 168 to 170°C.

7β-OH-DHA was given by Dr. R.M. Dodson, M.P. 215 to 215.5°C.

7-keto-DHA was given by Dr. W. Klyne from the Medical Research Council Steroid Reference Collection, M.P. 232 to 234°C.

Androstenedione, obtained from Calbiochem, Los Angeles, California, Lot 32522.

Androsterone, obtained from Sigma Chemical Co., Lot No. A. 105-1.

Etiocholanolone, given by Dr. W. Klyne from the MRC Reference Steroid Collection.

3β-hydroxy-5α-androstan-17-one, given by Dr. W. Klyne and the MRC Reference Steroid Collection.

3β-hydroxy-5β-androstan-17-one, given by Dr. W. Klyne and the MRC Reference Steroid Collection.

Estrone, obtained from Sigma Chemical Co., St. Louis, Mo., Lot No. E95-44, M.P. 260°C.

Reagents: Chemicals were reagent grade except for methanol which was spectral grade and chloroform which was washed and redistilled (152). The mid-portion of the third distillate at reduced pressure of 2-furaldehyde was stored at -14°C. A solution of 1% (v/v) in 50% acetic acid (w/v) was stable at 4°C. The tetramethyl-ammonium hydroxide solution[1], was 1% (w/v) in 90% ethanol. Blue tetrazolium reagent [3:3' - dianisolebis - 4:4' (3:5-diphenyl) tetrazolium chloride][2] was a 0.07% (w/v) solution in 95% ethanol. SU-9055[3], [3-(1,2,3,4-tetrahydro-1-oxo-2 naphthyl)-pyridine] was dissolved in 10^{-2} *M* tartaric acid. The crystalline human chorionic gonadotrophin (HCG)[4] in a vial containing 5000 I.U. was weighed and portions corresponding to 100 and 500 I.U. were weighed and dissolved in the incubation media.

E. Miscellaneous procedures.

Melting points (uncorrected) were obtained using a Kofler hot stage microscope. Infrared spectra of steroids in KI pellets were determined with a Perkin-Elmer Model 221 instrument utilizing KB-015 microframes[5]. Acetylation of steroids was done overnight at room

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[1]Eastman Organic Chemicals, Rochester, N.Y.

[2]Mann Research Laboratories, Inc., N.Y., N.Y.

[3]A gift from CIBA Pharmaceutical Co., Summit, N.J.

[4]Upjohn Co., Kalamazoo, Michigan

[5]Western Scientific Associates, San Ramón, California

temperature in 0.1 ml. acetic anhydride and 0.1 ml. of pyridine. The Oertel-Eik-Nes reaction (117) was at room temperature using 2 ml. of reagent (sulfuric acid:ethanol/1:2). At 15 and 120 minutes thereafter, the spectrum was read in a Cary model 15 recording spectrophotometer. The concentrated sulfuric acid spectra were read after a two-hour reaction at room temperature. The microanalytical Zimmerman reaction (154) was modified to utilize 95% ethanol for the preparation of the reagents and no ascorbic acid was added to stabilize the 2.5 KOH. The Pettenkofer reaction (75) was also done using 1% furaldehyde-acetic acid mixture and 17 *N* sulfuric acid but with heating for 12 minutes at 67°C.

Additional reagents for identification of compounds on thin-layer chromatograms and the conditions for color development include:

1. Anisaldehyde reagent - 1% (w/v) solution of distilled anisaldehyde in glacial acetic acid to which 2% (v/v) of concentrated sulfuric acid was added immediately prior to spraying. Thereafter, the plates were heated about 6 to 8 minutes at 85°C (97).

2. Antimony trichloride reagent - 20% (w/v) solution of antimony trichloride in chloroform. After spraying the chromatographed residues, the plates were heated to 85°C for 15 minutes (113).

3. Pettenkofer reagent - (the preparation is described in the section on quantitation) The plates were sprayed and heated to 85°C for 6 minutes (18).

4. Blue tetrazolium reagent - a mixture (1:9) of 1% (w/v) solution of blue tetrazolium in distilled water and a 2 *N* NaOH in methanol was freshly prepared and sprayed on the plates which were then heated to 85°C for 5 minutes (115).

III. RESULTS

The organization of this section roughly follows the experimental design which included studies of biological differences of microsomal hydroxylations and *N*-demethylation and alteration, in the rat, of those enzymatic activities after drug treatment. Other experiments tested some kinetic parameters in the relationship between the enzymes. Also, physicochemical alteration of the membranes with deoxycholate, a non-ionic detergent, was the basis for another type of comparison. Finally, the isolation and identification of 7β-hydroxylation of DHA as a new transformation *in vitro* of a steroid hormone is described.

A. Microsomal hydroxylation and *N*-demethylation.

The body and liver weights of the rats, and the protein contents of the hepatic subcellular fractions are in Tables No. 6 (total microsomal preparations) and 7 (subfractions). The data in those tables represent separate sets of experiments but they contain duplicate observations for some parameters such as the weight of the organs after the drug treatments. Except for the experiments with phenobarbital, where some animals died prematurely, all of the values in Section A represent at least duplicate experiments done on separate days with at least two adult animals in each experiment.

1. Protein, RNA, and cytochrome P-450 contents and succinic-cytochrome *c* reductase activity.

a. Body weight and protein content. The average weight of the male animals and their livers was 287 and 12.1 g. respectively and 175 and 8.8 g. for the females (see Table No. 6 which also shows

Table No. 6. Body and liver weights from rats and protein and cytochrome P-450 contents in microsomal fractions from rat liver. The values are mean values ± standard deviations, if given.

DESCRIPTIVE DATA* OF NONFASTED AND DRUG-TREATED RATS: PROTEIN AND CYTOCHROME P-450 IN HEPATIC MICROSOMAL FRACTIONS.

	Nonfasted		Drug-Treated					
			Phenobarbital		3-Methylcholanthrene		Phenylbutazone	
	♂	♀	♂	♀	♂	♀	♂	♀
Number of rats	13	8	2	3	4	4	4	4
Body weight (g.)	287	175	244	162	237	181	250	174
Liver weight (g.)	12.1	8.8	15.3	10.3	13.7	9.7	12.3	10.0
Microsomal protein: (mg./g. liver)	14.6 ± 3.2	11.3 ± 1.5	15.7	14.1	14.5	12.9	15.1	12.7
% increase:	–	–	8	28	-1	14	3	12
Microsomal cytochrome P-450 (μmoles/mg. prot.)	0.48 ± 0.22	0.40 ± 0.21	1.00	0.68	0.77	0.78	0.48	0.33
% increase	–	–	108	70	61	95	0	-18

* Values are means (± S.D. when given).

Table No. 7. Body and liver weights and protein content in smooth and rough subfractions of microsomes from rat liver. The values represent mean values ± S. D., if given.

DESCRIPTIVE DATA* OF NONFASTED, FASTED AND DRUG-TREATED RATS: PROTEIN CONTENT OF THE HEPATIC SUBCELLULAR FRACTIONS (INCLUDING SMOOTH AND ROUGH MICROSOMES).

	Nonfasted		Fasted		Drug-Treated					
					Phenobarbital		3-Methyl-cholanthrene		Phenylbuta-zone	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Number of animals	14	8	20	5	2	3	4	4	4	4
Body weight (g.)	294	207	308	200	284	203	299	171	276	170
Liver weight (g.)	10.0	6.8	8.7	7.7	13.8	7.4	13.9	7.8	11.9	7.6
Protein content (mg./g.):										
10,000 g. sediment	87.6	115.5	88.9	77.6	81.5	98.5	77.0	-	-	-
Smooth microsomes	1.9 ± 0.5	3.0 ± 1.8	2.7 ± 1.8	3.1 ± 2.1	14.9	8.5	4.4	4.8	4.7	5.6
% increase**					685	183	132	60	147	87
Rough microsomes	11.3 ± 3.1	5.9 ± 1.2	14.1 ± 4.0	10.3 ± 2.6	7.3	7.2	9.1	7.3	9.1	6.4
% increase**					-35	22	-19	24	-19	9

* Values are means (± S.D. when given).
** Based upon values for similarly nonfasted rats.

that the livers from animals treated with phenobarbital or 3-methylcholanthrene were heavier than those from the control rats which weighed more). The total microsomal protein content/g. liver was approximately equal in all of the rats, except the fasted ones which had a smaller organ and slightly more microsomal protein/g. fresh liver. The protein content of the total microsomal fractions from non-fasted male rats was 14.6 ± 3.2 mg./g. fresh tissue ($\bar{x}$ ± S.D.) and the value for female rats, 11.3 ± 1.5.

The smooth subfractions of liver from fasted and non-fasted males contained 2.65 ± 1.8 and 1.91 ± 0.5 and the values for females were 3.1 ± 2.1 and 3.0 ± 1.8 mg./g. liver (see Table No. 7). These values were much smaller and less variable than those for the respective rough subfractions which were 14.1 ± 4.0 and 11.28 ± 3.1 for fasted and non-fasted males and 10.3 ± 2.6 and 5.9 ± 1.2 respectively for females.

The increase in the total microsomal protein/g. liver resulting from phenobarbital treatment is selectively apparent in the smooth subfractions of both sexes, to 14.9 mg./g. for males and to 8.5 mg./g. liver for females; however, the content in the rough subfractions from males was simultaneously reduced with the phenobarbital treatments to 7.3 mg./g. Increases over the control values were less marked when 3-methylcholanthrene or phenylbutazone were injected. Male rat liver usually contained more total and rough microsomal protein than the female tissue but the latter usually had more protein in the smooth microsomal fraction and only the females responded to drug treatment by also increasing the protein content of their rough subfractions; it actually decreased in the males.

The smooth subfraction from male rabbit liver contained an average of 2.8 (range 1.8 to 4.2) mg. protein/g. of fresh tissue and the rough subfraction had 3.5 (range 3.0 to 4.2) mg. protein/g. in three experiments (see Table No. 8). The comparable values for female rabbits were 3.7 (range 2.4 to 5.0) and 3.3 (range 2.2 to 4.4) mg. protein/g. of fresh liver. These values represent the fasted state and the sums of the smooth and rough fractions are much lower than those observed from fasted rats.

b. RNA content. The ribonucleic acid (RNA) content of the microsomal subfractions was determined to confirm the accuracy of the separation of the lighter smooth microsomal membranes from the heavier rough microsomal membranes, although recent evidence (28) definitely indicates that significant amounts of RNA are present in the smooth membranes. The rough subfractions, presumably containing the attached ribosomes, had 79 ± 18 µg. RNA/mg. protein compared to 39 ± 10 µg./mg. for smooth subfractions from male rats (see Table No. 9). A similar relation existed for the females which had 88 ± 21 and 55 ± 16 µg./mg. protein respectively for rough and smooth subfractions. The variation among the samples is apparent from the size of the standard deviations and the overlap of the extended values; however, when paired values are compared, the mean difference between rough and smooth subfractions is significant in every instance where data from three or more experiments are available.

The RNA content in the rough and smooth microsomal subfractions from male rabbits was 86 vs. 58 µg./mg. protein, respectively (refer back to Table No. 8). The related values from females were 119 and 81. Both differences were significant at the 1 or the 5% probability level (33, 100,151).

Table No. 8. Comparison of protein and RNA content, and enzymatic transformations by microsomal subfractions of liver from fasted rats and rabbits.

COMPARISON OF PROTEIN AND RNA CONTENT*, AND ENZYMATIC TRANSFORMATIONS* BY MICROSOMAL SUBFRACTIONS OF LIVER FROM FASTED RATS AND RABBITS.

Subfractions	Sex	Rat			Rabbit		
		Smooth (S)	Rough (R)	Ratio S/R	Smooth (S)	Rough (R)	Ratio S/R
Protein (mg./g./fresh tissue)	M	2.65	14.08	0.19	2.80	3.50	0.80
	F	3.09	10.32	0.29	3.73	3.30	1.13
RNA (μg./mg. protein)	M	27	69	0.39	58	86	0.64
	F**	55	88	0.62	81	119	0.64
Enzymatic Transformations:[1] (mμmoles/mg./min.)							
N-Demethylation	M	2.28	2.25	0.92	4.61	3.89	1.38
	F**	1.63	1.84	0.83	4.94	3.85	1.32
p-Hydroxylation	M**	0.42	0.33	1.42	1.82	1.53	1.07
	F**	0.40	0.57	0.56	1.61	1.22	1.32
16α-Hydroxylation	M	1.20	1.40	0.94	0.84	0.71	1.23
	F**	0.04	0	–	1.00	0.64	1.90

* Mean values
** Nonfasted
1 Substrates were aminopyrine, acetanilide and dehydroepiandrosterone, respectively

Table No. 9. RNA (μg./mg. protein) in microsomal subfractions from liver of rabbits and rats.

RNA (μg./mg. protein) IN MICROSOMAL SUBFRACTIONS FROM LIVER OF RABBITS AND RATS.

	Sex	No. Expts.	Smooth* (S)		Rough* (R)		Ratio S/R	P**
			$\bar{X}$	S. D.	$\bar{X}$	S. D.		
rabbit (fasted)	M	3	58	–	86	–	0.64	< 0.05
	F	3	81	–	119	–	0.64	< 0.01
rat								
fasted	M	3	27		69	–	0.39	< 0.02
nonfasted	M	7	39	10	79	18	0.50	< 0.01
nonfasted	F	4	55	16	88	21	0.62	< 0.01
treated: ***								
phenobarbital	M	2	33	–	57	–	0.58	< 0.10
	F	2	71	–	135	–	0.54	< 0.10
3-methylcholanthrene	M	2	54	–	94	–	0.58	< 0.10
	F	2	47	–	118	–	0.39	< 0.10
phenylbutazone	M	2	47	–	80	–	0.59	< 0.10
	F	2	61	–	120	–	0.51	< 0.05

* the average content/duplicate determination was 2.35 mg. for smooth and 2.46 mg. for rough subfractions.

** calculated for individual pairs by $\frac{X_N + X_i - 2\mu}{X_N - X_i}$ and $\mu = o$; values represent maximum probabilities. (33, 100, 151)

*** nonfasted

c. Cytochrome P-450 content. In the total microsomal fractions (refer back to Table No. 6), male rats had 0.48 ± 0.22 mμmoles of cytochrome P-450/mg. protein and females had 0.40 ± 0.21. The values from males treated with phenobarbital, 3-methylcholanthrene and phenylbutazone were 1.00, 0.77, and 0.48 mμmoles/mg. respectively and represent average increases of 108, 61, and 0%. Female rats treated with phenobarbital, 3-methylcholanthrene, and phenylbutazone had an average of 0.68, 0.78, and 0.33 mμmoles of P-450/mg. microsomal protein representing average increases of 70, 95% by the former agents and a 18% decrease by phenylbutazone.

d. Succinic-cytochrome *c* reductase activity. This activity which characterizes mitochondrial subcellular fractions was determined in an effort to establish the amount of mitochondrial contamination in microsomal fractions from rat liver. In preparations utilizing a single 10,000 *g* centrifugation before further sedimentation, the respective mean enzymatic rates for cytochrome *c* reduction by 10,000 *g* sediment (mitochondria, unbroken cells, etc.), rough microsomes, smooth microsomes and supernatant were 124, 25, 15 and 3 mμmoles mg. protein/minute (see Table No. 10). When the 10,000 *g* supernatant was diluted to an isotonic solution and recentrifuged, those values were reduced to 94, 4 and 3 respectively (the supernatant was not analyzed). On the basis of this result, the dilution and recentrifugation procedure was systematically incorporated into the method for preparation of microsomal fractions.

Table No. 10. Effect of the second 10,000 g max. centrifugation on the succinic-cytochrome c reductase activity (mmoles/mg./min.) in subcellular fractions of rat liver.

EFFECT OF THE SECOND 10,000 $\underline{g}$ max. CENTRIFUGATION ON THE SUCCINIC-CYTOCHROME C REDUCTASE ACTIVITY (mμmoles/mg./max.) IN SUBCELLULAR FRACTIONS OF RAT LIVER.

Centrifugation of Homogenized Tissue	Mitochondria	Rough Microsomes	Smooth Microsomes	Supernatant
10,000 $\underline{g}$ max. x 35 min.				
$\overline{X}$	124	25	15	3
S. D.	41	21	9	4
% mitochondrial contamination	–	20	12	2
no. preparations	11	11	11	8
10,000 $\underline{g}$ max. x 35 min.; add 5 vol. 0.04 $\underline{M}$ KCl; repeat centrifugation				
$\overline{X}$	94	4	3	–
S. D.	36	2	1	–
% mitochondrial contamination	–	4	3	–
no. preparations	6	6	6	0

2. Subcellular distribution of steroid enzymatic activities; effect of lyophilization.

The specific activities of the steroid 7- and 16α-hydroxylase systems were greatest in the microsomal fraction (see Table No. 11). In two experiments, 48 to 50% of the total cellular 7α- and 16α-hydroxylase activities was detected in the microsomal fraction and 46 to 52% in the 10,000 *g* sediment. All of the 7β-hydroxylase activity was found in the microsomal fraction. Since no attempt was made to prepare mitochondrial fractions free of microsomes, the observed 16α-hydroxylase activity in the 10,000 *g* sediment cannot be ascribed to mitochondria alone.

In the earliest experiments with the microsomal 16α-hydroxylase system, batches of microsomes prepared from several rats were tested and the remainder was immediately lyophilized. After the lyophilized preparations were observed to retain their 16α-hydroxylase activity (µg./g. tissue/hour), they were used to study the optimal incubation conditions for the hydroxylation (see Table No. 12). The activity per mg. of powder appeared to decrease in the preparations stored *in vacuo* at room temperature; however, most of those stored at -14°C were stable or increased. Unfortunately, the water content of these preparations was not measured.

3. Effects of age, sex, species, and substrate modifications.

Liver microsomes from immature rats of either sex have a low activity (µg./g./hour) of 16α-hydroxylation. Subsequently, it appears to develop rapidly in male rats to a maximum coinciding with sexual maturation at about 50 to 60 days of age (see Figure No. 9). The activity was considerably less in liver microsomes from rhesus monkeys

Table No. 11. The 7- and 16α-hydroxylation of DHA: subcellular distribution in liver from male rats.

THE 7- AND 16α-HYDROXYLATION OF DHA: SUBCELLULAR DISTRIBUTION IN LIVER FROM MALE RATS.

Fraction	Expt. No.	Protein (mg./g. fresh tissue)	7α-		7β-		16α-	
			rate[1]	%[2]	rate[1]	%[2]	rate[1]	%[2]
10,000 xg max. sediment (cello, nuclei, mitochondria)	1	80.4	0.29	46	0	0	0.45	52
	2	70.0	0.23	50	0	0	0.28	46
78,000 xg av. sediment (microsomal)	1	17.9	1.39	49	0.12	100	1.82	48
	2	18.0	0.88	50	0.14	100	1.15	49
supernatant	1	47.0	0.06	5	0	0	0	0
	2	46.7	0	0	0	0	0.05	5

1. mμmoles/mg. protein/minute.
2. % of the total activities corresponding to one g. of fresh tissue.

Table No. 12. The stability of the 16α-hydroxylase system in lyophilized microsomal fractions. Preparations A through C are from male rats (A_1 and A_2 are from the same fraction) and those designated H_1 to H_3 are from two human male anencephalic fetuses (H_1 and H_2 are from the same fetus). The incubation mixture was microsomal powder equivalent to 1 g. tissue, 8.65 μmoles of DHA, 4.16 μmoles NADPH, 5 ml. of 0.25 M sucrose, and 15 ml. of Krebs-Ringer-phosphate buffer, pH 7.4; incubation time, 2.5 hrs.

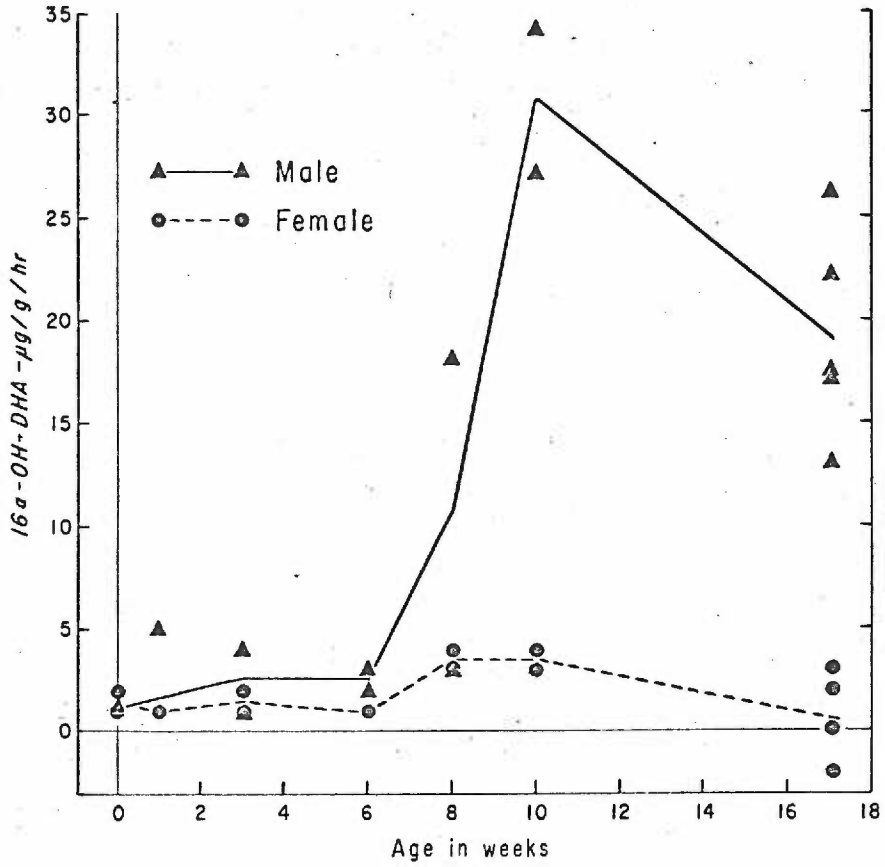
THE STABILITY OF THE 16α-HYDROXYLATION (μg. product formed/g. fresh tissue/hr.) IN LYOPHILIZED HEPATIC MICROSOMAL FRACTIONS FROM MALE RATS (A_1 THROUGH C) AND MALE HUMAN ANENCEPHALIC FETUSES (H_1 THROUGH H_3).

	Prep. No.	Age of Microsomes (weeks)							
		Fresh	1	2	3	4	8	16	30
Adult Male Rats	A_1*	23	26	16	18	18	18	14	13
	A_2	23	22	23	24	26	24	32	30
	B	27	21	17	19	17	17	15	30
	C	18	21	18	21	20	26	19	40
Fetal Male Anencephalic Humans	H_1	–	–	77	–	–	68	–	113
	H_2	–	92	–	–	–	62	–	118
	H_3	–	–	–	81	–	–	120**	–

* All fractions were stored in vacuo at -14° C except A_1 which was stored at 20° C.

** The activity was present over two years later.

Figure No. 9. Age and sex effects upon the 16α-hydroxylation of DHA by rat liver microsomes. The incubation mixture was microsomal powder equivalent to 0.5 g. tissue, 4.43 μmoles DHA, 2.08 μmoles NADPH, and 5.0 ml. of Krebs-Ringer phosphate buffer, pH 7.4; incubation time, 2.5 hours.


Male
Female
16α-OH-DHA -μg/g/hr
Age in weeks
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18

fetuses than that in most of the newborn animals up to six weeks. Although the values for the latter group varied widely, some were equivalent to adult values and others were at an intermediate level. The respective rates for tissue from adult male and female monkeys were 34.7 ± 11.0 and 40.5 ± 9.6 µg./g./hour (see Table No. 13).[1]

The mean rates of 16α-hydroxylation by smooth and rough microsomes from mature rabbits of either sex were comparable (see Table No. 8, p.55). The values were 0.84 and 0.71 for males and 1.00 and 0.64 mµmoles/mg./minute for female rabbits. The respective S/R ratios were 1.23 and 1.90.

In contrast to the values observed from newborn rats and monkeys, the values from lyophilized microsomes from two male human newborn monsters (77 to 180 µg./g./hour), were large and exceeded the largest ones observed from any of the adult animals (see Table No. 12). Freshly incubated microsomes from a female monster also had an active 16α-hydroxylase system (data given in Table No. 35).

Some C_{19}- and C_{18}-steroids were tested in a preliminary experiment as substrates for the hepatic 16α-hydroxylase system. The value (mµmoles/mg./minute) for DHA was higher than for other unsaturated steroid hormones (see Table No. 14). The rates of 16α-hydroxylation were higher for certain steroid substrates with a 3β-hydroxy configuration. The Δ^4-3 keto and phenolic configurations appeared to exert a negative influence under the conditions used.

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[1] I owe the rhesus monkey-liver samples to the cooperation of Dr. H. Feder who studied steroid techniques for an academic term under Dr. Colás' supervision. Dr. Feder aided in some of the analyses with rhesus liver and in others on the age effects on the 16α-hydroxylation of DHA by rats.

Table No. 13. Age and sex effects upon the 16α-hydroxylation of DHA by rhesus liver microsomes. The incubation conditions were: protein equivalent to 0.5 g. liver, 8.65 μmoles of DHA, 4.16 μmoles NADPH, 5 ml. of 0.25 M sucrose, and 15 ml. of Krebs-Ringer phosphate, pH 7.4; incubation time, 2.5 hrs.

AGE AND SEX EFFECTS ON THE 16α-HYDROXYLATION OF DHA (μg./g./hr.) BY HEPATIC MICROSOMES FROM MACACA MULATTA.

Age		Males	Females
Fetuses	75-80 days	–	9.8
	124-127 days	1.3 1.3 6.2	2.6 – –
	150-160 days	1.5 0.8	0.5 3.1
Newborn	1 day	– –	11.0 9.5
	10-14 days	18.0 23.6	0.9 –
	6 (weeks)	–	23.0
Immature	3 (years)	7.0	–
Adults	> 5 (years)	27.0 23.2 29.0 36.0 37.0 57.0 28.0 40.0 – – –	35.0 37.8 45.0 32.0 50.0 45.0 25.0 44.0 30.0 57.0 45.0
		adult males ($\bar{X}$ = 34.7, S.D.= 11.0	adult females ($\bar{X}$ = 40.5, S.D. = 9.6

Table No. 14. 16α-hydroxylation of C_{19} and C_{18} steroids by rat liver microsomes. The incubated mixtures (2.5 hr., 37° C.) consisted of 0.313 mg. of substrate, 6.24 μmoles of NADPH, microsomal powder from 0.328 g. of tissue and 5 ml. of Krebs-Ringer phosphate buffer, pH 7.4.

16α-HYDROXYLATION OF C_{19} STEROIDS AND ESTRONE BY LIVER MICROSOMES FROM MALE RATS.

Substrate	No. Expts.	Activity $\overline{X}$	(mμmoles/mg./min.) range
3β-hydroxy-5β-androstan-17-one	2	0.91	(0.88 - 0.93)
3β-hydroxy-5α-androstan-17-one	2	0.77	(0.76 - 0.77)
DHA	9	0.47	(0.40 - 0.54)
3α-hydroxy-5α-androstan-17-one	2	0.38	(0.37 - 0.38)
3α-hydroxy-5β-androstan-17-one	2	0.32	(0.31 - 0.33)
androst-4-en-3,17-dione	4	0.23	(0.19 - 0.28)
estrone	4	0.07	(0.06 - 0.08)

4. Effect of drug-treatment on the enzymatic activities in total microsomal fractions.

All of the rats were killed in a non-fasted condition for this set of experiments.

a. Acetanilide *p*-hydroxylation. The activity (mμmoles/mg./minute) in 7 sets of untreated male rats was 0.37 ± 0.12 and was 0.28 ± 0.14 in 4 sets of untreated female rats (see Table No. 15). The mean ratio of the male/female activities (M/F ratio) was 1.32. Males treated with phenobarbital (100 mg./kg.) metabolized 0.46 mμmoles/mg./minute, a 24% increase from control values. The average rate in tissue from females was 0.83 which represents a 200% increase over the control values and the M/F ratio from phenobarbital-treated rats was reversed; the value was 0.56.

The respective activities in males and females treated with 3-methylcholanthrene (20 mg./kg.) were 1.35 and 1.63; these values represent average increases of 265 and 482% from control values. The M/F ratio was 0.83.

Rats treated with phenylbutazone (134 mg./kg.) had activities within the range of one S.D. from the average mean of control values.

b. Aminopyrine *N*-demethylation. The *N* demethylase activity (mμmoles/mg./minute) in 7 sets of untreated male rats was 2.62 ± 0.25 and was 1.70 ± 0.12 in 4 sets of untreated females (see Table No. 16). The mean ratio of the male/female activities (M/F ratio) was 1.54, a value which confirms a significant sex-difference (p = <.001). Males treated with phenobarbital metabolized 7.93 mμmoles/mg./minute which represents an increase of 202% of the control value. The average rate

Table No. 15. Acetanilide p-hydroxylation (mμmoles/mg./min.) by hepatic microsomal fractions from nonfasted and drug-treated rats.

ACETANILIDE p-HYDROXYLATION (mμmoles/mg./min.) BY HEPATIC MICROSOMAL FRACTIONS FROM NONFASTED AND DRUG-TREATED RATS.

Condition	Sex	No. Expts.	Activity			M/F ratio	
			$\bar{X}$	S. D.	% increase	$\bar{X}$	range
Nonfasted	M	6	0.37	0.12	-	1.32	(1.0 - 2.10)
	F	4	0.28	0.14	-		
Treated:							
phenobarbital	M	2	0.46		24	0.55	(0.47, 0.65)
	F	2	0.83		200		
3-methylcholanthrene	M	2	1.35		265	0.83	(0.78, 0.90)
	F	2	1.63		482		
phenylbutazone	M	2	0.36		-3	1.09	(0.46, 2.08)
	F	2	0.33		18		

Table No. 16. Aminopyrine N-demethylation (μmoles/mg./min.) by hepatic microsomal fractions from nonfasted and drug-treated rats.

AMINOPYRINE N-DEMETHYLATION (mμmoles formaldehyde/mg./min.) BY HEPATIC MICROSOMAL FRACTIONS FROM NONFASTED AND DRUG-TREATED RATS.

Condition	Sex	No. Expts.	Activity			M/F ratio	
			$\bar{X}$	S.D.	% increase	$\bar{X}$	range
Nonfasted	M	7	2.62	0.25	–	1.54*	(1.30 - 1.96)
	F	4	1.70	0.12	–		
Treated:							
phenobarbital	M	2	7.93		202	1.20	(1.09 - 1.19)
	F	2	6.62		289		
3-methylcholanthrene	M	2	2.47		-6	1.05	(0.86 - 1.26)
	F	2	2.35		38		
phenylbutazone	M	2	3.75		43	1.38	(1.38)
	F	2	2.72		60		

*Values from M are significantly higher ($p = < .001$) than those from F.

in tissue from females was 6.62 mμmoles/mg./minute which indicates a 289% increase. The M/F ratio was 1.20.

The respective activities in males and females treated with 3-methylcholanthrene were 2.47 and 2.35; these values represent an average of 5 and 38% increases from control levels and the M/F ratio was 1.05.

Male rats treated with phenylbutazone had an activity of 3.75 which indicates a 43% increase over the control levels and females metabolized 2.72 mμmoles/mg./minute, an average increase of 60%. The M/F ratio was 1.38.

c. DHA 16α-hydroxylation. Microsomes from non-fasted male rats 16α-hydroxylated DHA at the rate of 1.41 ± 0.32 mμmoles/mg./minute (see Table No. 17). Pretreatment of the male animals with phenobarbital resulted in an average decrease to 0.77 mμmoles/mg./minute. Similarly, values decreased after 3-methylcholanthrene and phenylbutazone pretreatments to 0.67 and 0.56. The microsomal 16α-hydroxylase activity from non-fasted female rats was 0.10 ± 0.07 mμmoles/mg./minute. After phenobarbital treatment, the mean activity increased to 0.32; from 3-methylcholanthrene, no change; and from phenylbutazone, the mean activity was 0.14.

d. DHA 7-hydroxylation. The 7α-hydroxylation rate for microsomes from male rats was 1.33 ± 0.82 mμmoles/mg./minute and for females the significantly lower rate (p = <.002) was 0.41 ± 0.34 (see Table No. 18). The respective average values for microsomes from males pretreated with phenobarbital, 3-methylcholanthrene or phenylbutazone were 2.04, 1.75 and 1.96. The corresponding values from the

Table No. 17. DHA 16α-hydroxylation (mμmoles/mg./min.) by hepatic microsomal fractions from nonfasted and drug-treated rats.

16α-HYDROXYLATION OF DHA (mμmoles/mg./min.) BY HEPATIC MICROSOMAL FRACTIONS FROM NONFASTED AND DRUG-TREATED RATS.

Condition	Sex	No. Expts.	Activity			M/F Ratio	
			$\bar{X}$	S.D.	% increase	$\bar{X}$	range
Nonfasted	M	6	1.41	0.32	–	14.10*	(7.5 - 19.2)
	F	4	0.10	0.07	–		
Treated:							
phenobarbital	M	2	0.77	–	-45	2.40	(1.89 - 3.14)
	F	2	0.32	–	220		
3-methylcholanthrene	M	2	0.67	–	-52	6.70	(3.2 - 21.5)
	F	2	0.10	–	0		
phenylbutazone	M	2	0.56	–	-60	4.00	(3.6 - 4.3)
	F	2	0.14	–	40		

* Values from M are significantly higher ($p = < .001$) than those from F.

Table No. 18. DHA 7-hydroxylations (μmoles/mg./min.) by hepatic microsomal fractions from nonfasted and drug-treated rats.

7-HYDROXYLATION OF DHA (mμmoles/mg./min.) BY HEPATIC MICROSOMAL FRACTIONS FROM NONFASTED AND DRUG-TREATED RATS.

Condition		Sex	No. Expts.	Activity $\bar{X}$	Activity S.D.	Activity % increase	M/F Ratio $\bar{X}$	M/F Ratio range
Nonfasted		M	6	1.33	0.82	–	3.24 *	(1.48 - 7.94)
		F	4	0.41	0.34	–		
7α-/	Treated:							
	phenobarbital	M	2	2.04	–	53	2.44	(2.01 - 5.05)
		F	2	0.87	–	112		
	3-methylcholanthrene	M	2	1.75	–	32	2.11	(1.51 - 3.70)
		F	2	0.83	–	102		
	phenylbutazone	M	2	1.96	–	47	4.17	(3.51 - 5.62)
		F	2	0.47	–	15		
Nonfasted		M	6	0.09	0.07	–	1.13	(0.60 - 1.64)
		F	4	0.08	0.05	–		
7β-/	Treated:							
	phenobarbital	M	2	0.20	–	122	0.87	(0.78 - 1.50)
		F	2	0.23	–	188		
	3-methylcholanthrene	M	2	0.15	–	66	1.00	(0.90 - 1.22)
		F	2	0.15	–	84		
	phenylbutazone	M	2	0.20	–	122	2.22	(2.0 - 2.85)
		F	2	0.09	–	13		

*Values from M are significantly higher ($p = < .002$) than those from F.

drug-treated female rats were 0.87, 0.83 and 0.47 mμmoles/mg./minute. The trend was for an increase in activity in both sexes after each of the drugs; however, only the females treated with phenobarbital and 3-methylcholanthrene had rates greater than one S.D. from the mean.

The DHA 7β-hydroxylation activities by microsomes from male and female rats were 0.09 and 0.08 mμmoles/mg./minute respectively. Treatment of the males with phenobarbital or phenylbutazone increased the rate to 0.20 which is greater than one S.D. from the mean. In females, treatment with phenobarbital and 3-methylcholanthrene increased the rates to 0.23 and 0.15 mμmoles/mg./minute, also greater than one S.D. from the average rate.

5. Effect of drug-treatment on enzymatic activities in microsomal subfractions.

The procedure for the separation of smooth and rough microsomal subfractions incorporates an additional 8 hour centrifugation. In order to avoid doing incubation experiments at night with the freshly prepared subfractions, I completed the tissue preparations, in early experiments, at a reasonable midnight hour and waited until the next morning to do the enzymatic studies. The results for acetanilide *p*-hydroxylation and DHA 16α-hydroxylation are given in the next two tables (Tables No. 19 and 20). The data are characterized by large standard deviations from the mean activities of each group. The variability is emphasized by the wide range of the smooth/rough ratios (Ratio S/R) and the lower values than obtained by immediate incubations. The steroid hydroxylase system was especially labile. By changing the preparation schedule so that I was incubating freshly-

Table No. 19. The effects of delaying the test of p-hydroxylation of acetanilide for 2-10 hours after preparation of the microsomal fractions from liver of fasted rats.

COMPARISON OF THE p-HYDROXYLATION OF ACETANILIDE[1] TESTED PROMPTLY OR DELAYED 2-10 HOURS AFTER PREPARATION OF THE MICROSOMAL FRACTIONS FROM LIVER OF FASTED RATS.

Test:	Smooth (S)		Rough (R)		Ratio S/R
	mg. prot.*	Rate (μmoles/mg./min.)	mg. prot.*	(μmoles/mg./min.)	
Immediate[2]	1.5	0.51	2.6	0.35	1.45
Delayed	1.8	0.31	2.8	0.32	0.97
	2.8	0.18	3.8	0.37	0.49
(Males)	1.1	0.20	3.0	0.16	1.25
	1.6	0.89	3.1	0.08	11.00
	1.8	0.40 ± 0.33	3.2	0.23 ± 0.13	3.45
Immediate[3]	2.1	0.40 ± 0.28	2.0	0.57 ± 0.05	0.70
Delayed	1.6	0.28	1.1	0.26	1.08
	2.3	1.11	3.7	0.94	1.18
(Females)	0.8	0.50	1.4	0.70	0.71
	1.0	0.21	2.3	0.03	7.00
	1.4	0.53 ± 0.41	2.1	0.48 ± 0.41	2.49

* Mg. protein/flask.
1. Mean (± S.D. when given).
2. Data from two experiments.
3. Data from four experiments with nonfasted animals.

Table No. 20. The effects of delaying the test of 16α-hydroxylation of DHA for 2-10 hours after preparation of the microsomal fraction from liver of fasted rats.

COMPARISON OF THE 16α-HYDROXYLATION OF DHA[1] TESTED PROMPTLY OR DELAYED 2-10 HOURS AFTER PREPARATION OF THE MICROSOMAL FRACTIONS FROM LIVER OF FASTED MALE RATS.

Test:	Smooth (S)		Rough (R)		
	mg. prot.*	Rate (mμmoles/mg./min.)	mg. prot.*	Rate (mμmoles/mg./min.)	Ratio S/R
Immediate[2]	2.2	1.20 ± 0.22	2.4	1.40 ± 0.33	0.86
Delayed	14.8	0.17	14.8	0.15	1.13
	12.5	0.06	12.5	0.06	1.00
	7.0	0.31	8.5	0.45	0.69
	5.6	0.65	8.6	0.76	0.86
	3.1	1.74	1.9	2.23	0.78
	1.0	1.23	2.1	1.44	0.85
	2.9	0.32	2.2	0.38	0.84
	3.5	0.05	4.8	0.17	0.29
	2.7	0	7.5	0.04	–
	–	–	3.1	0.35	–
	3.1	0	3.1	0.45	–
	5.6 ± 4.6	0.45 ± 0.55	6.3 ± 4.4	0.59 ± 0.67	0.82 ± 0.25

* Mg. protein/flask.
1. Mean (± S. D. when given).
2. Represents data from four experiments.

prepared tissue, the results were reproducible. Thereafter, that procedure was adopted for all of the experiments.

In the earliest experiments with the subfractionation, the rough fraction from rat liver usually had the greatest DHA 16α-hydroxylase activity, the opposite of reported results obtained with drug-metabolizing enzymes in rabbit liver. Because of some uncertainty about the completeness of the subfractionation, in spite of confirmation from the RNA data, I tested the procedures as I had been using them by attempting to reproduce published results from experiments with rabbits. These results are given in Table No. 21.

The consideration of primary interest from the rabbit experiments was whether the enzymatic activities were greater in the smooth subfraction as had been reported. The average ratio of the activities in smooth/rough fractions (Ratio S/R) ranged from 1.18 to 1.56. The conclusion was that the tissue fractionation method gave the expected and reproducible results. Not only the drug-metabolizing enzymes but also the DHA 16α-hydroxylase system had its greatest specific activity in smooth microsomes from rabbits. For rat subfractions, the S/R ratio had been less than 1.0, except for *p*-hydroxylation by tissue from males, indicating that the enzymatic distribution in rats was different from that of rabbits.

a. Acetanilide *p*-hydroxylation. The average acetanilide *p*-hydroxylase activities (mμmoles/mg./minute) in smooth and rough microsomes from male rabbits were 1.82 and 1.53 and those for females were 1.61 and 1.22 respectively. The corresponding data from non-fasted rats was 0.42 ± 0.19 and 0.33 ± 0.14 for males and 0.40 ± 0.28

Table No. 21. Enzymatic transformations by microsomal sub-fractions of liver from rabbits.

ACETANILIDE p-HYDROXYLATION, AMINOPYRINE N-DEMETHYLATION AND DHA 16α-HYDROXYLATION BY MICROSOMAL SUBFRACTIONS OF RABBIT LIVER.

Activity	Sex	Smooth (S)		Rough (R)		Ratio S/R	
		Rates*	$\bar{X}$	Rates*	$\bar{X}$	$\bar{X}$	range
p-Hydroxylation	M	0.53 1.52 3.40	1.82	1.08 1.52 1.98	1.53	1.19	(0.49 - 1.72)
	F	1.20 2.06 1.57	1.61	1.03 1.21 1.42	1.22	1.32	(1.11 - 1.70)
N-Demethylation	M	4.90 5.50 3.43	4.61	4.80 5.20 1.66	3.89	1.19	(1.02 - 2.06)
	F	4.80 7.30 2.73	4.94	5.00 4.70 1.86	3.85	1.28	(0.96 - 1.55)
16α-Hydroxylation	M	0.81 1.00 0.71	0.84	0.59 1.00 0.53	0.71	1.18	(1.00 - 1.37)
	F	1.09 1.17 0.73	1.00	0.46 0.81 -	0.64	1.56	(1.14 - 2.36)

* μmoles/mg. protein/min.

and 0.57 ± 0.05 for female rats (see Table No. 22). The *p*-hydroxylase activity and the S/R ratio was slightly greater in male rats which had been fasted.

The average activities in smooth and rough subfractions from phenobarbital-treated male rats were higher at 0.59 and 0.98 mμmoles/mg./minute than control value, but those of females decreased in comparison to the values for untreated females to 0.38 and 0.39.

Rats treated with 3-methylcholanthrene had large increases in *p*-hydroxylation. The smooth microsomes of the males had the greatest average activity but in females the rough fraction was most active. The respective values were 2.44 and 1.57 for males and 2.21 and 2.93 for females.

The average values for both smooth and rough fractions from phenylbutazone-treated male rats were decreased but for the female rats both were greater than their respective control values. The average of the measured activities was 0.20 and 0.16 for males and 0.68 and 0.89 mμmoles/mg./minute for female rats treated with phenylbutazone.

b. Aminopyrine *N*-demethylation. The average activities (mμmoles/mg./min.) for smooth and rough microsomal subfractions from male rabbits were 4.61 and 3.89, compared to 4.94 and 3.85 for tissue from females. The corresponding values from non-fasted rats were 2.18 ± 0.52 and 2.40 ± 0.54 for males and 1.63 ± 0.25 and 1.84 ± 0.31 for female rats (see Tables No. 21 and 23). Fasted males had similar values as non-fasted animals, 2.28 ± 0.96 and 2.25 ± 0.86 mμmoles/mg./minute respectively, for smooth and rough subfractions, but the S/R ratio was larger.

Table No. 22. Acetanilide p-hydroxylation (μmoles/mg./min.) by hepatic microsomal subfractions from nonfasted, fasted and drug-treated rats.

ACETANILIDE p-HYDROXYLATION (mμmoles/mg./min.) BY HEPATIC MICROSOMAL SUBFRACTIONS FROM NONFASTED, FASTED, AND DRUG-TREATED RATS.

Condition	Sex	No. Expts.	Smooth (S)		Rough (R)		Ratio S/R	
			$\bar{X}$	S. D.	$\bar{X}$	S. D.	$\bar{X}$	range
Fasted	M	2	0.51	–	0.35	–	1.45	(1.09 - 2.10)
Nonfasted	M	7	0.42	0.19	0.33	0.14	1.27	(0.52 - 2.33)
	F	4	0.40	0.28	0.57	0.05	0.70	(0.30 - 1.05)
Treated:								
phenobarbital	M	2	0.59	–	0.98	–	0.60	(0.20 - 1.49)
	F	2	0.38	–	0.39	–	0.97	(0.75 - 1.15)
3-methylcholanthrene	M	2	2.44	–	1.57	–	1.55	(1.20 - 1.65)
	F	2	2.21	–	2.93	–	0.76	(0.67 - 0.84)
phenylbutazone	M	2	0.20	–	0.16	–	1.25	(∞ - 0.88)
	F	2	0.68	–	0.89	–	0.76	(0.72 - 0.90)

Table No. 23. Aminopyrine N-demethylation (μmoles/mg./min.) in hepatic microsomal subfractions from nonfasted, fasted and drug-treated rats.

AMINOPYRINE N-DEMETHYLATION (μmoles formaldehyde/mg./min.) IN HEPATIC MICROSOMAL SUBFRACTIONS FROM NONFASTED, FASTED AND DRUG-TREATED RATS.

Condition	Sex	No. Expts.	Smooth (S)		Rough (R)		S/R ratio	
			$\bar{X}$	S. D.	$\bar{X}$	S. D.	$\bar{X}$	range
Fasted	M	4	2.28	0.96	2.25	0.86	1.01	0.44 - 1.27
Nonfasted	M	7	2.18*	0.52	2.40**	0.54	0.91	0.70 - 1.15
	F	4	1.63*	0.25	1.84**	0.31	0.89	0.75 - 0.93
Treated:								
phenobarbital	M	2	6.75	–	6.65	–	1.01	0.84 - 1.20
	F	2	5.31	–	6.13	–	0.87	0.80 - 0.95
3-methylcholanthrene	M	2	2.95	–	2.84	–	1.04	0.98 - 1.10
	F	2	1.75	–	2.14	–	0.82	0.78 - 0.87
phenylbutazone	M	2	2.15	–	2.58	–	0.83	0.80 - 0.86
	F	2	2.25	–	1.95	–	1.15	1.00 - 1.34

* $p = < 0.1$ for the sex difference.
** $p = < 0.05$ for the sex difference.

Large increases in *N*-demethylase activity in both sexes resulted from phenobarbital pretreatment. Values in smooth and rough subfractions from males were 6.75 and 6.65; females metabolized 5.31 and 6.13 mμmoles/mg./minute. The average *N*-demethylase activities in animals treated with 3-methylcholanthrene and phenylbutazone were roughly similar to control values for both subfractions and both sexes.

c. DHA 16α-hydroxylation. From fasted male rabbits, 16α-hydroxylation rates (mμmoles/mg./minute) in smooth and rough subfractions were 0.84 and 0.71; the respective values for female rabbits were 1.00 and 0.64. Smooth microsomes from non-fasted male rats hydroxylated 1.09 ± 0.44 mμmoles/mg./minute compared to 1.35 ± 0.33 for the rough subfraction (see Tables No. 21 and 24). The corresponding values from fasted male rats were 1.20 ± 0.22 and 1.40 ± 0.33. The S/R mean ratios for non-fasted or fasted males were 0.81 and 0.86, respectively. In contrast, only the smooth microsomes from female rats had detectable activity, 0.04 mμmoles/mg./minute indicating the sex-related difference.

The average activities in smooth and rough fractions after phenobarbital treatment to males were reduced to 1.0 and 0.47 mμmoles/mg./minute. Treatment of males with 3-methylcholanthrene resulted in a small increase in the activity of the smooth subfraction, to 1.24, but that in the rough subfraction was only 0.72. In phenylbutazone-treated males, activities of both subfractions were reduced to 0.15 and 0.54 mμmoles/mg./minute, respectively.

The response of the female rats to phenobarbital treatment was to give an equal activity, 0.22 mμmoles/mg./minute, in both subfractions.

Table No. 24. DHA 16α-hydroxylase activity (μmoles/mg./min.) in hepatic microsomal subfractions from nonfasted, fasted and drug-treated rats.

16α-HYDROXYLATION OF DHA (mμmoles/mg./min.) BY MICROSOMAL SUBFRACTIONS OF LIVER FROM NONFASTED, FASTED AND DRUG-TREATED RATS.

Condition	Sex	No. Expts.	Smooth (S) $\bar{X}$	Smooth (S) S.D.	Rough (R) $\bar{X}$	Rough (R) S.D.	S/R ratio $\bar{X}$	S/R ratio S.D.
Nonfasted	M	7	1.09*	0.44	1.35*	0.33	0.81	0.21
	F	4	0.04*	0.02	0 *	–	–	0.32
Fasted	M	4	1.20	0.22	1.40	0.33	0.86	
Treated:								
phenobarbital	M	2	1.00		0.47		2.13	
	F	2	0.22		0.22		1.00	
3-methylcholanthrene	M	2	1.24		0.72		1.72	
	F	2	0		0		–	
phenylbutazone	M	2	0.15		0.54		0.28	
	F	2	0		0		–	

*Values for M are significantly higher ($p = < .001$) than those from F.

Table No. 25. DHA 7-hydroxylation (μmoles/mg./min.) in hepatic microsomal subfractions from nonfasted and drug-treated female rats.

No 16α-hydroxylation was detected in any of the subfractions from 3-methylcholanthrene or phenylbutazone-treated female rats.

d. DHA 7-hydroxylation. These activities were measured in only two experiments with microsomal subfractions because the identification of the compounds and the development of the analytical procedure were not completed until late in the course of this work. From non-fasted females, the rates of 7α-hydroxylation were 0.41 and 0.54 mμmoles/mg./minute (see Table No. 25). After 3-methylcholanthrene the respective average values were 1.13 and 0.77. Smooth and rough microsomes metabolized 0.91 and 0.28 mμmoles/mg./minute, respectively, after phenylbutazone treatment.

The 7β-hydroxylase activities in smooth and rough subfractions from female rats were 0.05 and 0.03. The rates increased in both fractions to 0.16 and 0.28 mμmoles/mg./minute after 3-methylcholanthrene treatment. Only the smooth subfraction had 7β-hydroxylase activity after phenylbutazone treatments.

7-HYDROXYLATION OF DHA (mμmoles/mg./min.) BY HEPATIC MICROSOMAL SUBFRACTIONS FROM NONFASTED AND DRUG-TREATED FEMALE RATS.

		Smooth (S)		Rough (R)		S/R Ratio	
		$\bar{X}$	(values)	$\bar{X}$	(values)	$\bar{X}$	(values)
7α-	nonfasted	0.41	(0.47, 0.34)	0.54	(0.48, 0.60)	0.76	(0.98, 0.57)
7α-	3-methylcholanthrene	1.13	(1.08, 1.18)	0.77	(0.71, 0.82)	0.47	(1.52, 1.43)
7α-	phenylbutazone	0.91	(1.13, 0.68)	0.28	(0.10, 0.46)	3.25	(11.30, 1.47)
7β-	nonfasted	0.05	(0, 0.09)	0.03	(0, 0.06)	1.67	(0, 1.50)
7β-	3-methylcholanthrene	0.16	(0.11, 0.20)	0.28	(0.24, 0.32)	0.57	(0.46, 0.63)
7β-	phenylbutazone	0.11	(0.11, 0.12)	0	(0, 0)	–	–

B. Inhibition of enzymatic activities.

1. Reciprocal inhibition between steroids and drugs.

a. *N*-demethylation in the presence of DHA. In incubations incorporating two concentrations of aminopyrine, 2.5×10^{-3} *M* and 1.25×10^{-3} *M*, the addition of different amounts of DHA decreased the amount of formaldehyde liberated in every instance. See Table No. 26 which shows that the percentage of decrease of *N*-demethylation (mμmoles/mg./minute) was nearly identical for both concentrations of aminopyrine. A plot of the reciprocal of the velocities and the concentration of inhibitor (DHA) (34) (see Figure No. 10) shows that a best-fitting straight line does not describe the relationship between the two variables. No information about the type of inhibition was apparent using the incubation conditions of previous experiments.

b. 16α-hydroxylation in the presence of aminopyrine. The optimal concentration of DHA for 16α-hydroxylation, 1.83×10^{-4} *M*, and one-half of that amount were used to examine the effect of aminopyrine on the 16α-hydroxylation of DHA. The results in Table No. 26 show that the larger quantities of aminopyrine do inhibit 16α-hydroxylation at about the same rates for either concentration of DHA. See Figure. No. 11 which demonstrates that the reciprocal of the rates are similar but give no information about the type of inhibition. The percentage of inhibition produced by aminopyrine is less than that for DHA in the opposite experiment using similar quantities of inhibitor.

c. 7α-hydroxylation in the presence of aminopyrine. The effect of aminopyrine on 7α-hydroxylation was measured in the same flasks as for the 16α-hydroxylation data. At the higher concentration

Table No. 26. Reciprocal effects of DHA and aminopyrine on their rates of oxidation (mμmoles/mg./min.) by hepatic microsomes from male rats.

RECIPROCAL EFFECTS OF DHA AND AMINOPYRINE ON THEIR RATES OF OXIDATION (mμ moles/mg./min.) BY HEPATIC MICROSOMES FROM MALE RATS.

	N-Demethylation of Aminopyrine	
[10^{-4} M DHA]	[1.25×10^{-3} M]	[2.5×10^{-3} M]
0	3.42 (100)*	3.79 (100)*
1.38	1.27 (37)	1.44 (38)
2.75	1.04 (33)	1.01 (27)
5.50	0.89 (26)	0.69 (18)
10.90	0.74 (22)	0.64 (17)

	16α-Hydroxylation of DHA	
[10^{-4} M aminopyrine]	[0.92×10^{-4} M]	[1.83×10^{-4} M]
0	1.40 (100)	1.77 (100)
1.7	1.27 (91)	1.44 (81)
3.3	1.08 (77)	1.41 (80)
5.0	1.27 (91)	1.26 (71)
6.7	0.97 (69)	1.30 (73)
8.4	0.97 (69)	1.25 (71)

	7α-Hydroxylation of DHA	
[10^{-4} M aminopyrine]	[0.92×10^{-4} M]	[1.83×10^{-4} M]
0	2.43 (100)	2.36 (100)
1.7	1.63 (67)	3.08 (132)
3.3	0.98 (40)	2.88 (122)
5.0	0.77 (32)	2.54 (108)
6.7	0.74 (30)	2.41 (105)
8.4	2.11 (87)	1.75 (74)

*Values in parentheses are % of control values.

Figure No. 10. Effect of DHA on N-demethylation of aminopyrine (μmoles/mg./min.) by hepatic microsomes from male rats.

EFFECT OF DHA ON N-DEMETHYLATION OF AMINOPYRINE
BY HEPATIC MICROSOMES FROM MALE RATS

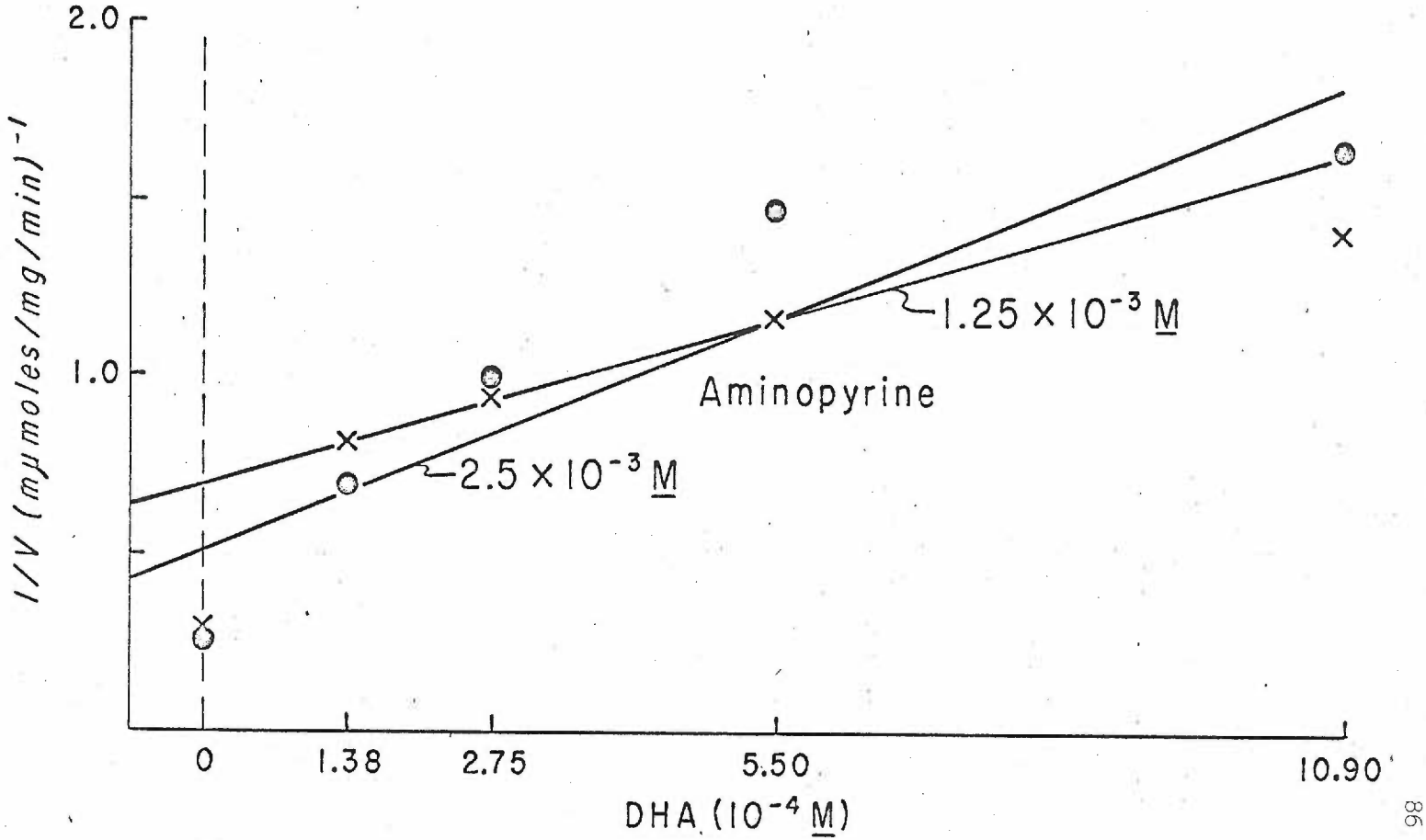
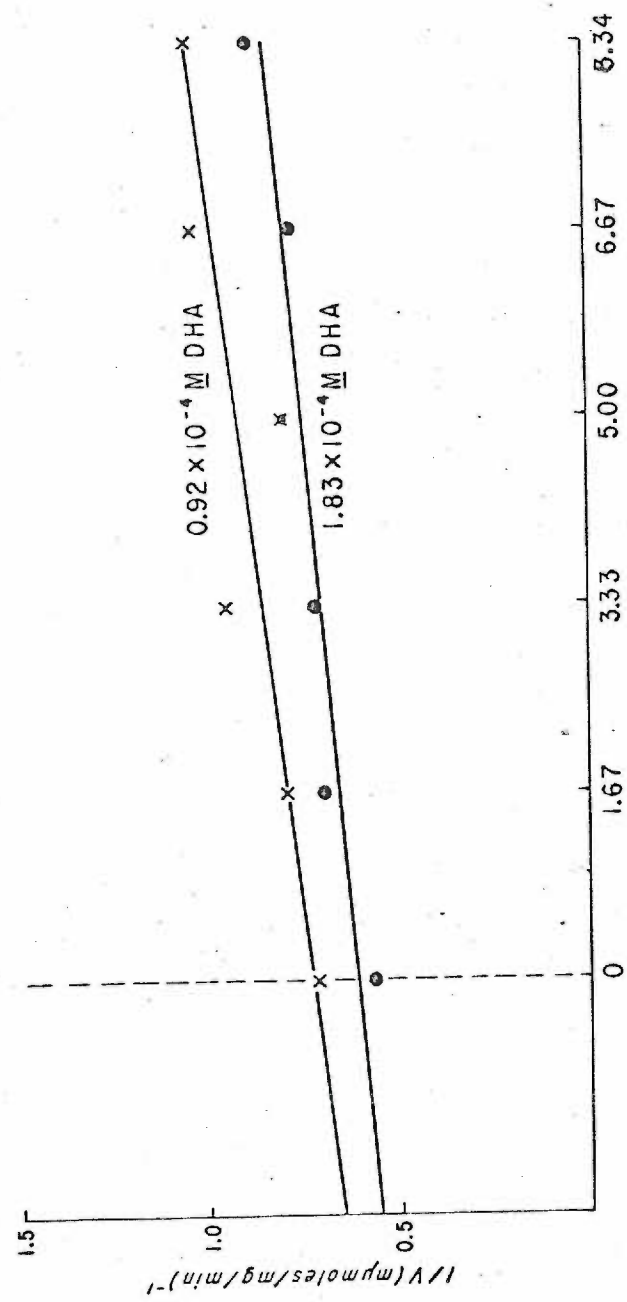


Figure No. 11. The effect of aminopyrine on 16α-hydroxylation (mμmoles/mg./min.) of DHA by hepatic microsomes from male rats.

EFFECT OF AMINOPYRINE ON 16α-HYDROXYLATION OF DHA
BY HEPATIC MICROSOMES FROM MALE RATS



of DHA substrate, addition of the smaller amounts of aminopyrine resulted in increased 7α-hydroxylation. In contrast, the transformation was inhibited when aminopyrine was added to the flasks containing the lower concentration of DHA. Figure No. 12 demonstrates the different activities of the 7α-hydroxylase system under these conditions and permits the calculation of an apparent K_i for aminopyrine (34).

2. Inhibition of enzymatic activities by SU-9055.

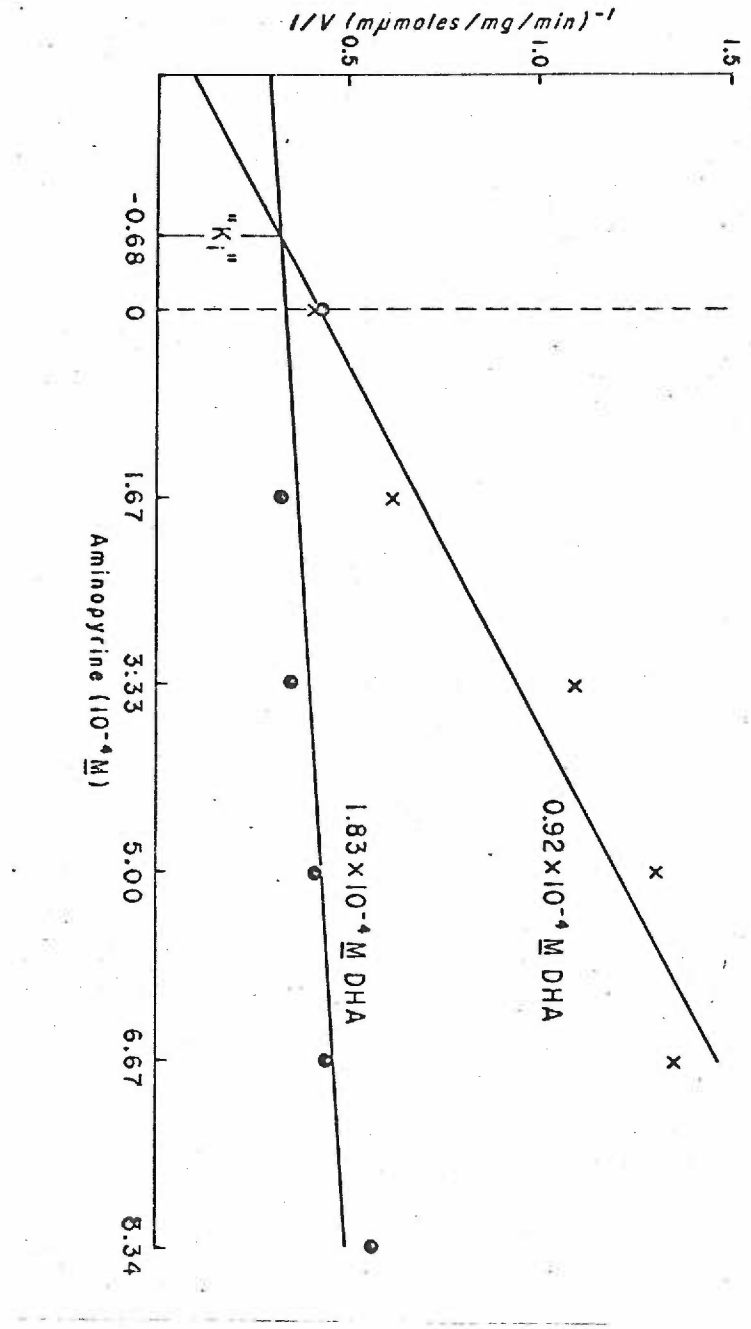
Because some of the data obtained from the preceding experiments were inconclusive, another approach to the problem was initiated using a known steroid hydroxylase-inhibitor.

a. *N*-demethylation in the presence of SU-9055. SU-9055 is a synthetic organic compound which inhibits the 16α-, 17α-, and 18-hydroxylations of steroids *in vitro* (20). Results in Table No. 27 and Figure No. 13 show that SU-9055 also inhibits *N*-demethylation of aminopyrine more profoundly than steroid hydroxylation. At high concentrations of SU-9055, the inhibition is linear and greater than 90% complete.

b. 16α-hydroxylation in the presence of SU-9055. At all concentrations of SU-9055, 16α-hydroxylation was inhibited although not linearly. The maximum average inhibition was 94% at the highest concentration and Figure No. 13 shows that the slope of the line in a plot of 1/v and [SU-9055] resembles that of *N*-demethylation more than that of 7α- + 7β-hydroxylation.

c. 7α- + 7β-hydroxylation in the presence of SU-9055. Because of an inadequate chromatographic separation of the 7α- and 7β-epimers in this experiment, probably due to a shortened stabilization

Figure No. 12. The effect of aminopyrine on 7α-hydroxylation of DHA (mμmoles/mg./min.) by hepatic microsomes from male rats.



EFFECT OF AMINOPYRINE ON 7α-HYDROXYLATION OF DHA BY HEPATIC MICROSOMES FROM MALE RATS

Table No. 27. Effect of SU-9055 on N-demethylase and steroid hydroxylase activities (mμmoles/mg./min.) in hepatic microsomes from male rats.

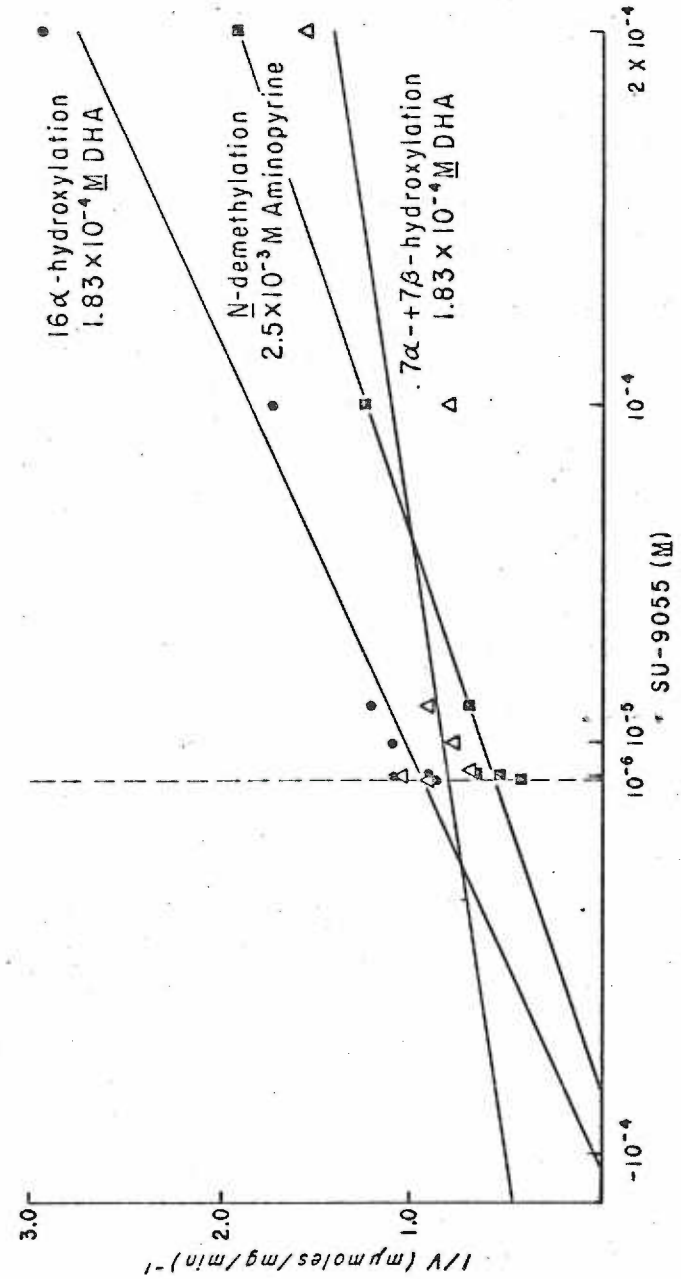
EFFECT OF SU-9055 ON N-DEMETHYLASE AND STEROID HYDROXYLASE ACTIVITIES IN HEPATIC MICROSOMES FROM MALE RATS.

[Su-9055]	N-demethylation		16α-Hydroxylation		7α- + 7β-Hydroxylation	
	Activity[1]	%[2]	Activity[1]	%[2]	Activity[1]	%[2]
0	2.40	100	1.15	100	1.01	100
10^{-6} M	1.88	78	0.93	81	0.96	96
2×10^{-6} M	1.57	65	1.11	97	1.55	155
10^{-5} M	*	–	0.91	79	1.29	129
2×10^{-5} M	1.45	60	0.83	72	1.10	110
10^{-4} M	0.80	35	0.58	50	1.26	126
2×10^{-4} M	0.52	21	0.56	49	0.65	56
10^{-3} M	0.23	9	0.07	6	0	0

1. mμmoles/mg./min.
2. % of control value
* flasks accidentally lost

Figure No. 13. Effect of SU-9055 on steroid hydroxylase and N-demethylase activities (mμmoles/mg./min.) in hepatic microsomes from male rats.

EFFECT OF SU-9055 ON STEROID HYDROXYLASE AND N-DEMETHYLASE ACTIVITIES IN HEPATIC MICROSOMES FROM MALE RATS



period for the system, the compounds were measured together. The rates of hydroxylation increased up to 55% at lower concentrations of SU-9055 and only at the highest ones was significant inhibition apparent. The slopes of the lines (relating 1/v to [inhibitor]) for 7-hydroxylation in the presence of SU-9055 or aminopyrine are very similar for the same concentration of DHA, 1.83×10^{-4} *M* (see Table No. 27 and Figure No. 13).

3. Inhibition of the chemical reduction of cytochrome P-450 by SU-9055.

The data which demonstrated SU-9055 inhibition of *N*-demethylase and 16α-hydroxylase activities suggested a common, or perhaps similar, mechanism for the inhibition. Therefore, some spectrophotometric studies were initiated with the SU-9055 and the protein solutions. Addition of varying amounts of SU-9055 to the protein resulted in difference spectra showing a trough at 383 mμ and a peak at 420 mμ (see Figure No. 14). The absorbancies at the wave length of both, the maximum and the minimum absorbance, were directly proportional (with one exception) to the inhibitor concentration. The largest absorbancy change, resulting from 5×10^{-3} *M* SU-9055, was 0.012 at 420 mμ. The change in absorbancy at 420 mμ was greater at low concentrations of SU-9055 than for the trough (A_{383}). Curves showing the rates of change are shown in Figure No. 15.

At the low concentrations of SU-9055[1], the P-450 content measured in microsomal solutions decreases proportionally. Figure No. 16

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[1] Difference spectra were obtained by adding tartaric acid to the reference cuvette when the effects of SU-9055 in tartaric acid were quantitated. The determination of P-450 in those solutions required 5 minutes of reaction time to get maximum reduction of P-450 with sodium dithionite.

Figure No. 14. Difference spectra after addition of SU-9055 (in 10^{-3} M tartaric acid) to hepatic microsomes from male rats (2.1 mg. protein).

DIFFERENCE SPECTRA AFTER ADDITION OF SU-9055 TO HEPATIC MICROSOMES FROM MALE RATS

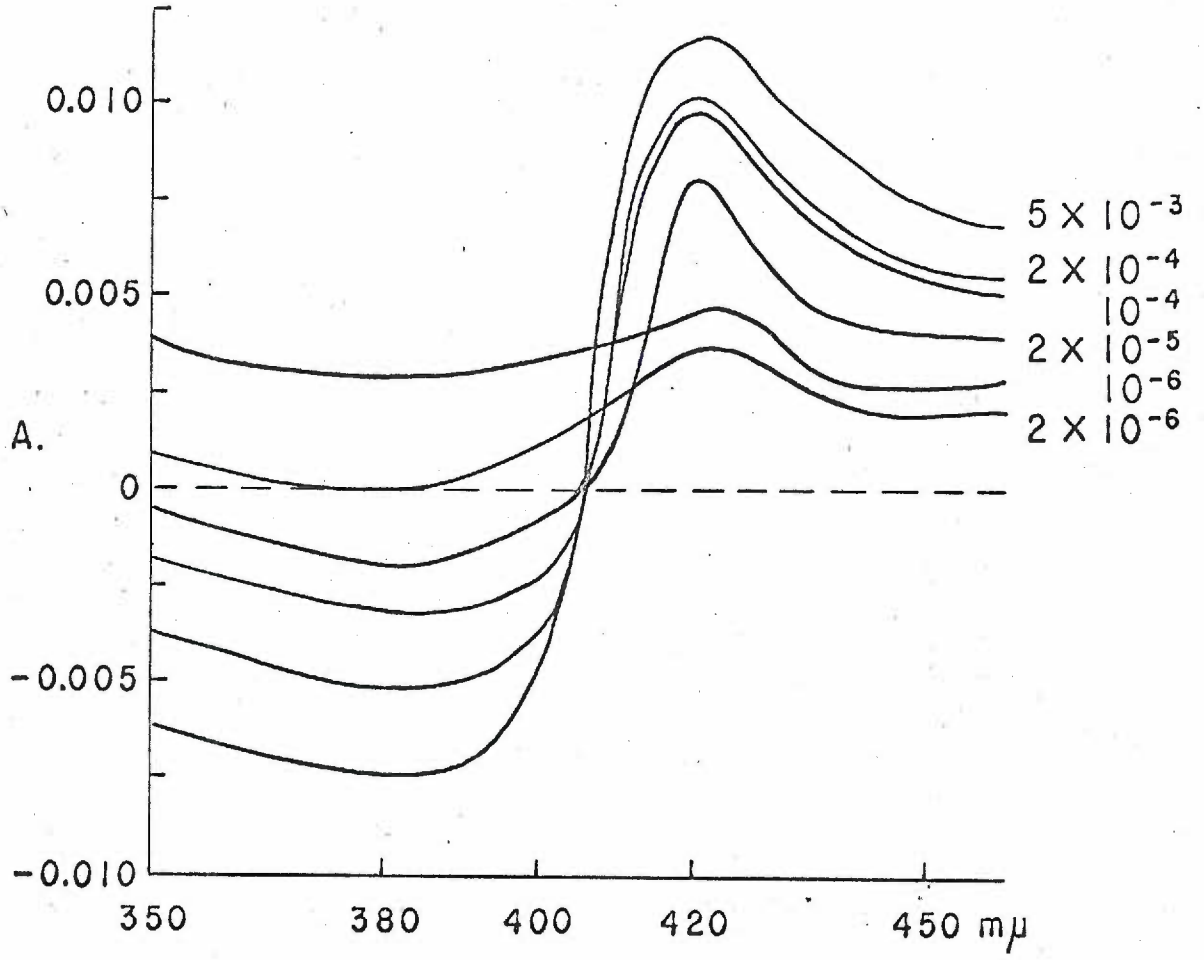


Figure No. 15. Relationship between peak (A_{420}) and trough (A_{383}) differential absorption and SU-9055 added to hepatic microsomes from male rats (2.1 mg. protein).

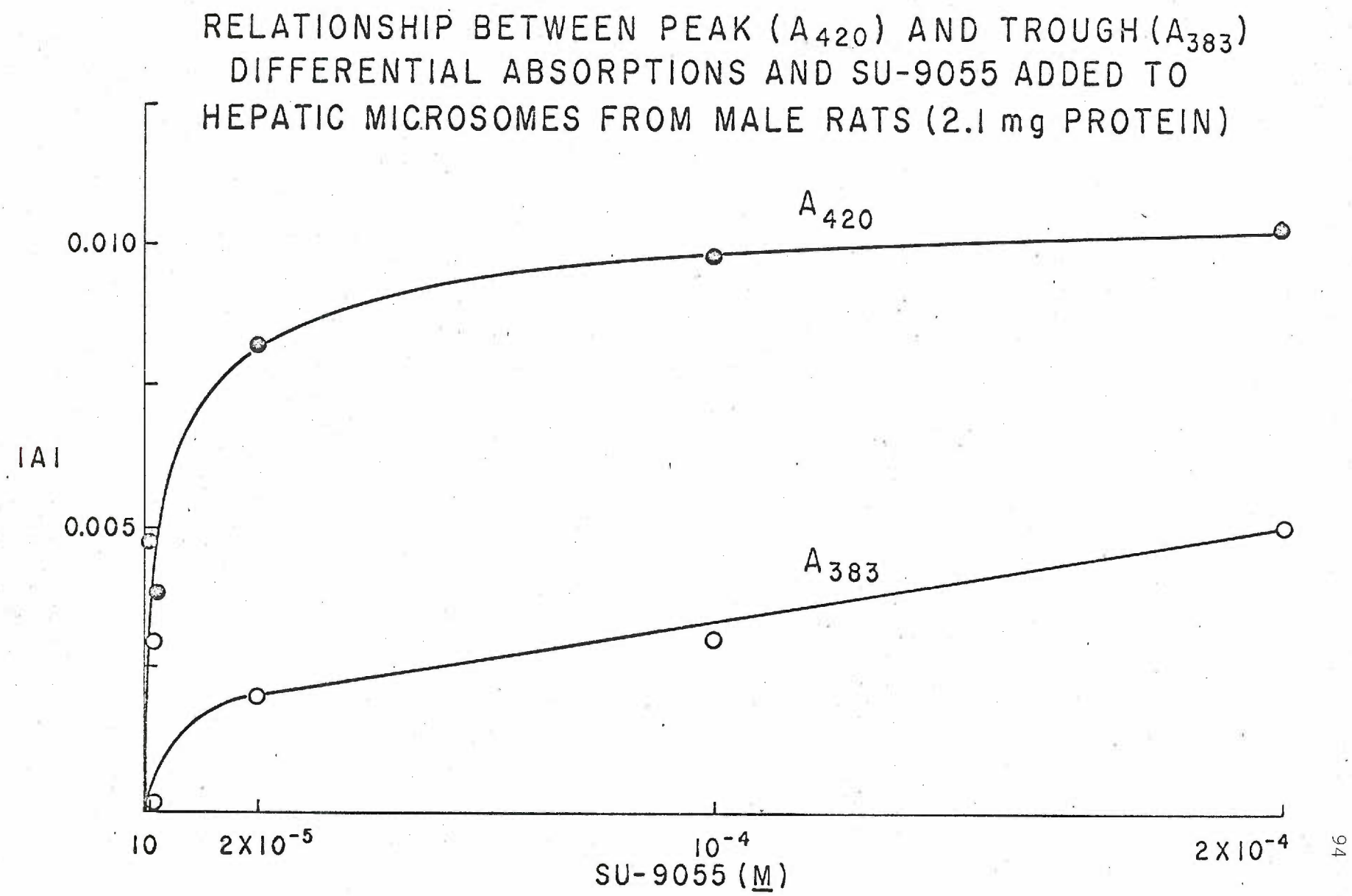
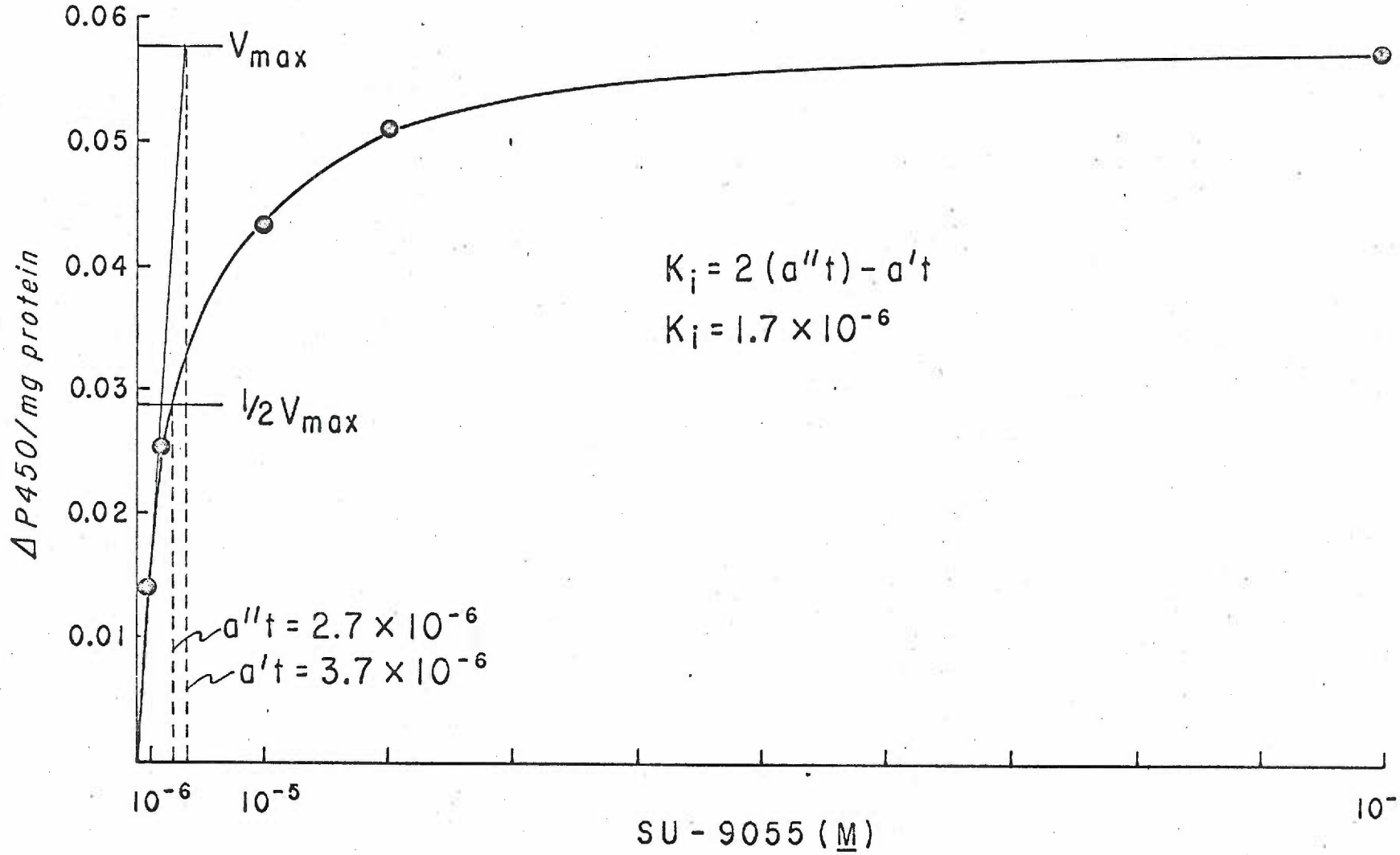

RELATIONSHIP BETWEEN PEAK (A_{420}) AND TROUGH (A_{383})
DIFFERENTIAL ABSORPTIONS AND SU-9055 ADDED TO
HEPATIC MICROSOMES FROM MALE RATS (2.1 mg PROTEIN)
A_{420}
A_{383}
0.010
0.005
|A|
10
$2X10^{-5}$
10^{-4}
$2X10^{-4}$
SU-9055 (M)

Figure No. 16. Graphic determination of "Ki" for SU-9055 and P-450 in hepatic microsomes from male rats.

GRAPHIC DETERMINATION OF "K_i" FOR SU-9055 AND P-450 IN HEPATIC MICROSOMES FROM MALE RATS



is the graphic determination (35) of the apparent K_i for the interaction between SU-9055 and P-450 which resulted in a value of 1.7×10^{-6} *M*. A modification of this procedure (87) yielded a value of 2.5×10^{-6} *M*. The validity of V_{max} was checked by finding that the rate (v) at 10 ($K_i + b_t$) was 89% of V_{max} (b_t is the total amount of enzyme, also determined graphically).

4. Inhibition of enzymatic activities by CO and KCN.

The results of the preceding set of experiments raised some doubt about the assumption that the supply of CO-sensitive cytochrome P-450 was a limiting factor for both the steroid 7- and 16α-hydroxylating systems.

The effect of a mixture of $CO:O_2$ (80:20) on drug-metabolizing enzymes and on the steroid hydroxylases was then analyzed and is given in Table No. 28. Enzymatic *N*-demethylation was decreased to 77% of the control and *p*-hydroxylation increased 52%. DHA 7α-hydroxylation fell to 62% of its control value and in the same flasks, 7β- and 16α-hydroxylation rates dropped to 22 and 20% of their control values, respectively.

In experiments carried out simultaneously, 10^{-3} *M* KCN reduced the N-demethylation to 77% and increased the *p*-hydroxylation to 150%, nearly exactly as the $CO:O_2$ mixture had done. The respective values for 7α-, 7β-, and 16α-hydroxylation in the presence of 10^{-3} *M* KCN represented 87, 75, and 60% in their control values.

Table No. 28. The effects of CO and KCN on aminopyrine N-demethylation, acetanilide p-hydroxylation, and DHA 7- and 16α-hydroxylations (mμmoles/mg./min.) by hepatic microsomes from male rats.

EFFECT OF CO AND KCN ON AMINOPYRINE N-DEMETHYLATION, ACETANILIDE, p-HYDROXYLATION AND DHA 7- AND 16α-HYDROXYLATIONS (mμmoles/mg./min.) BY HEPATIC MICROSOMES FROM MALE RATS.

Transformation	Control	CO (80%), O_2 (20%)		KCN [10^{-3} M]	
	rate	rate	% of Control	rate	% of Control
N-demethylation	2.73	2.10	77	2.10	77
p-hydroxylation	0.28	0.43	152	0.42	150
7α-hydroxylation	2.92	1.80	62	2.53	87
7β-hydroxylation	0.11	0.03	22	0.08	75
16α-hydroxylation	1.48	0.29	20	0.89	60

C. Physicochemical alteration of microsomal particles.

The preceding differences in activities of microsomal steroid hydroxylases and drug-metabolizing enzymes came to light in the course of comparative studies based upon biological differences, selective responses to treatments *in vivo* with enzyme-inducing drugs, or chemical inhibition of the reactions *in vitro*. The following experiments represent an effort to explore differences in the enzymic mechanisms by inducing physicochemical changes in the microsomal membranes with a detergent prior to the tests for enzymatic activity.

The addition of sodium deoxycholate [DOC (0.5 mg./mg. protein)] immediately reduced the turbidity of protein solutions observed visually. The only quantitative estimation, independent of the enzymatic activities, about the physicochemical alteration of the membranes was the residual content of cytochrome P-450 after the treatment. Inspection of Table No. 29 shows that the P-450 content (mμmoles/mg. protein) after the DOC-treatment was greatly reduced in both experiments. In experiment 3, where the P-450 decrease was least, about one-third of the *N*-demethylase and 16α-hydroxylase activities remained but neither *p*-hydroxylation nor 7α-hydroxylation were detectable.

In the microsomes treated with DOC, but in a N_2 atmosphere, the P-450 content was only partially reduced (the DOC treatment in experiment 3 was accidentally shortened from 30 minutes) and again, *N*-demethylation was observed but not proportional to the residual P-450 content; its rate remained near 30% of the control value. No steroid hydroxylations occurred in the valid experiment (No. 2) with anaerobic conditions for the DOC treatment but *p*-hydroxylation was unchanged.

Table No. 29. Cytochrome P-450 content and oxidase activities (mμmoles/mg./min.) after sodium deoxycholate treatment of hepatic microsomes from male rats.

P-450 CONTENT AND OXIDASE ACTIVITIES (mμmoles/mg./min.) AFTER DEOXYCHOLATE TREATMENT OF HEPATIC MICROSOMES FROM MALE RATS.

Treatment	Expt. No.	P-450 content (mμmoles/mg. prot.)	N-demethylation of aminopyrine	p-Hydroxylation of acetanilide	DHA hydroxylation	
					7α-	16α-
none	1	0.740	–	–	2.70	1.56
	2	0.585	2.70	0.15	1.79	1.00
	3	0.312	3.07	0.13	2.26	0.91
deoxycholate (DOC)	1	0.047	–	–	0	0.05
	3	0.064	0.92	0	0.06	0.30
N_2 + DOC	2	0.213	0.76	0.15	0.05	0
	3*	0.224	0.61	0	1.10	0.45
propylene glycol (P. G.) + DOC	3	0.392	0.67	0	0.06	0.14
DHA (in P. G.) + DOC	2	0.194	0.74	0.49	0.04	0.42
	3	0.405	0.76	0.38	0.23	0.37
A^4-3, 17-one (in P. G.) + DOC	3	0.288	0.50	0.04	0.06	0.24
N_2 + DHA (in P. G.) + DOC	1	0.142	–	–	0	1.02
	2	0.389	1.41	0.37	0.12	0.44

*15 minute exposure to DOC instead of the usual 30 minute period.

In the tissue to which propyene glycol was added before the DOC, more P-450/mg. protein was measured than in the control. In spite of that surprising result, *N*-demethylation rates remained at about 20 to 25% of control values, no *p*-hydroxylation was observed and steroid hydroxylations were not substantially protected by the steroid solvent, propylene glycol.

In contrast to propylene glycol alone, the addition of DHA in that solvent before the DOC treatment served to retain about 40% of the 16α-hydroxylase activity and to increase the *p*-hydroxylase activity by about 200%. *N*-demethylation still persisted but its rate remained at about 25% of the control and no substantial 7α-hydroxylation was detected in spite of a partial or no decrease of the P-450 content in that tissue.

Androstenedione in propylene glycol conferred less protection for all of the activities in comparison to the results with DHA and the *p*-hydroxylation was greatly diminished. No spots absorbed ultraviolet light on the thin-layer chromatograms which were obtained from the incubation of DHA with tissue which had been pretreated with androstenedione.

Finally, Table No. 29 shows that when DHA in propylene glycol was added to microsomes in a N_2-rich atmosphere and thereafter, the DOC was dissolved, the P-450 contents of two samples were reduced to about 20 and 65% of the controls, and the respective activities of the 16α-hydroxylase system were 65 and 44% of their control values. In those flasks, the rate of 7α-hydroxylation was low or undetectable. When the drug-metabolizing enzymes were tested, the residual *N*-

demethylation rate was about 45% of its control and *p*-hydroxylation was greatly increased as it had been in the presence of DHA in propylene glycol, but without N_2.

With the evidence that destruction of P-450 by DOC was accompanied by great decreases in *N*-demethylation and 7α-hydroxylation, and less for 16α-hydroxylation, it seemed logical to determine whether intact untreated membranes obtained from females would serve to supply P-450, or some other components, to the treated particles to restore the enzymatic functions. Table No. 30 shows the results of 7α- and 16α-hydroxylation from experiments wherein DOC-treated microsomes from male rats were incubated with intact ones from female rats. The rates calculated by subtraction of the female microsomal values from those obtained with the combined female and DOC-treated male preparation are given in addition to the results obtained separately.

The values in Table No. 30 for P-450 content of the DOC-treated tissue are identical to the ones given in Table No. 29 since the (male and female) values represent the combined individual amounts of male and female. The calculated values for the hydroxylations are of prime interest in comparison to the rates given by male microsomes alone. To the extent that those values agree, they are confirmatory. The differences would represent some facilitation or inhibition of the reaction as a result of a contribution from the female microsomes.

The rates for 7α-hydroxylation by untreated microsomes from males agree with the calculated male rates. With the DOC-treated microsomes, a minor quantitation error is apparent since the (male and female) value should be less than the female value alone if the male

Table No. 30. Cytochrome P-450 content and steroid hydroxylase activities by combined DOC-treated and intact microsomes.

P-450 CONTENT AND DHA 7α- AND 16α-HYDROXYLATION (mμmoles/mg./min.) BY THE COMBINED HEPATIC MICROSOMES FROM MALE AND FEMALE RATS.

Treatment	Expt. No.	P-450 Content (mμmoles/mg. prot.)				7α-Hydroxylation (mμmoles/mg./min.)				16α-Hydroxylation (mμmoles/mg./min.)			
		♂	♀	(♂+♀)	(♂+♀)-♀	♂	♀	(♂+♀)	(♂+♀)-♀	♂	♀	(♂+♀)	(♂+♀)-♀
none	1	0.740	0.590	0.670	0.740	2.70	0.34	1.51	2.70	1.56	0.17	0.89	1.56
	2	0.562	0.470	0.515	0.562	1.79	0.72	1.37	1.72	1.00	0.13	0.53	0.96
deoxycholate (DOC)	1	0.047	0.590	0.371	0.047	0	0.34	0.44	0.58	0.05	0.17	0.04	0
N_2 + DOC	2	0.206	0.470	0.376	0.206	0.05	0.72	0.18	0	0	0.13	0.18	0.24
DHA + DOC	2	0.186	0.470	0.364	0.186	0.04	0.72	0.33	0	0.42	0.13	0.20	0.30
N_2 + DHA + DOC	1	0.142	0.590	0.465	0.142	0	0.34	0.44	0.67	1.02	0.17	0.33	0.76
	2	0.374	0.470	0.436	0.374	0.12	0.72	0.30	0	0.44	0.13	0.25	0.44

value is actually 0. The value of 0.34 mμmoles/mg./minute by microsomes from female rats is slightly less than expected and, therefore, the residual value for the (male and female) female situation was probably reduced less than it probably should have been. This same consideration applies to experiment I in each instance. The data from experiment 2 show that there was no facilitation of 7α-hydroxylation by the addition of intact female microsomes to DOC-treated male microsomes.

With respect to 16α-hydroxylation, the data show no increases conferred by microsomes from female rats but the calculated rates represent confirmation that 16α-hydroxylation was not altered by DOC-treatment as much as the 7α-hydroxylase system.

D. Isolation and identification of 7β-OH-DHA.

Three products of the incubation of 3β-hydroxyandrost-5-en-17-one with liver microsomal fractions were consistently detected by using the picric acid spray on thin-layer plates chromatographed in system B. In addition to the red spot with the mobility (R_F 0.72) of the expected 16α-OH-DHA, there were two products, designated A_1 and A_2 (respective values, 0.31 and 0.35), which developed a vivid blue color immediately after the application of the spray. The only steroids reported (40) to give a blue color with the picric acid reagent are hydroxylated derivatives of parent compounds which display various shades of red. This fact and the demonstrated ability of rat liver homogenates to 7α-hydroxylate DHA (143) suggested that A_1 and A_2 might be 7-hydroxy derivatives of DHA. The ΔR_{Mg} values (97) for 7α- and 7β-hydroxylation, calculated from the chromatographic data of A_1 and A_2, and the reference steroids (see Table No. 31) were compatible with that hypothesis (70).

By pooling residues eluted from the chromatographic areas with the mobility of A_1 in system B, enough amorphous material was accumulated after several incubations with smooth and rough fractions of rat liver microsomes to perform the following tests: thin-layer chromatography in systems C, B, and M; overnight acetylation of eluates from these chromatograms followed by thin-layer chromatography in system C (R_F of the acetylated product, 0.67; blue color with the picric acid reagent); and anisaldehyde-concentrated H_2SO_4 (blue-gray), $SbCl_3$ (blue), blue tetrazolium (negative), and Zimmerman (positive) tests on the unacetylated compound. In addition, the latter was

Table No. 31. R_F and ΔR_{Mg} (7-H $\longrightarrow$ 7-OH) values derived from thin-layer chromatography of A_1, A_2, and reference steroids. $R_{Mg} = \log (\frac{1}{R_F} - 1)$;

ΔR_{Mg} (7-H $\longrightarrow$ 7-OH) = R_{Mg} (7-H)-R_{Mg} (7-OH).

Table No. 32. Spectrophotometric data (mμ) for the 7α-, and 7β-hydroxy derivatives of DHA, A_1, A_2 in concentrated sulfuric acid.

R_F and ΔR_{Mg} (7-H ⟶ 7-OH) Values Derived from the Thin-Layer Chromatography of A_1, A_2, and Reference Steroids.

Compound	System C		System B		System M	
	R_F	ΔR_{Mg} +	R_F	ΔR_{Mg}	R_F	ΔR_{Mg}
A_1 3β,7α-dihydroxy-androst-5-en-17-one:	0.04	1.362	0.31	0.994	0.20	0.949
This data	0.04	1.362	0.31	0.994	0.20	0.949
Data from (97)	0.02	1.382	–	–	0.17	0.908
A_2 3β,7β-dihydroxy-androst-5-en-17-one:	0.06	1.176	0.35	0.926	0.26	0.801
This data	–	–	0.35	0.926	0.26	0.801
Data from (97)	–	0.926 *	–	–	–	0.515 *

+ Subject to large errors because of very low R_F values.

* Values calculated for androst-5-ene-3β,17β-diol ⟶ androst-5-ene-3β,7β,17β-triol.

Spectrophotometric Data (mμ) for the 7α-, and 7β-hydroxy derivatives of 3β-hydroxyandrost-5-en-17-one, A_1 and A_2 in Concentrated Sulfuric Acid.

Free Alcohols	Maxima	Minima
7α-OH	232, 299, 395	253, 335
A_1	232, 299, 395	253, 345
A_2	232, 300, 395	253, 355
7β-OH	230, 300, 400	260, 345
Acetylated Products		
7α-OH	232, 297, 395	215, 270, 350
A_1	230, 300, 395	222, 260, 350
A_2	240, 300, 395	230, 265, 350
7β-OH	250, 297, 400	230, 270, 330

chromatographed on Whatman No. 42 paper with the system benzene: methanol:water (10:7:3; by vol.), eluted, and submitted to the modified Pettenkofer reaction. A violet color ($\lambda_{max.}$ 580 mμ) developed instantly at room temperature and faded upon subsequent heating. This behavior is characteristic of 3β, 7-dihydroxy-Δ^5-steroids, and appears to be related to the test originally described by Lifschütz (94,95) and subsequently modified by others (4,150). In all the tests described above A_1 and 7α-OH-DHA behaved identically. The spectra of the free and unacetylated compounds in concentrated H_2SO_4 is given in Table 32.

Sufficient A_1 for identification was accumulated by using rabbit liver microsomes. In these incubations, higher yields of A_2 than of A_1 are the rule, while the opposite is true about incubations with rat liver preparations. Two open flasks containing a total of 420 mg. microsomal protein from female rabbit liver, 10 mg. DHA (in 0.4 ml. propane-1,2-diol), 0.3 mmole NADPH, and 130 ml. Krebs-Ringer phosphate buffer pH 7.4 were shaken for one hour at 37°C.[1] The contents were extracted twice with 1600 ml. saturated $NaHCO_3$, twice with 680 ml. of distilled water, and reduced to dryness (1.5 g.). Successive TLC of the residue in systems B and M resulted in the isolation of about 100 μg. of crystalline A_2 with m.p. 211 to 214.5°C not depressed by authentic 7β-OH-DHA. These two compounds, after acetylation and thin-layer chromatography in system C, yielded single

---

[1] Mr. Robert Mushen, a Pre-Doctoral Trainee in the Department of Obstetrics and Gynecology, cooperated in these batch-incubations and the related procedures, the experiments on the mechanism of the hydroxylation and the development of the quantitative method for the 7-hydroxylated products.

spots with the same R_F, 0.69. Another batch of A_2 similarly prepared, m.p. 213 to 215°, permitted the comparison of its infrared spectrum with that of the reference steroid (see Figure No. 17). The identical infrared spectra, melting points, chromatographic properties of the free and acetylated compounds, and color reactions (which were the same as those described for A_1 above) are convincing evidence that A_2 is 7β-OH-DHA.

Evidence for the direct 7β-hydroxylation of the substrate: To ascertain the possible mechanism for the formation of 7β-OH-DHA, heated and fresh microsomes from rabbit liver were individually incubated with DHA and its 7α-hydroxy, 7β-hydroxy, and 7-keto derivatives (steroid/microsomal protein ratio, 1/20). Examination of the incubation products by TLC in system M and detection by the anisaldehyde and picric acid reagents showed that no transformation occurred with microsomes previously placed in a boiling water bath for ten minutes. Three major products of DHA after the incubation with fresh microsomes were the 16α- and 7β-hydroxy derivatives and an unidentified metabolite of intermediate polarity (respective R_F values: 0.53, 0.26, and 0.40); only traces of the 7α-hydroxy compound were detected. Incubation of the 7α- or the 7β-hydroxysteroid resulted in very little transformation of the substrate into its epimer, and no other major transformation. Traces of both 7-hydroxy epimers were also detected when 7-keto-DHA was the substrate (see Figure No. 18). Reliable quantitation of these transformations was not possible because of insufficient amounts of the reference compounds.

Figure No. 17. Infrared spectra of A: 220 μg. of 7β-OH-DHA and B: 205 μg. of crystalline A_2. The steroids were mixed with 80 mg. KI for the preparation of the pellets.

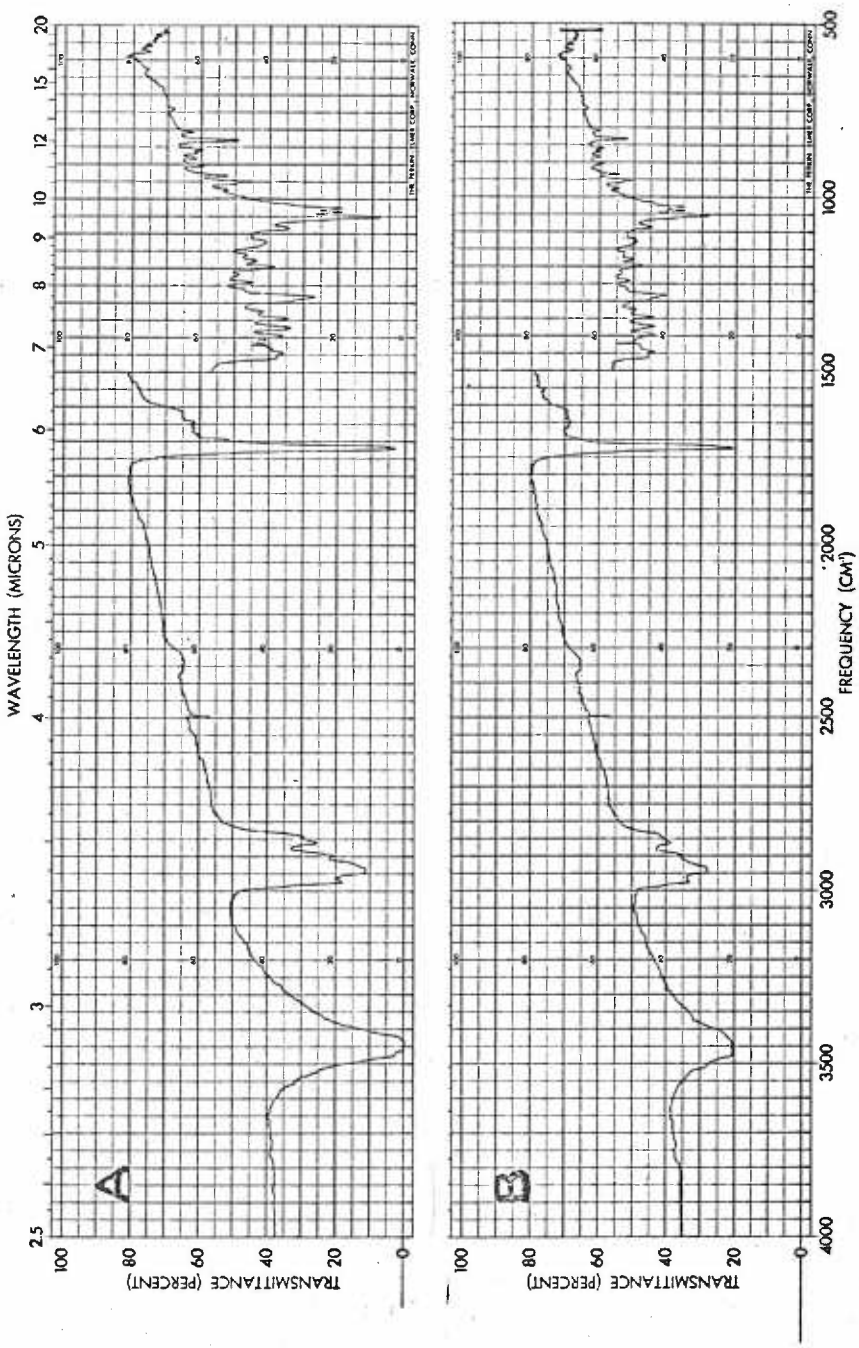
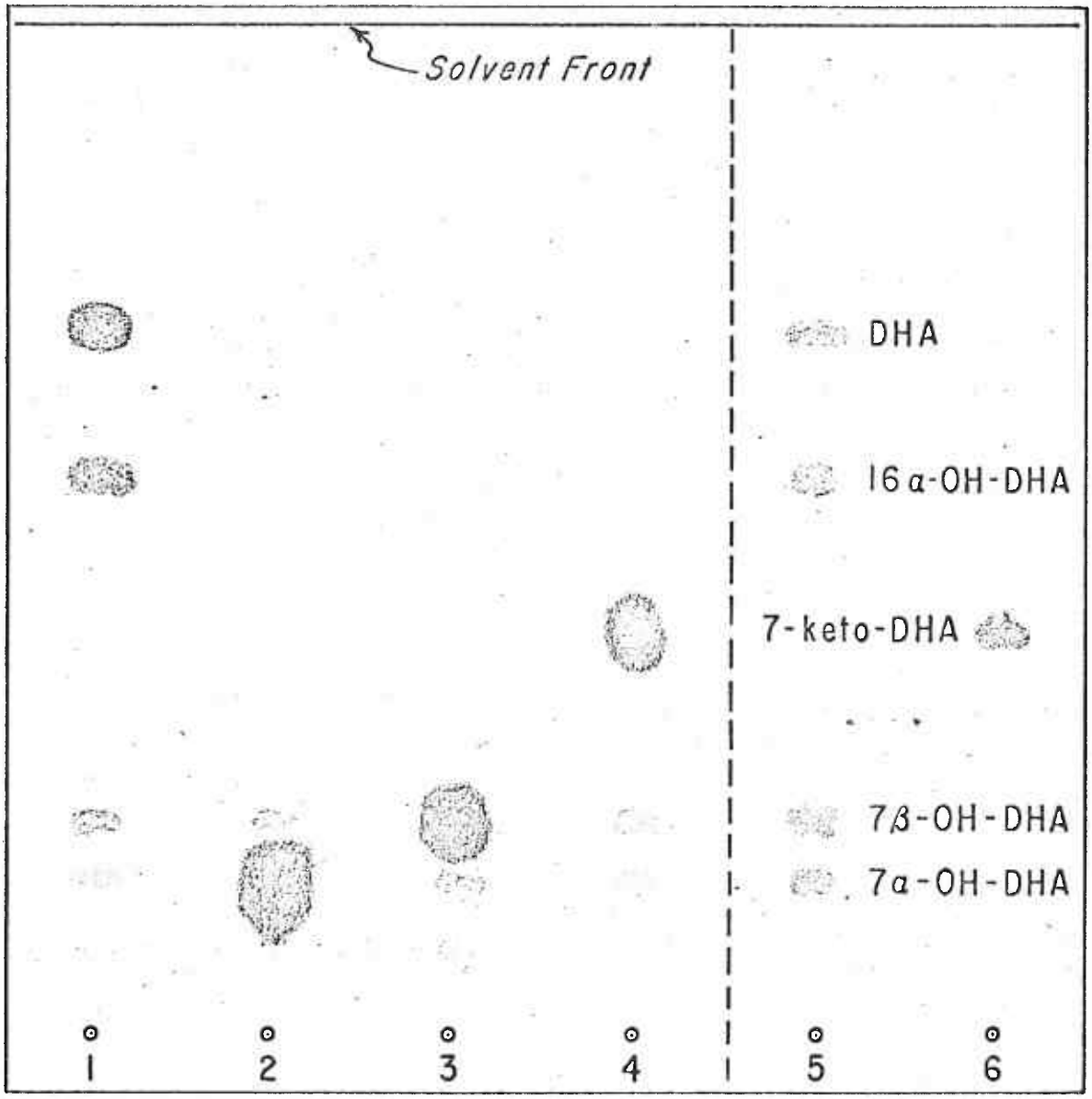

A
B
WAVELENGTH (MICRONS)
FREQUENCY (CM⁻¹)
TRANSMITTANCE (PERCENT)
TRANSMITTANCE (PERCENT)
2.5
3
4
5
6
7
8
9
10
12
15
20
2500
3000
3500
4000
2000
1500
1000
500
100
80
60
40
20
0

Figure No. 18. Chromatographic evidence for the direct 7β-hydroxylation of DHA by liver microsomes from male rabbits.

CHROMATOGRAPHIC EVIDENCE FOR THE DIRECT 7β-HYDROXYLATION OF DHA BY LIVER MICROSOMES FROM MALE RABBITS



Because 7β-hydroxylation was detected concurrently with the previously described experiments, and since the initial studies were related primarily to 16α-hydroxylation, quantitative data are unavailable for experiments relative to species, sex and age effects on 7-hydroxylations of DHA. Fortunately, however, photographic records of the chromatograms were made and semi-quantitative data was available from them. On the basis of the size of the spots and the intensity of the color reactions with picric acid reagent, the rates of transformations were graded 0 to +++ (see Table No. 33). The sex difference for 16α-hydroxylation in rat liver was clearly apparent and 7α-hydroxylation was the favored transformation at C-7. In rabbit liver, the 7β-hydroxylated product predominated and no sex difference was apparent for steroid hydroxylations. In contrast with the lack of 16α-hydroxylase activity in fetal monkey liver, both 7-hydroxylase systems were already detectable at this stage and were fully developed shortly after birth. Neither liver from a male nor a female human anencephalic fetus had 7-hydroxylation activity (see Table No. 34).

A search for 7-hydroxylation in various fetal organs yielded no activity in spleen, thymus, lung nor kidney (see Table No. 35). In the spleen and thymus gland, approximately 0.10 mμmoles/mg./minute of 16α-hydroxylation were observed. Comparison of the P-450 data from liver with that of the 16α-hydroxylation rate shows a lack of correlation but the 7-hydroxylation was absent when the P-450 content was reduced. From another type of experimental situation, Table No. 36 shows that the 7-, and 16α-hydroxylation activities were very low in

Table No. 33. Effects of species, sex and age on some hydroxylations of DHA by liver microsomes. The rate of each transformation is graded 0 to +++ on the basis of intensity and size spots in thin-layer chromatograms. The No. of preparations are in brackets.

EFFECT OF SPECIES, SEX AND AGE ON SOME HYDROXYLATIONS OF 3β-HYDROXYANDROST-5-EN-17-ONE BY LIVER MICROSOMES.

TISSUE SOURCE	No.	7α-OH-	7β-OH-	16α-OH-
ADULT RAT (Smooth microsomal subfraction):				
Male	[59]	+++	+	+++
Female	[16]	+++	+	0
(Rough microsomal subfraction):				
Male	[59]	+++	+	+++
Female	[16]	+++	+	0
ADULT RABBITS (Smooth microsomal subfraction):				
Both sexes	[3]	+	+++	+++
(Rough microsomal subfraction):				
Both sexes	[3]	+	+++	+++
RHESUS (Total microsomal fraction):				
Immature fetus (70-75 days) - female	[1]	+	+	0
Mature fetuses (124-160 days) - both sexes	[8]	++	+	0
Newborn (< 42 days) - both sexes	[6]	+++	+++	++
Mature (> 4 years) - both sexes	[4]	+++	+++	+++
HUMAN ANENCEPHALIC FETUSES (Total microsomal fraction):	[2]	0	0	+++

Table No. 34. 16α-hydroxylation by hepatic microsomes from male and female human anencephalic fetuses. The adjacent values represent duplicate incubations under the standard conditions calculated using the equivalent weight of liver or the mg. of biuret protein.

Table No. 35. Cytochrome P-450 and steroid hydroxylations in rat liver and in various organs of a human female anencephalic fetus.

16α-HYDROXYLATION BY HEPATIC MICROSOMES FROM A MALE AND A FEMALE HUMAN ANENCEPHALIC FETUS.

Source	Rates	
	µg./g./30 min.	mµmoles/mg./min.
♂; lyophilized tissue, H_3: (30 months old)	155.0	4.01
	180.0	4.70
♀; fresh tissue	83.0	0.87
	97.0	1.02

CYTOCHROME P-450 AND STEROID HYDROXYLATIONS IN RAT LIVER AND IN VARIOUS ORGANS OF A HUMAN ANENCEPHALIC FETUS.

	Cytochrome P-450 (mµmoles/mg. prot.)	Steroid Hydroxylations (mµmoles/mg./min.)		
		16α-	7α-	7β-
♂ rat liver	0.304	0.87	1.88	0.25
♀ rat liver	0.276	0.05	1.11	0.25
♀ human liver	0.066	0.95	0	0
" spleen	–	0.10	0	0
" thymus	–	0.09	0	0
" lung	–	0	0	0
" kidney	–	0	0	0

Table No. 36. Cytochrome P-450 content, N-demethylation, p-hydroxylation, and steroid hydroxylations by hepatic microsomes from a pregnant rat (est. 20 days gestation) and after addition of human chorionic gonadotrophin (HCG).

CYTOCHROME P-450 CONTENT, N-DEMETHYLATION, p-HYDROXYLATION AND STEROID HYDROXYLATIONS BY HEPATIC MICROSOMES: A PREGNANT RAT AND SUPPLEMENTATION WITH HUMAN CHORIONIC GONADOTROPHIN (HCG).

	♂	Preg. ♀	♂	♂ 100 i.u. HCG	♂ 500 i.u. HCG
Cytochrome P-450: (mμmoles/mg. prot.)	0.748	0.214			
Enzymatic activities: (mμmoles/mg./min.)					
p-hydroxylation	0.51	0.45	–	–	–
N-demethylation	3.79	0.91	–	–	–
7α-hydroxylation	2.36	0.03	1.39	1.69	1.88
7β-hydroxylation	0.15	0	0.14	0.05	0.05
16α-hydroxylation	1.77	0.05	1.71	1.58	1.76

hepatic microsomes from the pregnant rat which contained 0.214 mμmoles/mg./protein of P-450. The latter amount is slightly more than one-half of the mean value from non-pregnant rats. In the presence of human chorionic gonadotrophin, the 7α-hydroxylation rates were greater than for the control value but 7β-hydroxylation was reduced. Addition of 100 I.U. reduced the 16α-hydroxylation; however, the average rate in the presence of 500 I.U. was unchanged.

IV. DISCUSSION

The DHA 7α- and 16α-hydroxylase systems are active in hepatic microsomal fractions which also contain many other oxido-reductases. The identification of 7β-OH-DHA as another metabolic product of DHA in the incubations of microsomal fractions from rat, rabbit and monkey liver reveals a new transformation of steroid hormones by mammalian preparations. The criteria for identification were chromatographic mobilities, several color reactions, melting behavior, concentrated H_2SO_4 and infrared spectra. The rabbit liver in this study also had a higher rate of 7β-hydroxylation in comparison to that in the 7α-configuration confirming the same distribution of activity when 3β-OH-Δ^5-cholenic acid was the substrate (156). In rat liver, the opposite distribution observed with cholesterol-26-^{14}C as the substrate (107), was also confirmed using DHA. The accumulation of both 7-epimers of DHA appeared equal in monkey liver and none was detected from human fetal liver incubations.

Two facts point to a direct hydroxylation mechanism for 7β-OH-DHA. Autoxidation was not responsible since boiled preparations yielded no product, confirming observations with cholesterol (107). The possibility of the operation of a 7β-oxidoreductase, which seems likely with the C_{27} substrate, but not with C_{18} substrates (96), is unlikely with C_{19} substrates, since neither 7-keto-, nor 7α-OH-DHA yielded more 7β-OH-DHA than did DHA, although they would be expected to be intermediates in such conversion.

Aside from its participation in the formation of bile acids from cholesterol, little is known about the precise biological significance of steroid 7-hydroxylation (149). A precursor role is possible for 3β-7-dihydroxy-Δ^5-C_{19} steroids in the formation of 7-hydroxylated estrogens and steroids with an aromatic ring B (145). It is interesting that 7β-hydroxylated bile acids were found only in the gall bladders of infants or younger children at autopsy (8) but steroid 7β-hydroxylation is not limited to infants since the transformation was also demonstrated *in vivo* following the administration of etiocholanolone or testosterone to adult males (54).

The colorimetric method for the quantitation of both 7-hydroxylated epimers has precedent in tests which also utilize concentrated sulfuric acid and have a λ_{max} at 580 mμ (150,8,4). The modified Lifschütz reaction is highly specific for 3β, 7-dehydroxy-Δ^5-steroids and the chromogen is stable for at least 20 minutes (150). The chromogens formed by the 7-hydroxy epimers of DHA with the modified Pettenkofer reagent are less stable, but they have molar extinction coefficients in the range 17-20,000 compared to 7500 given by 3β, 7α-dihydroxy-Δ^5-cholenic acid in the Lifschütz conditions. Also, the Pettenkofer reagent has the convenience of detecting 7-hydroxylated 3β-OH-Δ^5-steroids separately from others in the same solution merely by recording the absorbancies before and after heating which destroys the chromogen of the 7-hydroxy compounds. Inadequate amounts of standard compounds were available to do a complete analytical study, but the sensitivity calculated for the Pettenkofer reagent using the data reported with the Lifschütz reagent is high enough to distinguish 0.3 μg of product.

The rates of the steroid hydroxylations, expressed as mμmole/mg./minute or μg./g./hour, are net rates which apply specifically to the conditions employed and are not strictly comparable to those determined with other steroid substrates or with different time intervals. The experimentally determined conditions, which have precedent in many reports of *in vitro* steroid metabolism, do not account for the metabolism of the products to any other compounds with chemical configurations which might alter their detectability based upon the specificity of the colorimetric methods. If, for example, 16α-OH-DHA was further hydroxylated, the product might be measured as one of 16α-hydroxylation as long as the α-ketolic configuration remained intact (the molar extinction coefficients may change appreciably, however). Nevertheless in this study, most of the added 16α-OH-DHA could be accounted for as α-ketols at the end of the incubation so that any transformations subsequent to 16α-hydroxylation are probably minimal. The inhibiting effect of a 16α-hydroxyl function on further metabolic conversions has been recognized already from studies *in vivo* (53).

Alternatively, to measure initial rates, which would have been ideal, would have required that the experimental conditions be repeatedly determined and changed when different species and sexes and drug-treatments were evaluated. The chemical induction of such interrelated and variable enzymatic systems as those contained in the microsomal membranes produces selective but unknown effects which might have direct, indirect or no effects on any part of the system. For example, the hepatic microsomal 16α-hydroxylation of adult male rats might be decreased after drug treatments because of an induction of enzymes

which compete for the substrate, or might be decreased as a result of the induction of other enzymes active in the metabolism of 16α-hydroxylated products. To meet the objectives of this study, it was necessary, therefore, to choose conditions where the rate of the transformation was linear with respect to the amount of enzyme; the substrate and cofactors were present in excess; and where the amount of product formed was adequate for accurate measurements. These conditions were met. Furthermore, it was determined that most of the product formed was not metabolized and that 70 to 90% of the substrate remained at the end of the incubation period when non-treated tissues were being tested. As shown in the curve of the rates of 16α-hydroxylation in incubations of 30 minutes and longer, those for the former period are the greatest and certainly periods longer than 30 minutes cannot be safely used. The rates are of the same order of magnitude as those determined for hydroxylations of other steroid substrates in conditions that utilized 5, 7, 10 or 15 minute incubation periods, but where imprecise detection methods were employed (see Table No. 1). Comparisons with data reported by the same workers in 1967, where specific hydroxysteroids were measured, shows that the rate of 16α-hydroxylation of DHA (1.41 mμmoles/mg./minute) is nearly identical to that for testosterone 16α-hydroxylation (1.5) for which the 7 minute incubation period applies.

The preliminary data related to the 16α-hydroxylation rates of some C_{19} steroids and estrone are subject to reservation because they also represent net rates. It is possible that the compounds for which the highest rates were observed are those which simply are not metabolized

further in those conditions, rather than reflecting any selectivity for the 16α-hydroxylase system. In addition, those rates may be incorrect to the extent that the various 16α-hydroxylated products have different extinction coefficients in the BT reaction from that of 16α-OH-DHA, the colorimetric standard.

The DHA 16α-hydroxylation was decreased but 7-hydroxylation was increased in hepatic tissue from male rats which had been treated with phenobarbital, 3-methylcholanthrene, or phenylbutazone. In contrast to the result in males, the 16α-hydroxylase activity was increased slightly in the tissue from females after phenobarbital treatment. The decrease in the activity was limited to the rough subfraction after phenobarbital or 3-methylcholanthrene, so that the smooth/rough ratios increased to over 1.0. The opposite distribution prevailed after phenylbutazone therapy and no other major products of DHA were observed after that agent.

In no instance were the rates of 7α-, or 7β-hydroxylation reduced by drug treatments. The small number of experiments in each group does not permit the calculation of reliable significance levels but the rate increases ranged from 13 to 188%. The average age of the animals that contributed the control data was older and the large S.D. is probably a result. The largest increase occurred after phenobarbital treatment, just as was observed with *N*-demethylation. The 7-hydroxylations by the smooth subfractions were selectively stimulated by 3-methylcholanthrene and phenylbutazone; therefore, the smooth/rough ratios increased to more than 1.0. The 7β-hydroxylase activity, which represented only about 10 to 20% of that for 7α-hydroxylation and is

therefore less accurately measured, followed exactly the same trend. The expected increases in cytochrome P-450 content and the stimulations of the drug-metabolizing enzymes were confirmed and it was also observed that the mature females were stimulated more than the males, confirming previous data (85). These results of enzymatic activities after drug treatments indicate that the 16α-hydroxylase system responds in an opposite way from the 7-hydroxylase which mimics the increases in the cytochrome P-450 content and the activity of drug-metabolizing enzymes. Therefore, if the observed parallelism of the changes can be taken as an indication, DHA 7-hydroxylation is more likely to be dependent on cytochrome P-450 than is DHA 16α-hydroxylation. Table No. 37 tabulates the comparisons between cytochrome P-450, steroid hydroxylations and drug metabolism.

This failure to stimulate the 16α-hydroxylation of steroid substrates by drug treatments is contrary to the published reports (153). Significant differences in the experimental conditions are possibly the basis for the different results. Although those authors limited the incubation periods to 7 minutes, the ratio of substrate/microsomal protein was much smaller and they used a NADPH generating system. Their rates could hardly be initial rates since about 70% of the substrate, testosterone, was metabolized in that period. Even so, the rate of 16α-hydroxylation measured by these authors from adult male rats (nutritional status not reported) can be calculated as 1.5 mμmoles/mg./minute, assuming 20 μg. microsomal protein/g. liver. In the present study, the rate of 16α-hydroxylation was 1.41 ± 0.32 mμmoles/mg./minute from unfasted animals. The rate of 7α-hydroxylation calculated from

Table No. 37. Comparison of the cytochrome P-450 content, steroid hydroxylations and drug metabolism by hepatic microsomes from rats.

COMPARISON OF PROPERTIES OF HEPATIC SYSTEMS INVOLVED IN THE METABOLISM OF STEROIDS AND DRUGS.

		Cytochrome P-450	DHA Hydroxylation 7α-	7β-	16α-	p-Hydroxylation	N-Demethylation
Present in immature rats		yes	yes	–	no	yes	yes
Sex dependent in adult rats		no	yes	no	yes	no	yes
Stimulated in adults by:							
phenobarbital	M	yes	yes	yes	no[1]	no	yes
	F	yes	yes	yes	yes	yes	yes
3-methylcholanthrene	M	yes	yes	yes	no[1]	yes	yes
	F	yes	yes	yes	no	yes	yes
phenylbutazone	M	no	yes	yes	no[1]	no	yes
	F	no	yes	yes	no	yes	yes
Inhibition by:							
aminopyrine		–	yes	–	yes	–	–
DHA		–	–	–	–	–	yes
SU-9055		yes	yes	yes	yes	–	yes
CO		–	yes	yes	yes	no	yes
KCN		–	yes	yes	yes	no[2]	yes
pregnancy		yes	yes	yes	no	no	yes
Destruction by deoxycholate:							
alone		yes	yes	–	yes	yes	yes
DHA plus DOC		yes[3]	yes	–	yes[3]	no[2]	yes

1. actually reduced
2. actually increased
3. but some protection

the reported data was 0.54 mμmoles/mg./minute compared with 1.33 ± 0.82 in this study. This difference is an example of the limitation imposed upon any comparisons of data collected under different conditions such as substrate, cofactor, time and preparation of the tissue. A factor which may be important--and yet has not been studied--in comparisons of the induction of steroid hydroxylase systems is the age of the animals. The experiments that demonstrated the induction of the 16α-hydroxylase were carried out using rats younger than those used in the present experiments wherein animals with a maximum, natural development of the enzymatic activity were selected. The stimulation of drug-metabolizing enzymes by 3-methylcholanthrene or barbital treatments is much greater in immature males than in mature ones (27,89) and the same response might be true for steroid hydroxylases.

Aminopyrine inhibited competitively the DHA 7-hydroxylase system in reciprocal kinetic studies and the drug affected 16α-hydroxylation to a much less extent. In the tests of *N*-demethylation, DHA proved to be a potent inhibitor. Based upon the smaller apparent K_m values obtained previously for steroid substrates, one would expect that DHA would inhibit the drug-metabolism (148). The difference in the degree of competition of the drug and steroid for 16α- and 7α-hydroxylations was unexpected but the data are consistent with the hypothesis of different mechanisms for these two reactions. Furthermore, the graphical plot, indicating a competitive inhibition by aminopyrine on 7α-hydroxylation in the presence of low concentrations of DHA, also suggests that their reactions are related. The apparent K_i, 0.68

10^{-4} *M*, is in the range of reported values (148). At the higher concentrations of DHA, the effect of aminopyrine was opposite in direction and remains unexplained.

Kinetic studies with SU-9055 show that this steroid hydroxylase inhibitor decreases both *N*-demethylation of aminopyrine and 16α-hydroxylation of DHA, but in equimolar concentrations, the former reaction was inhibited over 90% whereas 16α-hydroxylation sustained only a 50% inhibition. The reported inhibition of testicular 16α-hydroxylation of progesterone by SU-9055 (10^{-4} *M*) was over 75% complete (20). The 7-hydroxylation reaction was not inhibited at low or moderate concentrations of SU-9055, which rather increased the activity. After it was determined that SU-9055 produces a low amplitude spectral response, as had drugs or steroid substrates when they were added to microsomes, it was observed that the amount of cytochrome P-450 remaining varied inversely with the concentration of SU-9055. The apparent K_i of the interaction determined graphically, 1.7×10^{-6} *M*, is an order lower than the apparent K_m's reported for the group of steroid substrates (148). At 2×10^{-6} *M*, the concentration of SU-9055 which produced a 50% inhibition of P-450, *N*-demethylation was inhibited 65%, 16α-hydroxylation was not affected and 7-hydroxylation was increased 50%, but as was mentioned, at equimolar concentrations of inhibitor and substrate, the cytochrome P-450 content and *N*-demethylation were decreased over 90% but 16α-, and 7-hydroxylation were inhibited only by about 50%. The obvious interpretation of these data is that the steroid hydroxylases studied are less, if at all, dependent on P-450 than is *N*-demethylation. Alternatively, if they are P-450

dependent, the turnover rate is very high, a different mechanism of electron transfer is operative, or different species of P-450 with varied specificities, perhaps, are present. SU-4885,[1] another substituted pyridine, appears from indirect data to inhibit partially the steroid 11β-hydroxylation by adrenal mitochondria by an interaction with cytochrome P-450 (131). Evidence for more than one cytochrome P-450 is accumulating from several different kinds of experiments (73, 74,108,139).

In preliminary studies included only for completeness, the 80% CO:20% O_2 gas mixture inhibited *N*-demethylation and 7α-hydroxylation by less than 40% and 16α-hydroxylation by nearly 80%. KCN inhibited the latter transformation by 40% but the former ones by less than 25%. Besides pointing to one different response of the steroid hydroxylases, these data show again a similarity of the 7α-hydroxylase with the *N*-demethylase systems. In regard to the sensitivity to KCN, the 16α-hydroxylase is analogous to the stearyl-CoA desaturase, a hepatic microsomal mixed function oxidase, which apparently is independent of P-450 and is sensitive to KCN (122). The 7-hydroxylation of 3β-OH-Δ^5-cholenic acid by rabbit liver microsomes was inhibited 65% by KCN (150). The increase in *p*-hydroxylation in these experiments confirms KCN inhibition data obtained with rabbit microsomes (105). The stimulation of *p*-hydroxylation by CO may have some relation to the same response by the stearyl-ACP desaturase from yeast (109).

A 30 minute treatment of hepatic microsomal membranes obtained from male rats with deoxycholate (DOC) abolished the 7α-hydroxylation

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[1] 2-methyl-1, 2-bis (3-pyridyl) -1-propanone

and *p*-hydroxylation, and destroyed 90% of the P-450, 70% of the *N*-demethylation, and 67 to 97% of the 16α-hydroxylation. Addition of DHA before the DOC resulted in destruction of only 33 to 58% of the 16α-hydroxylation but changed little the 7α-hydroxylation and *N*-demethylation. Under the same conditions, *p*-hydroxylation was stimulated by about 200% and cytochrome P-450 was either protected partially from the action of DOC or increased slightly. N_2, propylene glycol or androstenedione did not affect the hydroxylations but appeared to protect the P-450. The addition of untreated microsomes from female rats did not facilitate either steroid transformation.

Interpretation of these results is difficult because some important samples were lost or the importance of including others could not be anticipated. Nevertheless, they indicate too that 7α-, and 16α-hydroxylation rates are not solely dependent on the cytochrome P-450 content. Also, the interaction of DHA with the 16α-hydroxylase partially prevents the destructive action of DOC but the 7α-hydroxylase remains vulnerable, or perhaps an additional co-factor required for the action of the latter is not influenced by DHA. Further experiments will be necessary to show if more than one P-450 and/or another redox component(s) are active in steroid and drug metabolism, and, if so, whether they are destroyed selectively by DOC. The 7α-hydroxylation of DOC has been reported and one might speculate that this transformation inhibits the DHA 7α-hydroxylation, or that after the enzyme interacts with the DOC, further hydroxylations are impossible because of structural changes in the enzyme (5). The present data confirm the retention of 20 to 25% of the *N*-demethylase activity found after

snake venom solubilization of microsomes from either control or phenobarbital-treated rats (120). The same % of the *N*-demethylation of *N*, *N*-dimethylalanine was retained after DOC treatment (157). Addition of untreated microsomes to the tests of benzpyrene hydroxylation in Triton N - 101 - treated microsomes did not stimulate the reaction either (137).

Species, age, and sex-related differences in the steroid 7-, and 16α-hydroxylase activities are greater than has been appreciated previously. In the fetal condition, for example, the only detected transformation of DHA by human fetal microsomes was 16α-hydroxylation, but in the fetal monkey, the latter transformation was nearly absent whereas the 7-hydroxylated epimers were found. The very low concentration of cytochrome P-450 found in the hepatic microsomes from the human fetus parallels the reduced 7-hydroxylation activities. Similarly, microsomes from a pregnant rat were inactive in the tests for the 7-hydroxylations and less active in *N*-demethylation, but the cytochrome P-450 content was 45% of the values of nonpregnant rats. Pregnancy has been observed to decrease also the alanine transaminase activity in liver from rats near term gestation (66). Newborn rat liver contains 25% as much cytochrome P-450 as that from adults and it increases progressively to 75% of adult levels during the first week of life. In the same time period, the *N*-demethylase activity increases to 35% of adult values (29). The latter transformation also is sex-dependent in rats (p = <0.001) with a male/female ratio (M/F) of 1.62 (83). Although the DHA 7α-hydroxylation rates were not studied with respect to the age of rats (Table No. I confirms the activity

from 40 to 50 g. immature rats), microsomes from mature males were more active in this respect too, than those from females (p = <0.002). The M/F ratio was 3.24. In contrast, the 16α-hydroxylation became really active only with the sexual maturity of males. The M/F ratio was 14.1. Taken together, these comparative findings from several biological situations suggest that 7α-hydroxylation is probably dependent on cytochrome P-450 for reducing equivalents to a greater extent than 16α-hydroxylation. Acetanilide p-hydroxylation, also dependent on cytochrome P-450, is not sex-dependent in rats nor is it affected by pregnancy (85,90).

Biological variations of the group of the microsomal mixed function oxidases from mammalian liver are not limited to enzymatic transformations. As an example of another type of variation, microsomal fractions obtained from rabbit liver appear to have less mitochondrial contamination than those prepared similarly from rats (112). It was possible in this study to reduce greatly the amount of mitochondrial fragments, determined by a marker enzyme, succinic-cytochrome c reductase, in the microsomal fractions from rats by introducing a second 10,000 g centrifugation of the diluted, isotonic supernatant. Incorporation of this procedure and the practice of using non-fasted animals, whose hepatic protein content is lower than is obtained from fasted ones, served to reduce by about 25% the content of protein (mg./g.) in the microsomal fractions. A comparative study of rabbit and rat microsomes showed that either pre-incubation for one hour or prolonged centrifugations affect rat microsomes more than those from rabbits, especially in the smooth subfractions (51). The

separation methods utilized for the comparative study with drug-metabolizing enzymes from rats were incorporation of an addition of $CsCl_2$ to increase the particle density or dilution of the fractions to hypotonic solutions; in the present study the comparable solution was made isotonic. Also in the comparative study cited, the incubation mixtures were supplemented with 105,000 *g* supernatant as a partial source of the NADPH-generating system and the reactions were carried out in an O_2 atmosphere. This study utilized incubation mixtures with an excess of NADPH in a buffer medium, and incubations in air. In spite of the modifications employed here, the acetanilide and the DHA-16α-hydroxylases from the rat liver microsomal subfractions also gave erratic and variable results if the tests of enzymatic activity were delayed between 2 to 10 hours.

The protein content in the smooth and rough subfractions of rabbits or rats represents another species difference. Both subfractions from rabbits contain 2 to 4 mg. of protein per g. of fresh tissue whereas the rough subfraction from rats has three to five-fold more protein than the smooth one. The extrapolation to the situation in the intact animal of the facts in conjunction with other data (62) showing that the smooth subfraction from rabbits is the one more active in oxidative metabolism, but that the rough one from rats is usually more active (28), would indicate that the SER of rabbits, but the RER of rats, is responsible for the quantitative transformations per g. of liver tissue from normal animals. The protein content from fasted rats was slightly higher, probably because of glycogen depletion from the livers of those animals.

Another variation observed was a sex difference in the hepatic protein content in rats after the drug treatments. The amount of protein in the smooth subfractions increased in both sexes, and did so in the rough subfraction from females, but less protein was measured in that subfraction from males after all three types of treatment. The latter observation is consistent with the hypothesis that SER is formed in some fashion from RER (29). The accurate separation of the subfractions is supported by electron microphotography of the pellets, RNA predominance in the rough subfractions, and the confirmation of published data which correlated the enzymatic activities with morphologic parameters (50,125).

V. SUMMARY AND CONCLUSIONS

Three hydroxylations of dehydroepiandrosterone (DHA) were studied in hepatic microsomal fractions from rats, rabbits, rhesus monkeys and human anencephalic fetuses. In addition to known 7α-, and 16α-hydroxylation, DHA 7β-hydroxylation was identified as a new transformation of steroid hormones by mammalian preparations. The steroid transformations showed a number of biological differences related to species, age and sex. Separation of microsomal fractions into smooth and rough subfractions demonstrated that the smooth microsomes of the rabbit, but not of the rat, are more active to carry out steroid hydroxylations, *p*-hydroxylation of acetanilide, and *N*-demethylation of aminopyrine.

Tissue from mature rats which had been treated with phenobarbital, 3-methylcholanthrene, or phenylbutazone was characterized by increased rates of 7-hydroxylation and oxidative metabolism of acetanilide or aminopyrine, but 16α-hydroxylation was decreased. The cytochrome P-450 content was increased as expected.

Aminopyrine inhibited competitively the DHA 7α-hydroxylation but not that at C-16. SU-9055 inhibited all of the enzymatic transformations, although at different rates; the apparent K_i for the SU-9055-cytochrome P-450 interaction was 1.7×10^{-6} *M*. KCN or CO inhibited 16α-hydroxylation more than the other enzymatic activities.

Treatment of microsomal membranes with deoxycholate destroyed most of the cytochrome P-450 and the enzymatic activities, but the 16α-hydroxylase system was protected to the extent of 40% or more by

the addition of DHA prior to the treatment. The *p*-hydroxylation of acetanilide was unaccountably stimulated 200% after the same treatment.

These data emphasize a number of differences among steroid hydroxylases and drug-metabolizing enzymes. The great similarity observed between DHA 7α-hydroxylation and aminopyrine *N*-demethylation suggests that they share a closely-related mechanism; however, DHA 16α-hydroxylation and acetanilide *p*-hydroxylation differ so much from each other and from the former transformations that a multiplicity of specific enzymes, redox components, or mechanisms must be postulated to explain them.

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