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The Metabolism Of Estrone, Estradiol And Their 3-sulfates By Guinea Pig Liver Preparations

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THE METABOLISM OF ESTRONE, ESTRADIOL AND THEIR 3-SULFATES BY GUINEA PIG LIVER PREPARATIONS

by

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ABSTRACT

Estrogens, be they conjugated or unconjugated, are metabolized in tissues mainly by two enzymic processes: dehydrogenases which add or remove two hydrogen atoms and hydroxylases which add molecular oxygen to the steroid molecule. Although the biological activity of the hydroxylated estrogens is largely unknown, a few hydroxylated estrogens, mainly 2 and 16α-hydroxylated estrogens, have previously been shown to possess biological activity other than the typical estrogenic activity. In view of the apparent importance of at least some hydroxy phenolic steroids, it seemed important to develop a model to study their control and define their biosynthesis.

Guinea pig liver tissue (slices and microsomes) was employed in the investigation. Early studies were involved in comparing the metabolism of the estrogen conjugates, estrone sulfate and estradiol sulfate with that of the corresponding unconjugated estrogens, estrone and estradiol. Metabolites were identified by employing Sephadex A_{25} chromatography, specific enzymatic incubations of the conjugated products or solvolysis, Girard reaction and Sephadex LH_{20} chromatography.

Final verification was obtained by crystallization to constant specific activity of the isolated steroids and of acetate and, in some cases, acetonide derivatives of the steroids.

Metabolites identified from microsomal incubations indicated that 16β-hydroxylated products (16β-hydroxyestrone and 16-epiestriol) arose mainly from the free estrogens, while 16α-hydroxy steroid sulfates (16α-hydroxyestrone-3-sulfate and estriol-3-sulfate) were predominantly formed from the sulfated estrogens.

The 16α-hydroxylation of estrone sulfate by guinea pig liver microsomes (one of the major pathways of estrogen hydroxylations in man) offered a useful model to study this enzymatic activity under circumstances uncomplicated by the presence of additional hydroxylases which act upon the substrate. Although 16α-hydroxy metabolites of the estrogens have been recognized for more than forty years and are of major quantitative significance in the human, little specific information has been available regarding the properties of the hydroxylase(s) responsible for their formation. Employing guinea pig liver microsomes, properties of the 16α-hydroxylation of estrone-3-sulfate were investigated. Studies were also performed to determine the effect of

synthetic steroids, such as those contained in the oral contraceptives, and of other natural steroids on the 16α-hydroxylation pathway of estrone sulfate.

Following a microsomal incubation with estrone sulfate, the monosulfates, separated by Sephadex A_{25} chromatography, were investigated for the presence of 16α-hydroxyproducts by incubation with mylase P, a phenolsulfatase preparation, followed by Sephadex LH_{20} chromatography. Formation of 16α-hydroxyestrone-3-sulfate plus estriol-3-sulfate gave the total 16α-hydroxylation of estrone-3-sulfate. The optimum conditions of the 16α-hydroxylase activity were found to be: incubation time, 30 min., microsomal protein concentration, 6-8 mg/5 ml of incubation mixture and pH 7.5. Employing these conditions, Km for estrone-3-sulfate was determined to be 3.55×10^{-5} and that for the cofactor NADPH 4.18×10^{-4}M. The synthetic steroids, norethynodrel and ethinyl estradiol, were found to inhibit the 16α-hydroxy pathway to the extent of 35-40%.

The guinea pig liver system has been found to be a suitable model to study estrogen hydroxylations (particularly 16α-hydroxylation). Results from the microsomal incubations showed that the sulfate moiety at position 3 of the steroid directs 16-hydroxylation from the β- to the α-configuration. Inhibition caused by ethinyl estradiol seemed to be competitive in nature.

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I dedicate this thesis to my parents in recognition and sincere appreciation of their personal sacrifices on behalf of my education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

STEROID NOMENCLATURE

Trivial and systematic names of steroids used or mentioned in this thesis.

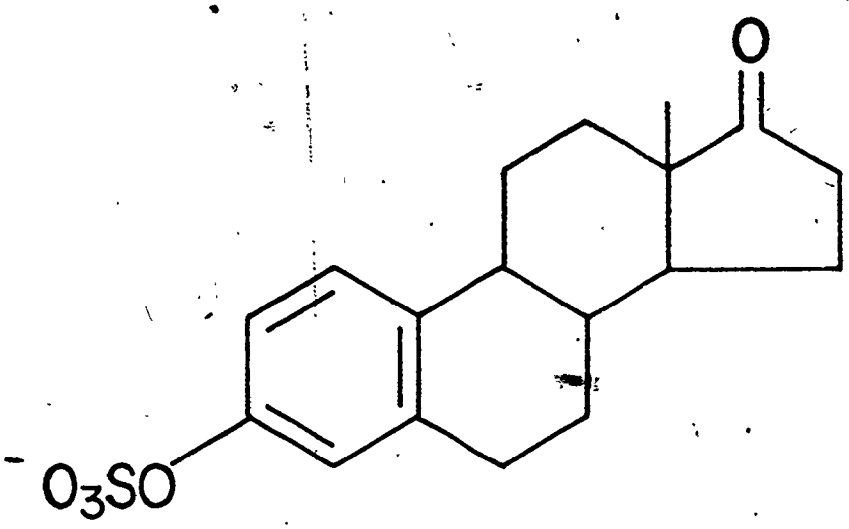
Trivial Names	Systematic Names
C_{18} Steroids:	
Estrone (E_1)	3-Hydroxyestra-1,3,5(10)-trien-17-one
16α-Hydroxyestrone ($16\alpha OHE_1$)	3,16α-Dihydroxyestra-1,3,5(10)-trien-17-one
16β-Hydroxyestrone ($16\beta OHE_1$)	3,16β-Dihydroxyestra-1,3,5(10)-trien-17-one
Estradiol-17β (Estradiol, E_2 $E_2 17\beta$)	Estra-1,3,5(10)-triene-3,17β-diol
Estradiol-17α ($E_2 17\alpha$)	Estra-1,3,5(10)-triene-3,17α-diol
16-ketoestradiol ($16ketoE_2$)	3,17β-Dihydroxyestra-1,3,5(10)-trien-16-one
Estriol (E_3)	Estra-1,3,5(10)-triene-3,16α,17β-triol
16-Epiestriol ($16EpiE_3$)	Estra-1,3,5(10)-triene-3,16β,17β-triol
Estrone-3-glucuronide ($E_1 3G$)	17-keto-estra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate
Estrone-3-sulfate (estrone sulfate, $E_1 3S$)	17-keto-estra-1,3,5(10)-trien-3-yl-sulfate
16α-Hydroxyestrone-3-sulfate ($16\alpha OHE_1 3S$)	16α-Hydroxyestra-1,3,5(10)-trien-17-one-3-yl-sulfate
2-Hydroxyestrone sulfate	2-Hydroxyestra-1,3,5(10)-trien-17-one-3-yl-sulfate

Trivial Names	Systematic Names
2-Methoxyestrone sulfate	2-Methoxy-estra-1,3,5(10)-trien-17-one-3-yl-sulfate
16α-Hydroxyestrone-16α-glucuronide ($16\alpha OHE_1 16G$)	3-Hydroxyestra-1,3,5(10)-trien-17-one-16α-yl-β-D-glucopyranosiduronate
16α-Hydroxyestrone-3-sulfate-16α-glucuronide ($16\alpha OHE_1 3S16\alpha G$)	17-keto-estra-1,3,5(10)-trien-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate
16α-Hydroxyestrone-3,16α-disulfate ($16\alpha OHE_1 3,16diS$)	17-keto-estra-1,3,5(10)-triene-3,16α-di-yl-sulfate
Estradiol-3-glucuronide ($E_2 3G$)	17β-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate
Estradiol-17β-glucuronide ($E_2 17G$)	3-Hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate
Estradiol-3,17-diglucuronide ($E_2 3,17diG$)	Estra-1,3,5(10)-triene-3,17β-di-yl-β-D-glucopyranosiduronate
Estradiol-3-sulfate (estradiol sulfate, $E_2 3S$)	17β-Hydroxyestra-1,3,5(10)-trien-3-yl-sulfate
Estradiol-17-sulfate ($E_2 17S$)	3-Hydroxyestra-1,3,5(10)-trien-17β-yl-sulfate
Estradiol-3,17β-disulfate ($E_2 3,17\beta diS$)	Estra-1,3,5(10)-triene-3,17β-di-yl-sulfate
Estradiol-3,17α-disulfate ($E_2 3,17\alpha diS$)	Estra-1,3,5(10)-triene-3,17α-di-yl-sulfate
Estradiol-3-sulfate-17-glucuronide ($E_2 3S17G$)	Estra-1,3,5(10)-trien-3-yl-sulfate-17β-yl-β-D-glucopyranosiduronate
Estradiol-3-phosphate	17β-Hydroxyestra-1,3,5(10)-trien-3-yl-phosphate

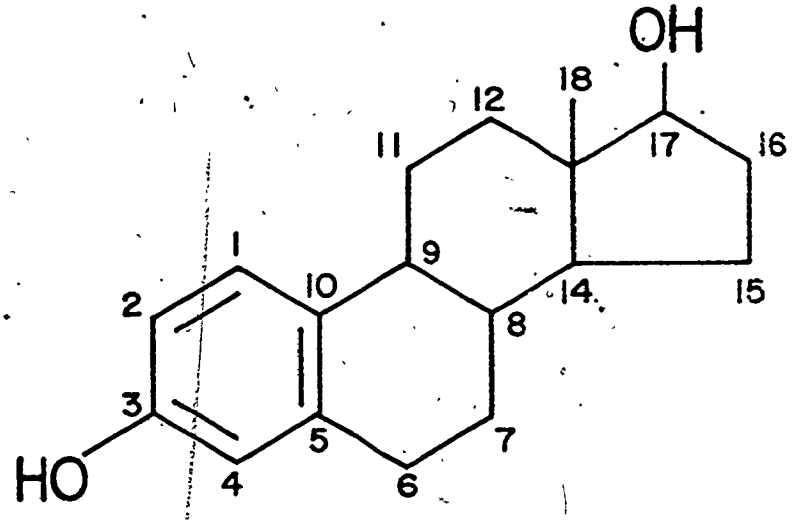
Trivial Names	Systematic Names
Estradiol-17-phosphate	3-Hydroxyestra-1,3,5(10)-trien-17β-yl-phosphate
Estradiol-3,17-diphosphate	Estra-1,3,5(10)-triene-3,17β-di-yl-phosphate
16-ketoestradiol-3-sulfate (16KetoE$_2$3S)	17β-Hydroxyestra-1,3,5-(10)-trien-16-one-3-yl-sulfate
Estriol-3-glucuronide (E$_3$3G)	16α,17β-Dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate
Estriol-16α-glucuronide (E$_3$16αG)	3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl-β-D-glucopyranosiduronate
Estriol-3-sulfate (E$_3$3S)	16α,17β-Dihydroxyestra-1,3,5(10)-trien-3-yl-sulfate
Estriol-16α-sulfate (E$_3$16αS)	3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl-sulfate
Estriol-3,16α-disulfate (E$_3$3,16αdiS)	17β-Hydroxyestra-1,3,5(10)-triene-3,16α-di-yl-sulfate
Estriol-3-sulfate-16α-glucuronide (E$_3$3S16αG)	17β-Hydroxyestra-1,3,5(10)-trien-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate
16-Epiestriol-3-glucuronide (16epiE$_3$3G)	16β,17β-Dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate
16-Epiestriol-3,16β-disulfate (16-epiestriol disulfate)	17β-Hydroxyestra-1,3,5-(10)-triene-3,16β-di-yl-sulfate
Ethinyl Estradiol	19-nor-17α-pregna-1,3,5-(10)-trien-20-yne-3,17-diol

Trivial Names	Systematic Names
Mestranol	19-nor-17α-pregna-1,3,5-(10)-trien-20-yne-3,17-diol-3-methyl ether
Norethynodrel	17α-ethyl-17β-hydroxy-19-norandrost-5(10)-en-3-one
C_{19} Steroids:	
Testosterone	17β-Hydroxy-4-androsten-3-one
Androsterone	3α-Hydroxy-5α-androstan-17-one
Isoandrosterone	3β-Hydroxy-5α-androstan-17-one
Dehydroepiandrosterone (DHA)	3β-Hydroxy-5-androsten-17-one
Dehydroepiandrosterone sulfate (DHAS)	17-keto-5-androsten-3β-yl-sulfate
Androstanediol disulfate (5α-Androstane-3α,17β-diol-3,17-disulfate)	5α-Androstan-3α,17β-di-yl-sulfate
C_{21} Steroids:	
Pregnenolone	3β-Hydroxy-5-pregnen-20-one
Pregnenediol	5-Pregnene-3β,20α-diol
Allopregnane-3β,20β-diol	5α-Pregnane-3β,20β-diol
21-Hydroxypregnenolone-3,21-disulfate	20-Keto-5-pregnene-3β,21-di-yl-sulfate
Medroxyprogesterone acetate	6α-Methyl-4-pregnene-3,20-dione-17α-yl-acetate
Deoxycortiscosterone	21-Hydroxy-4-pregnene-3,20-dione

Trivial Names	Systematic Names
Tetrahydrocortisone	3α-17α-21-Trihydroxy-5β-pregnane-11,20-dione
Deoxycortiscosterone sulfate	3,20-Diketo-4-pregnen-21-yl-sulfate
Other:	
Cholesterol	Cholest-5-en-3β-ol
Cholesterol sulfate	Cholest-5-en-3β-yl-sulfate



ESTRONE 3 SULFATE



ESTRADIOL-17β

Figure 1. Structure of the main estrogens involved in the investigation.

CHAPTER 1

HISTORICAL REVIEW

1.1 INITIAL ISOLATION OF ENDOGENOUS ESTRONE, ESTRADIOL AND ESTRIOL

Before success can be attained in the isolation and purification of biological components, two barriers must be overcome. These are a rapid quantitative assay and an economical source that is relatively rich in the desired compound. These two barriers were surmounted for the estrus producing hormone by the introduction of a bioassay by Allen and Doisy (1923), and the demonstration in 1927 of large amounts of an estrus active principle in the urine of pregnant women (Aschheim and Zondek, 1927). The importance of this latter discovery of a cheap and easily available source of the hormone is revealed by the difficulty encountered in isolating estrogens† from mammalian tissues. Earlier work on the isolation of the active principle from the ovaries was abandoned, due to the low concentration of the estrus producing material from this tissue, in favour of the more readily available material found in the urine of pregnant women and mares.

A historical account of the purification of estrone (E_1) and estriol (E_3) from human pregnancy urine is given

†"Estrogens" throughout this thesis refers to phenolic steroids be they conjugated or unconjugated.

by Marrian (1933, 1966). E_1 was isolated towards the end of 1929 and early in 1930 independently by Doisy, Veler and Thayer (1929, 1930a), Butenandt (1929, 1930) and Dingemanse, De Jongh, Kober and Laqueur (1930). Isolation of E_3 from the urine of pregnant women soon followed (Marrian 1930a, b; Doisy et al, 1930b).

These two compounds isolated at that time were not recognized to be E_1 and E_3 but were classified as active estrus-substances. Scientists of this period were reluctant to accept that the body could secrete chemically different hormonal substances possessing similar physiological activities. Doisy et al (1930a) noted that their previous procedures used with extracts of liquor folliculi could not be used on hormone solutions derived from urine. They stated that the possible reason was due to chemical differences between the physiologically active substances from the two sources or due to a difference in the nature and amount of contaminating substances to be removed. They believed that the latter was the probable cause. Marrian (1930b), in trying to explain the existence of two estrogenic compounds in the urine, suggested that they were not the hormone itself but rather different inactive compounds each associated with minute amounts of the real active principle. It seemed unlikely to him that estrogenic activity could be an intrinsic property of dif-

ferent molecules. The evidence that both E_1 and E_3 coexisted in the urine was provided by Butenandt and Hildebrant (1931). These workers discovered E_3 in one of the discarded fractions of Butenandt's method (1929, 1930) for isolating E_1. They also found that E_1 could be extracted from the mother liquors after the isolation of E_3 by Marrian's method (1930a, b). Conversion of the triol into the hydroxyketone by dehydration with potassium bisulfate (Butenandt and Hildebrant, 1931) confirmed that two distinct estrogenic substances existed.

During this period of the early thirties, other scientists (Wintersteiner et al, 1935) were busy isolating a number of pure estrogens from the urine of pregnant mares. With the isolation of so many different estrogenic compounds, it seemed desirable to determine the nature of the active substance in the ovary. The question arose as to which one of the estrogens isolated in the urine, if any, was the true estrus producing hormone of the ovary. It was now conceivable that a group of substances possessing similar qualitative physiological properties but differing chemically and in quantitative biological activity could be the result of biological reactions occurring in the body after the liberation of the hormone from the ovary. There was no reason to suppose that the ovary secreted estrogens in the form in which they appeared in the urine. This

question could not be answered until the active estrus hormone was isolated from the ovary and compared to those contained in the urine. This task was accomplished by MacCorquodale, Thayer and Doisy (1935, 1936). This feat required four tons of sow's ovaries. Complete recovery from the four tons of ovaries that were processed would have given no more than 25 mg of the hormone. The hormone identified as dihydrotheelin, now known as estradiol (E_2), was more potent than any previous substance isolated in producing cornification in the vagina of ovariectomized rat or mouse. Isolation and identification of E_2 as the active estrus hormone, and still looked upon today as the most biologically active naturally occurring estrogen, concluded the work begun in 1923 by Allen and Doisy, a period of over ten years.

1.2 ESTROGEN CONJUGATES

The main forms of steroids in biological fluids are water soluble conjugates rather than the free compounds. During the initial isolations of estrogens from urine, it was observed that yields obtainable from human urine could be considerably increased by a preliminary treatment with acid (Marrian, 1930c, 1933; Doisy et al, 1930a; Glimn and Wadehn, 1929). Marrian (1933) also noted that yields were increased greatly by allowing bacterial decomposition of the urine to occur. A considerable portion of estrogenic activity in the urine of pregnant women was shown to be in a form in which it was non-extractable by ether (Glimn and Wadehn, 1929). Similarly, while working on the isolation of estrogens from the urine of pregnant mares, Zondek (1930) observed that estrogens in this fluid could not be extracted by chloroform. Thus, it was generally assumed that much of the estrogen in the urine was present as a water-soluble, ether-insoluble ester. It was even suggested at this time that the unextractable estrogen might be excreted in combination with glucuronic or sulfuric acids (Marrian, 1933; Glimn and Wadehn, 1929).

During this same period, Collip (1930a, b) reported the existence of an active fraction in the aqueous extract of human placenta. His group (Collip et al, 1932) also

reported that during pregnancy the urine of women contained an ether-insoluble estrogenic substance similar to that occurring in the placental extracts. The crystals obtained from both sources following acid hydrolysis appeared to be identical to the estrus compound (E_3) isolated by Marrian (1930a, b) and Doisy et al (1930b). The identity of the placental hormone with E_3 was confirmed a few years later by the same group of workers (Collip et al, 1934). Their crystals from the ether-soluble fraction of the placenta were identical in properties to E_3, while the ether-insoluble fraction was a complex of E_3 with some unknown material.

Isolation and identification of estrogen conjugates was difficult. By the early fifties, only four intact steroid sulfates and three glucuronides had been isolated from urine and fully characterized compared to approximately 150 free steroids that had been isolated and identified up to that time (Siiteri, 1970). No steroid conjugate had yet been isolated from other sources, such as the plasma or bile. Of the estrogens, only one glucuronide [see section 1.3] and one sulfate [see section 1.5] had been isolated. Final proof of the nature of estrogen conjugates was delayed until the development of isolation experiments. One of the major problems was the lack of procedures to synthesize estrogen conjugates to serve as internal stand-

ards to correct for methodological losses and to act as authentic reference compounds. The absolute identification of steroid conjugates was difficult due to inadequate experimental procedures available in early times. Methodology was limited to acid or enzymic hydrolysis followed by extraction of the liberated estrogens with organic solvents. The disadvantage associated with acid hydrolysis was that the estrogens were partly destroyed (Cohen and Marrian, 1935; Katzman et al, 1954). Enzymic hydrolysis usually did not destroy estrogens but it resulted generally in relatively incomplete cleavage. Inhibitors that interfere with the hydrolytic action of both β-glucuronidase (Slaunwhite and Sandberg, 1960) and sulfatase (Dodgson and Spencer, 1953) have been shown to be present in the urine. The inhibitory power of urine could vary from subject to subject at different times, resulting in a variable degree of estrogen conjugate hydrolysis and thus could cause erroneous quantitative data. Prior hydrolysis also did not allow of assigning a position of the conjugating molecule on the steroid.

It seemed very difficult to introduce any modification of the acid hydrolysis method in an attempt to prevent the destruction of the estrogens. Hydrolysis with HCl in the presence of zinc (introduced by Smith and Smith, 1937 to increase the yield of estrogens) was shown to yield some-

what higher values than those obtained with HCl alone but not appreciably higher than those obtained by enzymatic hydrolysis (Straw et al, 1955). This fact plus the advantage of specific cleavages offered by enzymes made investigations for improving the yield of enzymic hydrolysis more promising. The main problem was to find a simple procedure for the removal of the enzyme inhibitors present in the urine. Comparison of enzyme hydrolysis using whole urines and their butanol extracts indicated that somewhat higher estrogen yields were obtained with the butanol extracts (Straw et al, 1955).

The introduction of Sephadex columns (Beling, 1961, 1963; Hähnel, 1965) made it possible to obtain conjugated estrogens directly from the urine in a high degree of purity. Beling (1961, 1963), employing columns of Sephadex G-25, was able to separate estrogen conjugates of pregnancy urine into two main peaks. Gel filtration of 13 conjugated estrogens added to male urine (Beling, 1963) showed that estrone-3-glucuronide ($E_1$3G), estradiol-3,17-diglucuronide ($E_2$3,17diG) and estriol-3-glucuronide ($E_3$3G) were eluted in the first peak. The other estrogen conjugates, such as estradiol-17-glucuronide ($E_2$17G), estriol-16(17)-glucuronide ($E_3$16(17)G), estrone-3-sulfate ($E_1$3S), estradiol-3-sulfate ($E_2$3S), estradiol-17-sulfate ($E_2$17S), estradiol-3,17-disulfate ($E_2$3,17diS), estriol-3-sulfate ($E_3$3S), estradiol-3-phosphate, estradiol-17-phosphate and estradiol-

3,17-diphosphate were found in peak two.

The use of DEAE Sephadex as a means of separating estrogen conjugates was introduced by Hähnel et al (1965, 1966, 1967). Preliminary results (1965, 1966) separated free estrogens from conjugated estrogens, a feat that was not accomplished by Beling's column, as well as some estrogen conjugates from each other. This group of workers was also able to separate estrogen sulfates completely from estrogen glucuronides by a suitable adjustment of the NaCl gradient (Hähnel et al, 1967). In Beling's method (1963), the estrogen sulfates occupied the same position as the other conjugates with the exception of estrogen-3-glucuronides. Thus, urine could be applied to DEAE Sephadex columns to separate four groups of estrogens from each other at the same time; namely, free estrogens, estrogen-3-glucuronides, estrogen-16(or 17)-glucuronides and estrogen sulfates. Hobkirk et al (1969a) greatly improved the use of DEAE Sephadex for the separation of estrogen conjugates. A number of glucuronides and sulfates of labeled E_1 and E_2 were prepared in the laboratory and, together with certain commercially available compounds, their behavior on DEAE Sephadex columns was investigated. Column chromatography on DEAE Sephadex in a linear gradient of 0-0.8M NaCl followed by 0.8-2.0M NaCl resulted in a clear separation of E_2, $E_1$3G, $E_2$17G, $E_1$3S, $E_2$3S,

estradiol-3-sulfate-17-glucuronide ($E_2$3S17G) and $E_2$3,17diS. The presence of urinary residues caused some changes in the elution pattern but still allowed reasonable separation. Thus it was shown that not only is there the possibility of separating like steroids conjugated with the same acid at different carbon atoms of the steroid molecule but also very clear resolution can be obtained of steroids differing only slightly in structure and conjugated with the same acid at the same carbon atom. This latter method, as described by Hobkirk, Musey and Nilsen (1969), was employed extensively in the present investigation [see methods section 4.3(a)].

The development of Sephadex chromatography aided greatly the separation and identification of estrogen conjugates present in biological fluids. Today it is well known that conjugated steroids are predominantly either sulfates or glucuronides, although other molecules, such as amino acids, glucose and N-acetyl-glucosamine, may be employed for conjugate formation.

1.3 ESTROGEN GLUCURONIDES

Cohen and Marrian (1935), optimizing the conditions for the acid hydrolysis of the combined estrogens in urine, attempted to isolate the combined form of E_3. This work was stimulated by the preceding observations of Collip's group (1932, 1934) that an ether-insoluble complex of E_3 was contained in the human placenta as well as in pregnancy urine. The identity of the E_3 conjugate as a glucuronide was established in 1936 by Cohen and coworkers (Cohen and Marrian, 1936; Cohen, Marrian and Odell, 1936). Chemical and spectrographic evidence showed that the glucuronic acid moiety was combined with E_3 at C-16 or C-17 and not at the phenolic C-3. This was the first isolation of an estrogen conjugate. Conclusive evidence that the uronic acid of the conjugate was glucuronic acid was given by Grant and Marrian (1950). It was not until many years later that Hashimoto and Neeman (1963) established the absolute structure of this conjugate to be estriol-16α-glucuronide ($E_3$16αG). With the availability of ^{14}C-$E_3$16αG for internal standard purposes, Slaunwhite et al (1964) were able to estimate accurately the quantity of this conjugate present in the urine of pregnant subjects. The E_3 glucuronide was purified by the method of Hashimoto and Neeman (1963) and the final butanol extract assayed for radioactivity and Kober chromogens without employing hydrolysis. They

reported that daily values rose from 7 to 10 mg in the third month to 37 to 54 mg in the eighth month of pregnancy.

$E_3$16αG may be found conjugated to sulfuric acid. Straw et al (1955) presented evidence that indicated a small amount of E_3 was excreted as a double conjugate with sulfuric and glucuronic acids in human pregnancy urine. They observed that preliminary treatment of the urine with β-glucuronidase increased the estrogen liberated by mylase P, a phenolsulfatase preparation. Smith and Kellie (1967), employing gel filtration on Sephadex G-25 followed by further purification procedures, identified estriol-3-sulfate-16α-glucuronide ($E_3$3S16αG) in human pregnancy urine. These workers also identified 16α-hydroxyestrone-3-sulfate-16α-glucuronide (16αOH$E_1$3S16αG).

As stated earlier, the isolation and identification of estrogen conjugates was difficult. By the early fifties, $E_3$16αG from the human urine was the only estrogen glucuronide to be isolated and characterized, although other estrogen glucuronides were known to be present. Oneson and Cohen, for example, in 1952, presented data indicating that a large proportion of estrone in human pregnancy urine was conjugated as the glucuronide. Following the introduction of Sephadex columns [section 1.2], isolation and identification of estrogen conjugates be-

came more rapid. Beling, employing Sephadex G-25 columns (1963), detected the presence of $E_1$3G and $E_3$3G, as well as $E_3$16αG in pregnancy urine. Smith and Kellie (1965, 1967) also employed Sephadex G-25 columns to isolate estrogen glucuronides from pregnancy urine. Further purification by ion exchange chromatography on ECTEOLA-cellulose powder and subsequent partition chromatography on Celite and paper led to the identification of the following glucuronides: $E_1$3G, $E_2$3G, $E_3$3G, $E_3$16αG, 16-epiestriol-3-glucuronide (16epi$E_3$3G) and 16α-hydroxyestrone-16-glucuronide (16αOH$E_1$16αG) (1967). DEAE Sephadex chromatography, as employed by Hähnel et al (1965, 1966), separated estrogen glucuronides from the unconjugated estrogens in pregnancy urine. $E_3$16αG represented the main peak of the DEAE Sephadex chromatogram. Preliminary evidence revealed also the presence of $E_3$3G, $E_1$3G and two E_2 glucuronides. In a follow-up study, Hähnel (1967) presented quantitative data on twenty patients who were 32-40 weeks pregnant. In all patients, $E_3$16αG was the estrogen conjugate present in the largest portion averaging about 60% of all the estrogen glucuronides present. $E_3$3G showed an average of about 27%. These two E_3 glucuronides together formed about 87% of all the estrogen glucuronides found in the urine. $E_1$3G constituted about 10% of the total glucuronides, while the two estradiol glu-

curonides, probably $E_2$3G and $E_2$17G, contributed only about 2% each.

The second major glucuronide metabolite in the urine, $E_3$3G, was obtained in crystalline form by Ladany in 1968. The infra-red spectrum of the isolated conjugate was identical with that of a synthetic sample of $E_3$3G. Enzymatic cleavage of the isolated compound yielded E_3 and glucuronic acid.

1.4 BIOSYNTHESIS OF ESTROGEN GLUCURONIDES

The conjugation of various compounds with glucuronic acid takes place in the liver, kidney and gastrointestinal tract both in animals and in man (Dutton and Greig, 1957; Dutton and Stevenson, 1959; Dutton, 1959; Lehtinen et al, 1958a, b; Schachter et al, 1959; Dicfalusy et al, 1962). The reaction mechanism in the formation of glucuronides can be represented as follows:

Glucose-1-phosphate + UTP → UDP-glucose + PPi (1)

UDP-glucose + 2NAD → UDP-glucuronic acid (2)

UDP-glucuronic acid + ROH → UDP + R-O-glucuronate (3)

The formation of uridine diphosphate glucose, (UDPG) by reaction (1) is followed by the conversion of this by oxidation into uridine diphosphate glucuronate (UDPGA). The glucuronyl residue of UDPGA, an activated form of glucuronic acid, is then transferred to an appropriate acceptor (Roy, 1970).

The enzymes catalyzing these three reactions are respectively: UDPG-pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9); UDPG-dehydrogenase (UDP glucose: NAD oxidoreductase, EC 1.1.1.22) and UDP-glucuronyltransferase (UDP glucuronate glucuronyltransferase). A recent commentary on the control of this

latter enzyme, UDP-glucuronyltransferase, activity is given by Dutton (1975), one of the pioneers in the elucidation of the glucuronidation mechanism. Excellent reviews of the mechanism of glucuronide biosynthesis are also given by Dutton (1961, 1966).

(a) ELUCIDATION OF GLUCURONIDATION MECHANISM

Studies with ^{14}C labeled glucose in the rabbit (Eisenberg and Gurin, 1952) and guinea pig (Douglas and King, 1953a) indicated that the carbon chain of glucose served as the precursor of the glucuronyl moiety of urinary steroid glucuronides. The glucose molecule seemed to be converted directly to glucuronide without requiring separate 3-carbon compounds as an intermediate. It was subsequently shown in the guinea pig and rat that exogenous glucuronic acid was not utilized in appreciable quantities for direct conjugation but was metabolized largely to respiratory CO_2 (Douglas and King, 1953b). The precursor for glucuronide biosynthesis remained a mystery until the publications of Dutton and Storey (Dutton and Storey, 1951, 1953, 1954; Storey and Dutton, 1955). These workers adapted the method of Levvy and Storey (1949) for the determination of glucuronidation of O-aminophenol in mouse liver slices to liver homogenates (Dutton and Storey, 1951) in the hope of gaining insight into the mechanism of synthesis. No syn-

thesis of aminophenylglucuronide could be detected unless a boiled extract of liver was also added to the system. In subsequent work (Dutton and Storey, 1953, 1954; Storey and Dutton, 1955), purification of this factor showed it to contain uridine, stable phosphate, labile phosphate and glucuronic acid in the proportions 1:2:1:1. Evidence thus suggested that the factor had the structure of uridine diphosphate glucuronate. This active factor was not confined to one species but could be obtained from rabbit, rat, mice and guinea pig livers (Dutton and Storey, 1954). When incubated with liver homogenates and O-aminophenol, approximately one equivalent of corresponding glucuronide formed for each equivalent of uridylic acid. Thus uridine diphosphate glucuronic acid (UDPGA) was an active form of glucuronic acid and transferred the glucuronyl moiety to the aglycone under the influence of an enzyme. This enzymic activity was shown to be located in a particulate material (now known as the microsomes) of the cell cytoplasm (Dutton and Storey, 1954). The transfer of the glucuronyl residue from UDPGA to the acceptor ROH as shown in reaction (3) [section 1.4] seemed to represent the final stage in the formation of glucuronides observed in liver slices (Storey and Dutton, 1955).

The preceding reactions (1) and (2) [section 1.4] were

soon clarified to complete the picture of the mechanism of glucuronide biosynthesis. Strominger et al (1957) showed that the soluble fraction of guinea pig liver homogenates contained an enzyme, referred to as "uridine diphosphoglucose dehydrogenase", which catalyzed the oxidation of UDP-glucose to UDP-glucuronate in the presence of NAD. The conversion of α-glucose-1-phosphate in the presence of UTP to UDP-glucose by pyrophosphorylase was shown previously to occur by Munch-Petersen et al (1953).

The glucuronide conjugating reaction mechanism involving UDPGA and microsomal enzymes was shown to be a general mechanism. Studies showed (Isselbacher and Axelrod, 1955; Dutton, 1956) that a wide range of aglycones, both foreign and natural substances, could act as acceptors of glucuronate from UDPGA. These early studies included steroids. Corticosteroids, such as tetrahydrocortisone (Isselbacher and Axelrod, 1955) were shown to be glucuronidated in the presence of UDPGA and guinea pig liver microsomes. Dutton (1956) extended his studies to the steroids, androsterone and allopregnane-3β,20β-diol and showed that these substances also acted as substrates.

1.5 ESTROGEN SULFATES

In 1935, Cohen, Marrian and Watson reported that during the greater part of pregnancy over 99% of the total estrogenic material excreted in the urine was in a "combined" ether insoluble form which possessed only a low physiological potency. They noted that during the last few days of pregnancy in women, immediately before and during labor, there occurred a rapid fall in total amount of estrogen excreted simultaneously with a very great increase in the ratio of ether-soluble estrogen to conjugated ether-insoluble estrogen. Marrian, attempting to obtain further evidence to support the proposed theory that conversion of the less physiologically potent conjugated hormones to the free hormones might be a factor in the initiation of labor, extended his research to study and determine the amount of free and conjugated estrogen in the urine of several mares at different stages of pregnancy (Schachter and Marrian, 1936). Parturition in the horse was not accompanied by marked changes in the ratio of free to conjugated estrogens as occurred in the human, but this study led to preliminary evidence that estrogen was conjugated to sulfuric acid. This led to the first isolation of an estrogen sulfate a few years later in 1938 by Schachter and Marrian. They isolated and identified the potassium salt of $E_1$3S from pregnant mares' urine.

H. Cohen and Bates reported the first utilization of a phenolsulfatase preparation, mylase P, for the hydrolysis of estrogen sulfates from the urine of pregnant mares (1949a). This enzyme preparation is specific for the hydrolysis of phenolic sulfates and does not hydrolyze alcoholic sulfates, indicating that the sulfate ester linkage in estrogen sulfates must be with the phenolic hydroxyl group. Employing phenolsulfatase, these workers determined the proportion of estrogenic conjugates that occurred in pregnant women's urine as the sulfate ester (Cohen and Bates, 1949b). Among seven different urine samples, they found a variable proportion of from 5-89% of the E_3 fraction and 8-100% of the E_1-E_2 fraction to be conjugated as the sulfate. In contrast, Oneson and S. L. Cohen (1952) found that a large portion of E_1 in human pregnancy urine was conjugated as the glucuronide. Re-evaluation of the previous reported data by Bates and H. Cohen (1949b) indicated the possibility of erroneous conclusions. High values given for the presence of estrogen sulfates in the urine of pregnant women could have been due to contamination of mylase P preparations with glucuronidase. Treatment of the urine by boiling, reduced hydrolysis by mylase P indicating the possible presence of a glucuronidase in the urine. Katzman et al (1954) also reported that the major portion of estrogens is excreted in conjugation with

glucuronic acid and only small amounts of estrogens are excreted as sulfates.

McKenna et al (1961) obtained direct evidence for the presence of estrogen sulfate in the urine of pregnant women. The infra-red spectrum of the isolated material was identical with that of authentic $E_1$3S. Only 8% of the E_1 in the pooled pregnancy urine was conjugated to sulfuric acid. Smith (1966) reports that, of the total urinary estrogens (E_1+E_2+E_3) from human pregnancy urine, 75-97% was E_3 with 85-97% of this conjugated to glucuronic acid. E_1 represented 2-21% of the total estrogens with only 1.5-7.5% accountable to the sulfate fraction. E_2 was only 1-4% of the total. Ninety to ninety-eight percent of E_1 and E_2 were excreted as glucuronides. Hähnel et al (1967), employing DEAE-Sephadex columns to separate estrogen sulfates from the glucuronides in pregnancy urine, showed that the sulfate fraction contained $E_1$3S, $E_3$3S, $E_2$3S, 16-keto-estradiol-3-sulfate (16ketoE$_2$3S) and estriol-16α-sulfate ($E_3$16αS). No quantitative data, however, were given. Quantitative data have, however, been published by Tan et al (1971).

1.6 BIOSYNTHESIS OF ESTROGEN SULFATES

The overall pathway for the formation of steroid sulfates involves two essentially discrete stages. The first stage involves sulfate activation. The formation of 3' phosphoadenylyl sulfate, also known as adenosine 3' phosphate 5' phosphosulfate (PAPS), the active form of sulfate, occurs by reactions common to several pathways of sulfate metabolism. The second stage in sulfate conjugation is the transfer of the sulfate group from the activated sulfate to the appropriate acceptor through the action of steroid specific enzymes (Roy, 1970). The mechanism of the biosynthesis of steroid sulfates is as follows:

Sulfate Activating System:

$$ATP + SO_4^{-2} \rightarrow \underset{(APS)}{\text{adenosine 5' phosphosulfate}} + PPi \qquad (1)$$

$$APS + ATP \rightarrow PAPS + ADP \qquad (2)$$

Sulfate Transfer:

$$PAPS + \text{steroid } (R \cdot OH) \rightarrow \underset{(PAP)}{\text{adenosine 3'5' diphosphate}} + \text{steroid sulfate } (ROSO_3^-) \qquad (3)$$

The enzymes catalyzing these three reactions are respectively: ATP-sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4); Adenylyl sulfate kinase or APS-phosphokinase (ATP: adenylylsulfate 3' phosphotransferase, EC

2.7.1.25) and steroid sulfotransferases (3' phosphoadenylylsulfate: 3β-hydroxysteroid sulfotransferase, EC 2.8.2.2 and 3' phosphoadenylyl sulfate: estrone sulfotransferase, EC 2.8.2.4). In the adult human, steroid sulfotransferases occur in significant amounts only in the liver, the adrenal gland and the jejunal mucosa (Bostrom and Wengle, 1967).

(a) ELUCIDATION OF SULFATE CONJUGATION MECHANISM

Early theories that inorganic sulfate was the precursor in sulfate conjugation (Hele, 1931; Bernheim and Bernheim, 1943) were supported by "in vivo" studies with radioactive sulfate. Rats, given sodium sulfate labeled with radioactive sulfur, were shown to excrete labeled sulfate conjugates in the urine (Laidlaw and Young, 1948; Dziesiatkowski, 1949). De Meio and Arnolt (1944) showed, in the early forties, that phenol could be sulfated in rat liver, intestine and spleen tissue preparations. Other tissues tested were inactive in sulfate conjugate formation. De Meio and Tkacz (1950, 1952), employing rat liver homogenates fortified with α-ketoglutaric acid and AMP, increased phenyl sulfate formation. The sediment and supernatant obtained by centrifuging the homogenates were individually inactive. Activity could be restored, however, when these fractions were recombined, but not if the supernatant was previously boiled. These studies, plus

inhibition of phenol conjugation by 2,4, dinitrophenol, indicated that the process might involve phosphorylation (De Meio and Tkacz, 1952). Further studies by De Meio et. al (1953) showed that "microsome-free" supernatants from rat liver homogenates could also form phenyl sulfate when fortified with ATP and Mg^{+2}. The early observation that mitochondria were required for phenyl sulfate synthesis when the supernatant contained AMP and an oxidizable substrate such as α-ketoglutarate (De Meio and Tkacz, 1952) could now be explained. Mitochondria were needed to generate ATP by aerobic phosphorylation. The requirement for ATP for sulfurylation confirmed that phosphorylation was involved in the formation of phenyl sulfate. Bernstein and McGilvery (1952) also showed that enzymatic activation of inorganic sulfate by ATP was required as a preliminary step for sulfate conjugation. Before Bernstein and McGilvery's work, it was not clear whether ATP served to create an active form of the phenol which then could be a common precursor in either sulfate or glucuronide formation or whether the inorganic sulfate was the primary reactant implying a separate mechanism for the two conjugates. These workers, however, presented data indicating that sulfate conjugation required activation of the sulfate with ATP followed by a second phase of the transfer of sulfate to phenol (Bernstein and McGilvery,

1952). This was demonstrated by an accumulation of an active intermediate during preincubation of the supernatant containing sulfate, ATP and Mg^{+2} before the addition of m-aminophenol. Addition of phenol to the preincubated mixture resulted in an initial rapid sulfate conjugation, an effect not seen if phenol was preincubated in the presence of ATP and Mg^{+2} before the addition of sulfate. Segal (1955) presented kinetic data supporting these observations. Rate curves of the phenyl sulfate conjugating system of the rat liver were shown to conform to the kinetics of a two step reaction in which the activated form of sulfate was an intermediate. Further evidence supporting the two step mechanism of sulfate conjugation was presented by De Meio et al (1955). These workers were able to separate an activating enzyme responsible for the formation of an active sulfate intermediate from a transferring enzyme involved in the transfer of the sulfate from the intermediate to the substrate.

The identity of the active sulfate intermediate as adenosine-3'-phosphate-5'-phosphosulfate (PAPS) was elucidated by Robbins and Lipmann (1956, 1957). A historical account of the isolation and identification of this active sulfate is given by Lipmann (1958). The demonstration of two separately linked phosphates in the active sulfate indicated a two-phase reaction in the formation of this

intermediate. Supporting data were produced by Wilson and Bandurski (1956) who showed that at least two separate heat labile protein fractions were required for sulfate activation. In their model of the yeast, each of the purified fractions was inactive by itself or in the presence of boiled aliquots of the other. Active sulfate, however, was formed after the two fractions were recombined. These results were confirmed in Lipmann's lab (1958). The initial reaction, as depicted by reaction (1) [section 1.6], involves the attack of one of the oxygens of sulfate on the proximal phosphorus in ATP with the displacement of pyrophosphate by sulfate. The adenosine-5'-phosphosulfate (APS) thus formed reacts with another molecule of ATP for the synthesis of PAPS [reaction (2); section 1.6]. These two reactions make up the sulfate activating system as depicted from animal models. Very little, however, is known of the distribution or properties of the component enzymes in mammalian tissues.

Steroid sulfate formation was reported also to require Mg^{+2} ions and ATP. Thus an activating system [reactions (1) and (2)], as proven for the phenols, seemed to be required for steroid sulfurylation. The transfer of sulfate from PAPS to steroids was shown by a number of investigators. Segal, in 1955, showed that his system of rat liver preparations gave rise to $E_1$3S when ^{14}C-estrone

was employed as the substrate instead of phenol. Roy (1956) and De Meio and Lewycka (1955), also using their rat liver preparations, presented evidence for the synthesis of dehydroepiandrosterone sulfate (DHAS) from dehydroepiandrosterone (DHA). Schneider and Lewbart (1956) showed that testosterone, as well as deoxycorticosterone, could be sulfated indicating that esterification with sulfate was not limited to the hydroxy group at carbon 3. De Meio et al (1958) expanded their studies to other animals showing that DHA, testosterone, E_1 and E_2-17β sulfates were formed with microsome-free fractions from rat, rabbit and ox liver preparations.

Investigations by De Meio et al (1958) showed that the sulfate conjugation of DHA was partially inhibited by the presence in the medium of phenols. These observations suggested, but did not prove, that the phenol and steroid compete for the same enzyme system. These workers however, being familiar with the work of Gregory and Nose (1957), concluded that the two substrates competed for "active sulfate" (PAPS) and that the transferring enzymes were different. Gregory and Nose (1957) reported earlier their separation of two sulfotransferases (referred to as sulfokinases) from soluble rabbit liver extracts. One enzyme produced sulfates of steroids containing a β hydroxyl group at carbon 3 while the other enzyme transferred sulfate to many phenolic compounds. Estrogens of phenolic character

were also sulfated by the latter enzyme. Nose and Lipmann (1958) succeeded in separating two different steroid sulfotransferases, one specific for E_1 and the other reacting with DHA. The latter sulfotransferase fraction also reacted with a few other 3β-hydroxysteroids, such as pregnenolone and isoandrosterone. These two enzymes were distinct from a third protein fraction which reacted with a number of phenols. The work of Banerjee and Roy (1966) in guinea pig liver confirmed the separate identity of a DHA and an estrone sulfotransferase. It was also suggested that deoxycorticosterone sulfate was formed by another steroid sulfotransferase. Estrogen sulfotransferase (3'-phosphoadenylylsulfate: estrone sulfotransferase, EC 2.8.2.4) isolated from bovine adrenal glands was the first steroid sulfotransferase to be isolated in comparatively pure form (Adams and Poulos, 1967). The enzyme existed in two forms. Further investigation (Adams and Chulavatnatol, 1967) showed a variance in behavior of E_1 as compared to E_2 and E_3 obtained with the β or trimer form of the enzyme. An increased rate of sulfation of E_1 occurred at high substrate concentration as compared to a lowered rate with E_2 and E_3. The rate of sulfate synthesis at low substrate concentration was similar for all three estrogens. This suggested a specific role for E_1 as the possible true substrate for the enzyme. It was suggested that conversion to the other estrogens in vivo might occur after

the formation of $E_1$3S. The observation that E_1 was bound to the estrogen sulfotransferase following purification of the enzyme (Adams, 1967) also suggested that E_1 is the true substrate of the enzyme.

(b) ESTROGEN DISULFATES

In contrast to the view earlier expressed by Schneider and Lewbart (1956) that enzymic formation of steroid disulfates is unlikely when polyhydroxy steroids are incubated in vitro, Wengle and Bostrom (1963) showed that dihydroxy steroids, especially 3,17 and 3,20 dihydroxy steroids, could form disulfates when incubated with crude sulfotransferase preparations from rat liver. Two of the disulfates shown to be formed were those of estradiol-17β and estradiol-17α ($E_2$17α). The hen has been shown to produce both $E_2$3,17βdiS and estradiol-3,17α-disulfate ($E_2$3,17αdiS) after injection of ^{14}C-estradiol-17β (Mathur et al, 1969). More recently, estriol-3,16α-disulfate ($E_3$3,16αdiS) formation from exogenous E_3 has been shown to occur in guinea pig liver homogenates (Levitz et al, 1974), as well as in the intact animal in vivo (Kirdani and Sandberg, 1976). Recently Hobkirk et al (1975, 1977) have shown the formation of $E_3$3,16diS, as well as the disulfate of 16α-hydroxyestrone (16αOHE_1), when guinea pig liver slices were incubated with E_1, E_2 and $E_1$3S. Some evidence

was also obtained for the disulfate fractions in liver and bile after the injection of labeled E_1 into the whole animal (Hobkirk et al, 1977).

Disulfates of estrogens have not been detected in the human. However, other steroid disulfates, such as 21-hydroxypregnenolone-3,21-disulfate (Pasqualini and Jayle, 1962) and the disulfate of pregnenediol (Arcos and Lieberman, 1967) have been isolated from human urine.

1.7 THE SIGNIFICANCE OF STEROID SULFATES

The isolation of E_3 16αG from pregnant women's urine by Cohen, Marrian and Odell (Cohen and Marrian, 1936; Cohen, Marrian and Odell, 1936) and of E_1 3S by Schachter and Marrian (1938) from pregnant mares' urine demonstrated that estrogens and their metabolites may be excreted as water soluble conjugates of glucuronic or sulfuric acid. Early investigations demonstrated that conjugated forms of steroids possessed little biological activity (Cohen, Marrian and Odell, 1936; Odell et al, 1937; Venning et al, 1942). Together with observations that not only the urinary (Cohen et al, 1935; Oneson and Cohen, 1952; Katzman et al, 1954) but also the biliary (Sandberg and Slaunwhite, 1957; Adlercreutz et al, 1960) excretion of estrogens occurred as conjugates, the corresponding unconjugated steroid being present only in insignificant amounts, led to the general view that steroid conjugates were metabolic end products destined only for excretion. This theory was strongly supported by the role of conjugation as a detoxication mechanism for drugs. This seems to be the case for the glucuronides but a different picture is emerging for the steroid sulfates. The significance of steroid sulfates, as will be described below, with the emphasis on estrogen sulfates, supports the theory that sulfation of steroids can no longer be solely considered

as a detoxication mechanism.

(a) STEROID SULFATES OCCUR IN HIGHER CONCENTRATION IN COMPARISON WITH FREE STEROIDS IN SOME SPECIES

Early attempts to quantitate estrogens in plasma (Preedy and Aitken, 1957; Aitken et al., 1958; Slaunwhite and Sandberg, 1959; Oertel et al, 1959) and whole blood (Roy and Brown, 1960) employed hydrolytic procedures which precluded the characterization of conjugated forms. The possibility that estrogens could occur conjugated to glucuronic or sulfuric acid in plasma or blood, however, was suggested (Preedy and Aitken, 1957; Oertel et al, 1959; Roy and Brown, 1960). Steroids were shown also to be associated with plasma proteins (Antoniades et al, 1957).

Purdy et al (1959, 1961), using reverse isotope dilution technique and countercurrent distribution, found that $E_1$3S is the major estrogen circulating in the human organism and that $E_1$3G is present in much smaller amounts. Plasma obtained 2.5 hours after the administration of ^{14}C-E_2 to a woman was found to contain ^{14}C-$E_1$3S as the principal radioactive metabolite. The amounts of $E_1$3S and some other estrogens (E_1, E_2, E_3 and $E_1$3G) were then measured in a pool of third trimester pregnancy plasma. Results showed that the major estrogen occurred as $E_1$3S. In the pregnancy plasma $E_1$3S was present approximately four

times that of the concentration of unconjugated E_1 (Purdy et al, 1961).

Smith and Akai (1963), also studying the circulating forms of estrogens, reported that circulating E_1 and E_3 were almost entirely either protein bound or conjugated, whereas the major portion of E_2 in the plasma was free or unconjugated. The major portions of E_1 and E_3 (85-95%) were recovered only after hydrolysis. Touchstone et al (1963) reported that only 17% of the total E_3 found in the maternal plasma was in the free state. The conjugated fraction averaged 83% of the total. Studies employing enzymatic hydrolysis with β-glucuronidase followed by hydrolysis with phenolsulfatase and vice versa indicated that a large proportion (44%) of the conjugated fraction of E_3 in the plasma of pregnant women was present as a sulfoglucosiduronate diconjugate. A sulfate of E_3 and a glucuronide were also present and represented respectively 25% and 31% of the conjugated fraction of E_3. These results as well as those of Purdy et al (1959, 1961) were confirmed by Smith and Hagerman (1965) who developed a specific method for measuring the major forms of circulating estrogens in late pregnancy plasma. They reported results on four pools of plasma from 8 pregnant women. Nine to 16.9% of the E_1 was free and

4.4 to 12.8% was released by glucuronidase. E_1 was at least three-quarters in the sulfate form. The small amount of E_2 in the blood was two-thirds unconjugated. E_3, the major estrogen in the blood during pregnancy, was reported to be mainly conjugated to glucuronic acid (35-46% as E_3 glucuronides). E_3 circulating in the unconjugated form was only 3.3 to 15.6% of the total. Two other E_3 conjugates were also reported: $E_3$3S (15-22% of the total) and the double conjugate $E_3$3S16αG (25-43% of the total).

More recently results by Hawkins and Oakey (1974) indicated that $E_1$3S was the main circulating form of estrogen in men as well as in women. The concentration of $E_1$3S in the peripheral plasma of six men averaged 72 ng/100 ml compared to an E_1 concentration of 2.3 ng/100 ml and an E_2 concentration of 3.6 ng/100 ml. In two women at mid cycle, $E_1$3S reached a level 5-6 times the concentration of E_2 and about 20 times that of E_1. Their reported mean values of $E_1$3S, E_2 and E_1 were respectively: 308 ng/100 ml, 57 ng/100 ml and 15 ng/100 ml. $E_1$3S has also been reported to exceed the concentration of unconjugated E_2 and E_1 during the course of human pregnancy (Loriaux et al, 1972).

Thus, the presence of $E_1$3S as a major circulating estrogen in at least the human has been established. This

focused new light on the estrogen conjugates. The fact that $E_1$3S is the predominant estrogen metabolite in the blood suggested that conjugated forms of estrogens, such as various estrogen sulfates, may play an important physiological role.

(b) SULFATES ARE CLEARED FROM THE PLASMA MORE SLOWLY THAN GLUCURONIDES AND EVEN SUGGESTED TO BE REABSORBED BY RENAL TUBULES

At about the same time that estrogens were being identified in the plasma, a group of workers was attempting to identify the estrogens contained in the bile (Sandberg and Slaunwhite, 1957; Adlercreutz et al, 1960). The importance of biliary excretion of estrogens was demonstrated by Sandberg and Slaunwhite in 1957, who showed that, in patients with biliary drainage, 50% of the radioactivity of injected ^{14}C-E_1 or ^{14}C-E_2 was excreted in the bile. The appearance of 72% of the radioactivity in the urine suggested that reabsorption of estrogens occurred in the intestine. The isolation and identification of E_3 and E_1 in human pregnancy bile was reported for the first time by Adlercreutz et al (1960). Most of the E_3 occurred in conjugated form. Some evidence for the presence of E_2 and 16-epiestriol (16epiE_3) was also given. In a follow-up study, Adlercreutz (1962) further established the identity of estrogens present in the bile of pregnant and

nonpregnant subjects. No free unconjugated estrogen was detected in the bile in any of the subjects. Results from the investigation showed that the liver concentrated estrogens from the blood into the bile mainly in the form of E_3 metabolites occurring in the glucuronide fraction. Presumptive evidence was also obtained for the presence of $E_3 16\alpha G$ in the bile of a woman treated with E_3. Quantitatively the most important additional fractions found were E_1 and E_3 as sulfates followed by E_2 as sulfates. It was concluded that the inactivating capacity of the liver with regard to estrogens was at least partially due to the conversion of E_1 and E_2 to E_3 followed by conjugation.

Comparison of estrogens contained in different biological pools indicated the importance of 16α-hydroxylation followed by conjugation with glucuronic acid as essential steps prior to excretion. Brown et al (1960), measuring urinary and plasma estrogen levels in seven pregnant women, noted marked differences between the urinary/plasma (U/P) ratios of E_1 and $E_2 17\beta$ on the one hand, and of E_3 on the other. Mean values obtained for the U/P ratio of E_1, E_2 and E_3 were 3.3, 2.5 and 64.2 respectively. This suggested a difference in the renal handling of these steroids. The low clearance rates reported for E_1 and E_2 (mean values

of 15.6 ml/min and 12.0 ml/min respectively) in contrast to the mean E_3 clearance (296 ml/min) suggested that E_1 and E_2 may exist in a different physiochemical state in the plasma or that glomerular filtration may be followed by tubular reabsorption. The clearance rate of E_3 being twice the reported figures for the glomerular filtration rate in late pregnancy suggested that some component of total E_3 of plasma (eg. E_3 glucuronides) was secreted by the renal tubule. Results from other studies showed that sulfated estrogens were being retained and possibly even reabsorbed by the kidney tubules (Smith, O.W., 1966; Adlercreutz, 1964). Adlercreutz (1964) found that the glucuronides of all three classical estrogens were more concentrated in the bile than in the plasma during late pregnancy in the human, while the concentration of the sulfates was approximately the same in both. E_1 and E_2 glucuronides were only 3-4 times as concentrated in the bile as in the plasma, while E_3 glucuronides were 25-30 times higher in the bile than in the circulation. If the total concentration of the three estrogens were considered, 39% consisted of sulfates in the blood with only 3% in the bile. These findings signified that the sulfates were being retained with some leakage into the bile, whereas glucuronides were being eliminated. Smith, O.W. (1966) compared the free and conjugated estrogens found

in the blood and urine before and during parturition in normal human pregnancy. Her findings also showed a pronounced difference between the mechanism of excretion of estrogen glucuronides, sulfates and free steroids. Urine to plasma ratios indicated that glucuronides of the three classical estrogens were secreted by the kidney tubule, while sulfurylated and free forms were reabsorbed. Similar observations have been made with regard to the androgens (Keilie and Smith, E.R., 1957); that is, glucuronides were cleared from the plasma far more rapidly than the sulfates suggesting the reabsorption or retention of the sulfates. Studies of administering radioactive estrogens to humans supported the observation that glucuronides are the excretory form of estrogens (Twombly and Levitz, 1960; Levitz, 1965; Emerman et al, 1967; Kardani et al, 1968; Jirku and Levitz, 1969). Recently, a report has appeared challenging the suggestion of reabsorption of estrogen sulfates (Swapp et al, 1975). The renal clearance of unconjugated E_3, E_3 glucuronides ($E_3$3G, $E_3$16G and $E_3$3S16$_\alpha$G) and $E_3$3S by the kidney in late human pregnancy was compared with simultaneous measurement of inulin clearance. Results indicated for fractions not bound to protein that, after glomerular filtration, unconjugated E_3 was completely reabsorbed, the E_3 glucuronides were both filtered and excreted actively by the tubules and that $E_3$3S was filtered but not excreted or absorbed by the tubules.

(c). STEROID SULFATES HAVE A LONGER PLASMA HALF LIFE THAN HAVE THE CORRESPONDING FREE STEROID

The significance of steroid sulfates is also reflected in their relatively longer half life in the plasma as compared to the corresponding unconjugated steroids (Wang et al, 1967a, Wang et al, 1967b). In the case of $E_1$3S, the blood metabolic clearance rate (MCR) was shown to be less than 10% of free E_1 (Longcope and Tait, 1971; Longcope, 1972). The low MCR of steroid sulfates has been attributed to their binding to plasma proteins (Wang and Bulbrook, 1967). $E_1$3S, as well as the sulfates of E_2 ($E_2$3S, $E_2$17S, $E_2$3,17diS), have been shown to be strongly bound with high capacity to human serum albumin (Rosenthal et al, 1972, 1975). Studies in rats (Savu et al, 1973) have also shown an association of $E_1$3S with albumin. Thus it is believed that the clearance rates of steroid sulfates vary inversely with the tightness of their binding to albumin.

It has also been suggested (Longcope, 1972) that the very low MCR of sulfates is due not only to plasma protein binding but also to the fact that only a small fraction of the circulating sulfates can be metabolized. This may be due to inherently low activity of the metabolizing enzymes or to a low blood supply to tissues with the capacity to metabolize the sulfates. In support of this theory, Fishman and Hellman (1973) have shown in their study of the fate of E_1 and $E_1$3S administered simultaneously to man

that the unconjugated E_1 was metabolized more extensively than its sulfate ester. Urinary E_1 and E_2 glucuronides were derived to a greater extent from administered ^{14}C-E_1 than from ^{3}H-E_1 3S. They have suggested that the greater metabolism of E_1 in comparison with $E_1$3S may be the result of reactions at the C-3 hydroxy group which is not "protected" by sulfate conjugation. Thus the transformations of E_1 may contribute to its shorter plasma half life in comparison with $E_1$3S.

(d) STEROID SULFATES PARTICIPATE IN THE BIOSYNTHESIS, SECRETION AND METABOLISM OF STEROID HORMONES

(i) Secretion

Evidence for the secretion of steroid sulfates was presented by Baulieu in 1962 who demonstrated in vivo that the adrenal venous blood of two persons with an adrenal tumor contained more DHAS than that found in the peripheral blood. No unconjugated DHA could be found in either the adrenal tumor or in the venous plasma. These observations suggested the production of DHA occurred as a sulfoconjugate followed by its subsequent secretion. These results were confirmed a year later by Wieland et al (1963) who demonstrated the same phenomena in a normal subject. These latter workers, however, also showed that DHA was secreted. Support for the secretion of DHAS by the adrenals was also given by the demonstration of the synthesis of the sulfate ester by adrenocortical tissue (Wallace and

Lieberman, 1963). Prior to this finding, sulfate conjugation formation was shown to take place only in liver tissue. Thus, it seemed unlikely that the adrenal gland would synthesize and secrete a steroid sulfate merely for its immediate excretion into the urine. The production and secretion of DHAS must have a more significant physiological consequence. With the discovery that steroid sulfates are not merely excretory products but may be secreted from endocrine tissue, interest in steroids conjugated with sulfuric acid has increased.

A very recent report (Doouss et al, 1975) has appeared which, although showing that human adrenal had the ability to secrete DHAS, data suggested that its secretion was not as great as DHA secretion.

Direct secretion of $E_1$3S into the circulation has been reported to occur scarcely at all (Longcope, 1972; Ruder et al, 1972). Most of the $E_1$3S entering the blood each day seems to be a result of conversion from the free estrogens, E_1 and E_2.

(ii) Biosynthesis and Metabolism

The first indications that sulfated steroids may act as metabolic intermediates were observed by Tombly and Levitz (1960) working with estrogens and Roberts et al (1961) studying the androgens. These workers respectively

showed that administration of labeled E_1S or labeled DHAS to humans resulted in the excretion in the urine of labeled glucuronides. These observations showed that conjugation of steroids with sulfate does not necessarily destine the hormone for excretion. The sulfated steroid seemed to be in equilibrium with its unconjugated form.

Shortly thereafter, certain studies demonstrated that steroid sulfates could be metabolized without the splitting of the ester link (Baulieu et al, 1963; Calvin et al, 1963). Evidence that ^{35}S-DHAS could be synthesized in vivo in man from ^{35}S-pregnenolone sulfate (Calvin et al, 1963) without cleavage of the sulfate ester group indicated the existence of a biosynthetic pathway involving steroid sulfates as intermediates. The in vivo studies of Roberts et al (1964a, 1964b) with ^{3}H and ^{35}S labeled cholesterol sulfate added further support to the concept that sulfated intermediates were involved in the biosynthesis of the steroid hormones. When the ^{3}H and ^{35}S labeled cholesterol sulfate was injected into a woman with an adrenal tumor, ^{3}H and ^{35}S labeled DHAS with the same $^{3}H/^{35}S$ ratio as the administered cholesterol sulfate was isolated from the collected urine. This demonstrated that cholesterol sulfate without the removal of the sulfate could be a precursor of other steroid sulfates going through the same biochemical pathway as un-

conjugated cholesterol. In vitro studies (Calvin and Lieberman, 1964; Wu and Mason, 1965; Payne and Mason, 1965; Huhtaniemi, 1974) confirmed the existence of a sulfated pathway of steroid biosynthesis and metabolism. Thus, steroid sulfates can take part in metabolic pathways which parallel those for the free steroids, although the quantitative importance of this may be questioned.

Estrogen sulfates have also been shown to be metabolized without prior hydrolysis of the sulfate ester group in a number of animals both in vivo and in vitro, enhancing the view that sulfates are important substrates in the intermediary metabolism of steroids. The metabolism of phenolic steroid sulfates is not limited to oxido-reduction reactions but hydroxylations, as well as O-methylations, have been shown to occur with the intact sulfated steroid. Emerman et al (1965) have shown that the human fetus, when perfused with ^{3}H and ^{35}S labeled $E_1 3S$, extensively metabolizes the sulfated substrate without prior deconjugation. Among the water-soluble metabolites, $E_3 3S$, a 16α hydroxylated metabolite, was identified. Data reported by Schwers et al (1965) confirmed and extended these observations. Besides the previously reported reduction of $E_1 3S$ to $E_2 3S$ (Emerman et al, 1965), the metabolism of $E_1 3S$ was shown to involve

a series of hydroxylations particularly in the α configuration at carbons 15 and 16.

Fishman et al (1969) have suggested that $E_1$3S in the human may be the primary substrate for estrogen hydroxylations, particularly at C-2 and C-16. In support of this suggestion, Fishman, Yoshizawa and Hellman (1973) provided evidence that $E_1$3S in man is hydroxylated in vivo at carbon 2 to form 2-hydroxyestrone sulfate. The hydroxylated product of $E_1$3S was partially O-methylated to yield 2-methoxyestrone sulfate. Raud and Hobkirk (1968a), employing the hen, presented evidence that liver homogenates could convert ^{3}H-$E_1$3S to ^{3}H-$E_2$3S, an oxido-reduction reaction. Mathur (1969), also employing the hen, showed that $E_1$3S is metabolized in vivo to yield monosulfates and disulfates of both E_2 and $E_2$17α. Traces of 16-ketoestradiol (16ketoE$_2$) and 16epiE$_3$ were also believed to be formed directly from $E_1$3S. Recent studies in the guinea pig by Hobkirk et al have shown that this species is capable of interconverting $E_1$3S and $E_2$3S in the liver (Hobkirk, Nilsen and Jennings, 1975a; Hobkirk et al, 1977) and in the kidney (Hobkirk, Nilsen and Jennings, 1975b). $E_1$3S was also shown to be converted directly to 16α hydroxylated metabolites (Hobkirk, Nilsen and Jennings, 1975a; Hobkirk et al, 1977). Rat (Dolly et al, 1971) and mouse (Hobkirk and Nilsen, 1975) tissues have also been employed

to investigate the metabolism of $E_1$3S.

It has been shown only very recently (Gustafsson and Ingelman-Sundberg, 1975; Ingelman-Sundberg et al, 1975; Einarsson et al, 1976) that the hydroxylations of steroid sulfates, "the steroid sulfate pathway", differ both qualitatively and quantitatively from the hydroxylations of the corresponding unconjugated substrates, "the free steroid pathway".

1.8 METABOLISM OF ESTROGENS

Isolation of the estrogens aroused considerable interest in their metabolism, an interest that is still maintained today. A better understanding of the metabolism of these steroids may shed light on some of the very fundamental problems of cellular growth and division, as well as neoplastic transformations. A principal factor in estrogenic activity is clearly the quantity of hormone secreted. Its subsequent metabolic pathway or qualitative alterations in metabolism, however, represents a possible means of modulating this activity either extending or terminating it. The metabolism of estrogens is very complex, as is best shown by the great number of different metabolites that have been isolated. The liver plays the most important role in the metabolic processes relating to the estrogen molecule. The principal reactions involved in estrogen metabolism are the addition or removal of two hydrogen atoms by dehydrogenases and the addition of molecular oxygen by hydroxylases. It is the latter reaction which will be focused upon in this section. Hydroxylations may occur in the phenolic steroid at carbon atoms 2, 4, 6, 7, 11, 14, 15, 16 and 18 depending upon the species and tissue (see Figure 1). Carbon atoms 6, 7, 15 and 16 may be hydroxylated to produce an asymmetric carbon forming an α or β hydroxylated product which increases the complexity of estrogen metabolism.

(a) THE HYDROXYLASE SYSTEM

Mason (1957a, 1957b) proposed that molecular oxygen is metabolized by three types of enzymes. Oxygen transferases or oxygenases incorporate both oxygen atoms of O_2 in the product. Transfer of reducing equivalents to O_2, two-electron or four-electron reductions of oxygen via electron transfer oxidases, respectively forms a molecule of hydrogen peroxide or two molecules of water from the oxygen consumed. Insertion of one atom of O_2 into the substrate and reduction of the other oxygen atom to oxidize NADPH or NADH (usually the former) is catalyzed by mixed function oxidases or hydroxylases. The overall reaction catalyzed by this latter enzyme system is described by the following equation:

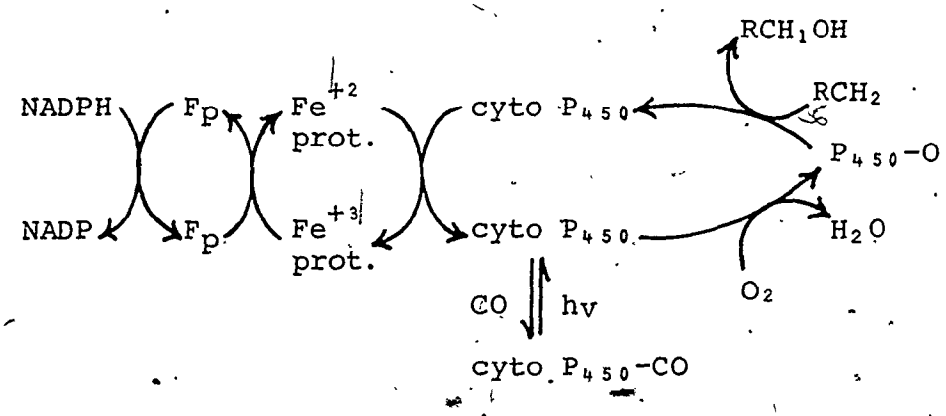
$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

RH represents the substrate undergoing hydroxylation. This is one of the principal reactions involved in steroid biosynthesis and metabolism.

Insight into the hydroxylating mechanism was derived from the 11β hydroxylation of deoxycorticosterone by adrenal cortical mitochondria. Early studies (Tomkins et al, 1957, 1958; Sweat and Bryson, 1962) on the 11β hydroxylase in the adrenal mitochondria showed that steroid hydroxylases are enzyme complexes which can be resolved into a

number of separate enzyme components.

Reconstitution experiments (Omura et al, 1965) with a particulate preparation of cytochrome P_{450}, a nonheme iron-protein and a FAD flavoprotein isolated from beef adrenal cortex mitochondria, suggested that the electron transport chain for hydroxylation reactions in this system is as illustrated:



Similar results from other reconstitution studies have been obtained for pig (Kimura and Suzuki, 1965, 1967) and rat (Harding et al, 1965) adrenal mitochondria.

Evidence that a similar electron transport scheme existed in microsomes† arose from studies showing that microsomes contained similar catalytic components as the mitochondrial hydroxylating system. For example, the FAD flavoprotein of mitochondria is similar to the NADPH cytochrome C reductase which has been purified and investiga-

†"Microsomes" are completely enclosed vesicles of endoplasmic reticulum membranes that are formed during the homogenization of a tissue.

ted from liver microsomes (Williams and Kamin, 1962; Kamin et al, 1965). Ryan and Engel (1957) showed that C-21 hydroxylation of 17-hydroxyprogesterone which occurred in beef adrenal microsomal preparations required NADPH and molecular O_2. They found that this hydroxylation was strongly inhibited by CO and that this inhibition could be partially reversed by light. In the following year, Klingenberg (1958) made the first observation of a "CO-binding pigment" now known as cytochrome P_{450} in rat liver microsomes. Cytochrome P_{450} has been purified and identified as a hemoprotein (Omura and Sato, 1964a, 1964b). The name cytochrome P_{450} originates from its ability when in the reduced form and combined with CO to form an intense absorption band with maximum at 450 nm. Cytochrome P_{450} is converted into an enzymically inactive form called cytochrome P_{420} (absorption maximum 420 nm) during purification procedures (Omura and Sato, 1964a, 1964b).

Treatment of rats with drugs, such as phenobarbital, which increases hydroxylating activity of liver microsomes, also produced increases in microsomal cytochrome P_{450} and NADPH-cytochrome C reductase (Ernster and Orrenius, 1965; Orrenius, 1965a, 1965b; Orrenius et al, 1965). This drug induction was sensitive to actinomycin D and puromycin (Orrenius et al, 1965), well known inhibitors of protein synthesis. Removal of the drug inducer resulted in a

parallel decrease of hydroxylating activity and microsomal levels of cytochrome P_{450} and NADPH-cytochrome C reductase (Ernster and Orrenius, 1965; Orrenius, 1965b). These studies provided evidence that the hydroxylating enzyme system of microsomes involved microsomal NADPH-cytochrome C reductase and the CO binding pigment, cytochrome P_{450}. Further evidence for the involvement of cytochrome P_{450} in the microsomal hydroxylating process comes from spectrophotometric observations. The photochemical action spectrum for the reversal of CO inhibition for the hydroxylation of a number of substrates by light occurred at a maximum of 450 nm (Estabrook et al, 1963; Cooper et al, 1965). This electron transport system is believed to be found with very few exceptions in all microsome fractions provided the microsomal fraction contains membranes (Siekevitz, 1965).

CHAPTER 2

PURPOSE OF INVESTIGATION

Hydroxylation at C-2 and at C-16 (α configuration) are the two major pathways of estrogen hydroxylation in man (Fishman et al, 1966, 1970). Fishman et al (1965) have provided evidence that hydroxylations at C-2 and C-16 are competing reactions for the available estrogen substrate. The rate and route of estrogen metabolism by hydroxylases, that is the relative quantities of 2-hydroxy and 16α-hydroxy products formed, is influenced by a variety of factors. For example, 2-hydroxy products are increased in man in vivo by high thyroxine levels so that these become the major estrogen metabolites. The amount of 16α-hydroxylated product decreases (Fishman et al, 1965; Brown and Strong, 1965), under these conditions. Decreased 2-oxygenation occurs regularly in cirrhosis and inconstantly in extrahepatic biliary obstruction. Depression of 16α-hydroxylation occurs regularly in both these conditions (Zumoff et al, 1968, 1970). Body weight has also been shown to influence the subsequent hydroxylation pathway. Brown and Strong (1965) found a direct correlation of urinary E_3 level, a 16α-hydroxylated product, with body weight. A recent study (Fishman et al, 1975) extended this finding indicating that increasing body weight was accompanied by a sharp decrease in 2-hydroxylation and

an increase in 16α-hydroxylation. Decreased body weight, as in anorexia nervosa, a condition in which malnutrition is accompanied by amenorrhea, increased 2-hydroxylation and decreased 16α-hydroxylation. Thus the nutritional status of an individual is related to the direction of estrogen metabolism. Synthetic steroids have also been shown to have a directing effect on the hydroxylation (Femino et al, 1974; Khoo and Mackay, 1969; Fishman and Hellman, 1976).

The importance of these metabolic changes may be understood by an understanding of the biological activity of these hydroxylated products. 2-Hydroxyestrogens and 16α-hydroxyestrogens seem to exhibit considerably divergent biological activities.

2-Hydroxyestrogen metabolites have been shown to possess much greater hypocholesteremic activity relative to estrogenic activity than for the classical estrogen, E_2, in some species (Gordon et al, 1964). It is well known that the enzymatic methylation and biological inactivation of neurotransmitters, the catecholamines by catechol-O-methyltransferase, is strongly inhibited by 2-hydroxy estrogens (Ball et al, 1972).

Recently, evidence has accumulated implicating a role for 2-hydroxyestrogens in gonadotrophin control mech-

anisms. Conversion of E_2 and E_1 to the catechol estrogens has been shown to occur in the rat hypothalamus (Fishman and Norton, 1975). These 2-OH estrogen metabolites were shown to compete very effectively for the E_2 receptor in the pituitary and anterior hypothalamus of the rat at a magnitude which could be biologically significant (Davies et al, 1975).

E_3 is looked upon as being a protective agent limiting or abolishing the action of E_2 on target tissues under certain conditions (Persianinov et al, 1975). In other circumstances where chronic exposure to E_3 prevails, E_3 is considered to be an active estrogen comparable to E_2 (Anderson et al, 1975; Clark and Peck, 1976).

Estrogen lack is an important contributing factor in the aetiology of postmenopausal osteoporosis. A recent study showed the inhibitory effect of E_3 on the development of experimental osteoporosis produced by low calcium diet in rats (Igarashi et al, 1974).

E_3 has been suggested to play a significant anticarcinogenic role in the human female (Lemon, 1970). Support for this suggestion is given by the protective effect of E_3 in rats against breast tumor induction by dimethylbenzanthracene (Lemon, 1975). Further support arises

from the observations that early pregnancy in humans seems to be protective, decreasing the risk of breast cancer, while in the mouse, pregnancy shows no such protection. During pregnancy in the human, E_3 is extremely elevated, but in the mouse it is E_1 and E_2, not E_3, that are elevated (cited in Cole and MacMahon, 1969).

Epidemiological studies, consisting of the measurement of the ratio of urine E_3 to E_1 plus E_2 (E_3 ratio), have shown higher values in Asian women whose mammary cancer rates are low. In comparison, lower ratios were found in Caucasian women of North America whose breast cancer rates are high (MacMahon et al, 1971; Dickinson et al, 1974). Asian women in Hawaii, whose breast cancer rates are intermediate between their homeland rates and those of Americans, had intermediate E_3 to E_1 plus E_2 urinary values (Dickinson et al, 1974). The lower E_3 ratio in American women was believed to be due to increased E_1 and E_2. The difference in ratios was distinct among younger women and slight among the older group (MacMahon et al, 1971). These observations supported the hypothesis that the estrogen profile (particularly the relative amount of a 16α-hydroxylated metabolite) during reproductive years is associated with subsequent

breast cancer risk.

Environmental factors which modify endocrine status and balance (eg. overnutrition, favoring endocrine hyperfunction followed by hyperstimulation of target organs) have been proposed to explain this estrogen ratio phenomenon (Berg, 1975).

A genetic explanation of the "estriol hypothesis" of mammary cancer has also been proposed. Lemon (1972) found that the Caucasian population could be divided into groups depending on their urinary E_3 ratio and plasma clearance rates of infused tritiated estrogens, E_1 and E_2. Breast cancer patients aggregated into the groups with the lowest E_3 excretion and lowest E_1 and E_2 clearance rates. Lemon proposed that a recessive mutant allele interfering with the formation of E_3 by reducing 16α hydroxylase activity was responsible for the observations. Such reasoning is not unrealistic in light of a similar finding with aryl hydrocarbon hydroxylase (AHH). Aryl hydrocarbon hydroxylase occurs in microsomal fractions of most tissues and is an inducible enzyme showing increased activity after the administration of a number of different agents. It catalyzes the conversion of environmental compounds to carcinogens (eg. epoxides) in the body during the detoxifi-

cation mechanism. The normal white population of the United States can be divided into three separate groups having low, intermediate and high inducible AHH activities. Data indicate that susceptibility to bronchogenic carcinoma is associated with higher levels of inducible AHH activity (Kellerman et al, 1973).

Thus an abnormal hormonal environment may be actively implicated in the etiology of human breast cancer. Whether hormones are responsible for the induction of the malignancy or whether they enable other unknown factors to cause transformations remains to be elucidated.

In view of the apparent importance of at least some hydroxy phenolic steroids, it would appear to be necessary to define their biosynthesis and obtain a model to study their control and factors responsible for altering their relative amounts or activities. Present models are disfavored due to the presence of multiple hydroxylases in some species, such as the rat (Ball et al, 1974) or mouse (Thorsen and Stoa, 1971) which possess the ability to hydroxylate estrogens in several positions, thus obscuring the study of any one. One may add that although human tissues should offer the most appropriate systems for study, availability of a continuing supply of normal material from this source constitutes a major problem.

The guinea pig liver has previously been shown to possess only two types of conjugating mechanism, glucuronidation (Sa'at and Slaunwhite, 1969; Goebelsman et al, 1965) and sulfurylation (Levitz et al, 1965). These two activities resemble the pattern seen in humans which also possess the ability to glucuronidate and sulfurylate estrogens. Formation of only two types of conjugates in the guinea pig simplifies the metabolic picture of estrogen transformation. The conjugation in several other species is complex and does not parallel the human situation. Early studies in the guinea pig in vitro (Quamme et al, 1972) and in vivo (Stoa and Borjesson, 1971; Sandberg et al, 1967) indicated that the unconjugated estrogens (E_1, E_2, E_3) employed in these investigations were rapidly glucuronidated. No evidence of hydroxylation of the estrogens was found. At about this same period, Fishman et al (1969, 1973) provided evidence and suggested that $E_1$3S was the primary substrate for estrogen hydroxylations in the human, to form both 2-hydroxy and 16α-hydroxy estrogen sulfates. Thus, the possibility existed that failure to detect hydroxylated estrogen in the guinea pig might be due to employing the improper estrogen substrate. $E_1$3S might be a potential estrogen substrate for possible hydroxylation in guinea pig tissues. The significance of steroid sulfates [as described earlier in section 1.7], particularly as metabolic intermediates, strengthened the case for employing $E_1$3S.

Initial studies were involved in comparing the metabolism of E_2 and $E_1$3S in guinea pig liver tissue slices. The work of Gustafsson et al (Gustafsson and Ingelman-Sundberg, 1974, 1975; Ingelman-Sundberg, Rane and Gustafsson, 1975; Einarsson, Gustafsson, Ihre and Ingelman-Sundberg, 1975) was unknown at the time and it was not certain whether there existed a qualitative difference in the metabolism of a sulfated steroid as compared to that of the free steroid. Fishman and Hellman (1973) did, however, point out that unconjugated E_1 was metabolized more extensively than its sulfate ester in vivo in man. Following the publications of the work of Gustafsson et al, the metabolism of E_2 and E_1 and their 3-sulfates by guinea pig liver microsomes was investigated. Employing guinea pig liver microsomes, properties of the 16α-hydroxylation of $E_1$3S were then investigated. Studies were also performed on how synthetic steroids, such as those contained in the oral contraceptive, as well as other natural steroids, may interfere with the 16α-hydroxylation pathway of $E_1$3S.

CHAPTER 3

EXPERIMENTAL MATERIALS

3.1 SOLVENTS AND REAGENTS

The following reagents were purchased from Fisher Scientific Co., unless otherwise stated.

(a) ORGANIC REAGENTS

1. Methanol, reagent grade and spectrograde
2. Ethanol was purchased from Commercial Alcohols Ltd., Gatineau, Quebec, Canada.
3. 1-Butanol, reagent grade
4. Tertiary butanol, reagent grade
5. n-Hexane, reagent grade
6. Chloroform, reagent grade
7. Ethyl acetate, reagent grade
8. Benzene, reagent grade and spectrograde
9. Cyclohexane, reagent grade
10. Pyridine, reagent grade
11. Acetic anhydride, reagent grade
12. Glacial acetic acid, reagent grade, was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.
13. Girard T. Reagent (Tri-methyl Acethydrazide Ammonium Chloride) reagent grade, stored in a desiccator.
14. Sodium bicarbonate, reagent grade
15. Nicotinamide was purchased from Matheson, Coleman and Bell Manufacturing Chemists, Norwood, Ohio, U.S.A.

16. Dextrose, reagent grade
17. Disodium Ethylenediamine Tetraacetate (EDTA), reagent grade
18. Diethyl ether (reagent grade)
19. L-ascorbic acid (reagent grade)

(b) INORGANIC REAGENTS

1. Potassium phosphate monobasic, reagent grade, was purchased from The McArthur Chemical Co. Ltd., Montreal, Quebec, Canada.
2. Potassium hydroxide pellets, reagent grade
3. Magnesium chloride hexahydrate crystals, reagent grade
4. Sodium chloride crystals, reagent grade
5. Potassium chloride crystals, reagent grade
6. Calcium chloride dihydrate crystals, reagent grade
7. Magnesium sulfate heptahydrate crystals, reagent grade
8. Concentrated hydrochloric acid, reagent grade
9. Concentrated sulfuric acid, reagent grade
10. Concentrated ammonium hydroxide, reagent grade
11. Chlorosulfonic acid was purchased from Matheson, Coleman and Bell Manufacturing Chemists, Norwood, Ohio, U.S.A.
12. Sodium hydroxide, 5 normal solution. Less concentrated solutions were prepared by diluting this stock.
13. Sodium borohydride (Powder, 98%) was stored in a desiccator.
14. Anhydrous sodium sulfate, reagent grade.

15. Sodium phosphate dibasic, reagent grade, was purchased from The McArthur Chemical Co. Ltd., Montreal, Quebec, Canada.

16. Anhydrous calcium sulfate "Drierite" was purchased from W. A. Hammond Drierite Co., Xenia, Ohio, U.S.A.

3.2 BIOCHEMICAL REAGENTS

(a) CHEMICALS

The following biochemical reagents were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

1. 7-Deoxycholic acid
2. Bovine serum albumin
3. Nicotinamide Adenine Dinucleotide Phosphate, reduced form (NADPH)
4. Nicotinamide Adenine Dinucleotide Phosphate, oxidized form ($NADP^+$)
5. Adenosine Triphosphate (ATP)

(b) ENZYMES

1. Mylase P, containing phenolsulfatase, was a product of Schwarz-Mann Research Labs., New York, U.S.A. It was stored in a desiccator at $-15^{\circ}C$ until needed.
2. Ketodase, a beef liver β-glucuronidase preparation, was purchased from Warner-Chilcott Labs., Morris Plains, N.J., U.S.A. It was refrigerated at $4^{\circ}C$ until required.

3.3 NONRADIOACTIVE STEROIDS

E_3 and 16epiE_3 were purchased from Schwarz-Mann Labs. Inc., Orangeburg, N.Y.

E_1 and E_2 were purchased from Ikapharm, Ramat-Gan, Israel.

16OHE_1, DHA, DHAS, ethinyl estradiol, mestranol, norethynodrel and medroxyprogesterone acetate were obtained from Steraloids, Inc., Wilton, N.H., U.S.A.

Unlabeled $E_1$3S was prepared chemically and purified as described in experimental methods section 4.1 (a).

3.4 RADIOACTIVE STEROIDS

Estrone-6,7-^{3}H of specific activity (S.A.) 47.9 Ci/mmol, estrone-4-^{14}C of S.A. 55.7 mCi/mmol and estradiol-4-^{14}C of S.A. 57.7 mCi/mmol were purchased from New England Nuclear, Canada (NEN), Dorval, Quebec. These radioactive steroids were purified as described by Hobkirk et al (1969) and stored in benzene methanol (1:1) at 4°C.

Estrone-6,7-^{3}H-3-sulfate of specific activity 42 Ci/mmol was purchased from New England Nuclear, Canada (NEN), Dorval, Quebec. An aqueous solution of this material was chromatographed on DEAE Sephadex in a linear concentration gradient of 0-0.8M NaCl. The main radioactive peak eluted between 0.3 and 0.4M NaCl, (about 85% of the original material) was pooled and recovered via Amberlite XAD-2 resin [see methods below]. Estrone-6,7-^{3}H-3-sulfate purity was further checked as described in experimental methods section 4.1 (a). It was stored in methanol with the addition of 1-2 drops of concentrated NH_4OH at -15°C.

Estradiol-6,7-^{3}H-3-sulfate was prepared by reducing estrone-6,7-^{3}H-3-sulfate with $NaBH_4$ followed by purification [see experimental methods below section 4.1 (b)].

Estrone-4-^{14}C-3-sulfate was prepared chemically from estrone-4-^{14}C and purified [see experimental methods section 4.1 (a)].

3.5 ABSOLUTE COUNTING STANDARDS

n-Hexadecane-1,2-^{3}H of S.A. 2.46 μCi/g and n-hexadecane-^{14}C of S.A. 0.781 μCi/g were purchased from The Radiochemical Centre, Amersham, Bucks, England. These were used to prepare absolute counting standards for efficiency determinations.

3.6 CHROMATOGRAPHIC MATERIALS

Diethylaminoethyl-Sephadex (DEAE-Sephadex A_{25}), an anion exchanger, Sephadex LH_{20}, a hydroxypropylated product of Sephadex G-25 and acrylic columns 1 x 60 cm (K 9/60) were purchased from Pharmacia Canada Ltd., Montreal, Quebec. Glass columns, 1 x 25 cm, equipped with teflon plug and an integral reservoir at top, which were employed for Sephadex LH_{20} chromatography, were purchased from O. H. Johns Glass Company Ltd., Toronto, Ontario.

Amberlite XAD-2 resin, a neutral cross-linked polystyrene polymer ion-exchange resin, was purchased from Rohm and Hass, Philadelphia, Pa., U.S.A.

Celite 545 was obtained from Johns Manville Co. Ltd. and prepared for use by steeping in concentrated HCl overnight followed by several washings with distilled water until the washings were neutral to pH paper. The Celite was then dried in an oven for a few days at 130°C and then kept in a desiccator until needed.

Precoated glass plates of silica gel impregnated with a fluorescent indicator for thin layer chromatography were purchased from Canlab, Toronto, Ontario.

3.7 ANIMALS

All guinea pigs (450-750g) used were of the English shorthair variety. Liver tissue was employed from mature males, nonpregnant and pregnant females. Pregnant females were of various gestational ages (35-65 days).

CHAPTER 4

EXPERIMENTAL METHODS

4.1 SYNTHESIS OF ESTROGEN SULFATES

(a) SYNTHESIS AND PURIFICATION OF ESTRONE-3-SULFATE AND OF ESTRONE-4-^{14}C-3-SULFATE

(i) Synthesis

Labeled and unlabeled $E_1$3S (Na^+ salt) were synthesized in the laboratory employing a methodology quite similar to that used by Fieser (1948) in his preparation of sulfate esters from alcoholic quinones.

Chlorosulfonic acid (0.1 ml) was added dropwise to pyridine (1.0 ml in a 100 ml round bottom flask) while stirring the solution in an ice bath. The mixture was left to stand until the cloudy vapour disappeared. E_1 (labeled or unlabeled), dissolved in 0.5 ml of cold pyridine, was added dropwise to the pyridine sulfate solution that was formed in the above mixture, followed by a second portion of 0.5 ml pyridine wash. The reaction mixture was warmed in a steam bath for 1-2 min, left at room temperature (~15 min) and then placed in an ice bath (~15 min). Water, (25 ml) was added to the solution and the pH adjusted to about 10 with NaOH 5.0M (or until deep red colour appears).

(ii) Purification of Estrone-3-Sulfate

Extraction and purification of unlabeled $E_1$3S was performed as described elsewhere (Raud and Hobkirk, 1968). Unreacted E_1 was removed by extracting the water solution with two portions (25 ml each) of diethyl ether. The water phase was then extracted with water-saturated n-butanol (3 extractions; 25 ml each). The butanol-soluble conjugated material was crystallized several times from methanol-diethyl ether.

Thin layer chromatography of the product employing the solvent system ethylacetate:cyclohexane (50:50) (Lisboa and Diczfalusy, 1962) indicated the absence of unconjugated E_1. A single band remained at the origin. On hydrolysis with mylase P sulfatase [as described in experimental methods section 4.3 (c) (ii)], the product gave rise to quantitative yields of E_1 as revealed by thin layer chromatography (employed the same system as described above).

The product $E_1$3S was stored in methanol with the addition of 1-2 drops of concentrated NH_4OH at -15°C and its purity was checked periodically.

(iii) Purification of Estrone-4-^{14}C-3-Sulfate

The reaction mixture was extracted with water-saturated n-butanol (ether extraction was omitted). Following the

evaporation of n-butanol via a rotary evaporator, the residue was redissolved in water and the solution was applied to a XAD-2 resin column which was then washed with H_2O to remove the unreacted pyridine sulfate salt. The radioactive material was then eluted from the column with methanol [see experiment methods section 4.3 (b)].

Sephadex A_{25} chromatography, as described in experimental methods section 4.3 (a), was then performed. The elution profile showed two peaks; one corresponding to unconjugated estrogens, presumably E_1 (4.7% of eluted material) and the main peak eluted between 0.3 and 0.4M NaCl (95.2% of total radioactive material eluted). This main radioactive compound estrone-4-^{14}C-3-sulfate was recovered through XAD-2 resin. It was stored in methanol with the addition of 1-2 drops of concentrated NH_4OH at -15°C.

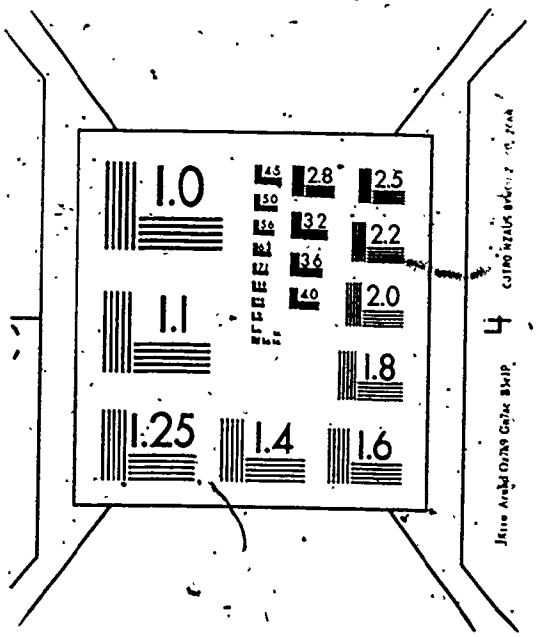
(b) SYNTHESIS AND PURIFICATION OF 17β-ESTRADIOL-6,7-^{3}H-3-SULFATE

17β-Estradiol-6,7-^{3}H-3-sulfate was prepared by reducing estrone-6,7-^{3}H-3-sulfate with $NaBH_4$ (Kirdani, 1965). Sodium borohydride (150 mg or 5 mg/ml MeOH) was added to estrone-6,7-^{3}H-3-sulfate and the reaction mixture was stirred for 60 min. The pH was lowered to 7.0 with the addition of 10% acetic acid to destroy excess $NaBH_4$. The

pH was then readjusted to 8.0 with 5M NaOH to stabilize the sulfated steroid. Methanol was evaporated off and the reaction residue washed by applying it to a XAD-2 resin column [experimental methods section 4.3 (b)]. Further purification was achieved by DEAE-Sephadex chromatography in a linear gradient of 0-0.8M NaCl [experimental methods section 4.3 (a)] which yielded two minor peaks and one major peak. The two minor peaks corresponded to unconjugated estrogens (0.3%) and $E_1$3S (1.0%), while the major peak eluted between 0.45 and 0.55M NaCl corresponded to 17β-estradiol-6,7-^{3}H-3-sulfate (98.0%). The product was recovered via XAD-2 resin and stored in methanol with the addition of concentrated NH_4OH (1-2 drops) at -15°C.

(c) IDENTIFICATION AND PURITY OF SYNTHESIZED ESTROGEN SULFATES

Previous procedures in the laboratory (Celite partition chromatography, melting point determinations, crystallization to constant specific activity with added carrier following hydrolysis or solvolysis) have shown that the estrogen sulfates prepared as described above are about 98-99% pure (Hobkirk, Nilsen and Blahey, 1969; Hobkirk, Musey and Nilsen, 1969). A repeat of these detailed procedures was therefore felt unnecessary.


1.0
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4

4.2 TISSUE INCUBATIONS

(a) Buffers

(i) Krebs-Ringer Phosphate Buffer

Krebs-Ringer phosphate buffer was prepared as described elsewhere (Umbreit et al, 1964) as follows:

1.	Sodium chloride (0.154M)	100 parts
2.	Potassium chloride (0.154M)	4 parts
3.	Calcium chloride (0.11M)	3 parts
4.	Magnesium sulfate $7H_2O$ (0.154M)	1 part
5.	Phosphate buffer (0.1M-pH 7.4)	20 parts

Phosphate buffer was composed of sodium phosphate dibasic ($Na_2HPO_4 \cdot 2H_2O$ - 17.8g) plus 20 ml of 1N HCl.

To simplify the preparation and handling of the solutions, it was found convenient to prepare five times the required concentration (stored at 4°C) making the appropriate dilution when needed.

(ii) Bucher Buffer

One litre of Bucher medium (pH 7.5±0.1) as described by Bergstrom and Gloor (1955) contained:

1.	Monobasic potassium phosphate (KH_2PO_4)	10.8 g
2.	Potassium hydroxide	3.2 g
3.	Magnesium chloride	1.0 g
4.	Nicotinic acid amide (nicotinamide)	3.6 g

(b) LIVER SLICES

Guinea pigs were sacrificed by cervical dislocation and their livers rapidly excised and rinsed in cold physiological saline. Liver slices (~250 mg wet weight and about 0.5 mm in thickness) were prepared using a Stadie-Riggs hand microtome. Tissue slices were added to Erlenmeyer flasks kept at 37°C each containing labeled substrate ranging from nanomolar to micromolar concentration in 10 ml of Krebs-Ringer phosphate buffer (pH 7.4) containing glucose (2 mg/ml). Incubations were performed in an atmosphere of air in a Dubnoff metabolic shaker for 2 hours at 37°C. In most cases, at the conclusion of the incubation, the tissue was separated from the medium and both were extracted with methanol. In other cases, tissue and medium were not separated but investigated for metabolites together. The methanol solutions were kept at -15°C at least overnight, filtered through Whatman No. 40 paper and the precipitates then washed carefully with cold methanol.

(c) SUPERFUSION OF LIVER SLICES

A perfusion apparatus for tissue incubations was assembled (with the aid of personal communications from Drs. J. K. Grant, E. Gurpide and M. Saffran) out of glass tubing (diameter 3 mm) as shown in Fig. 2. Tissue slices

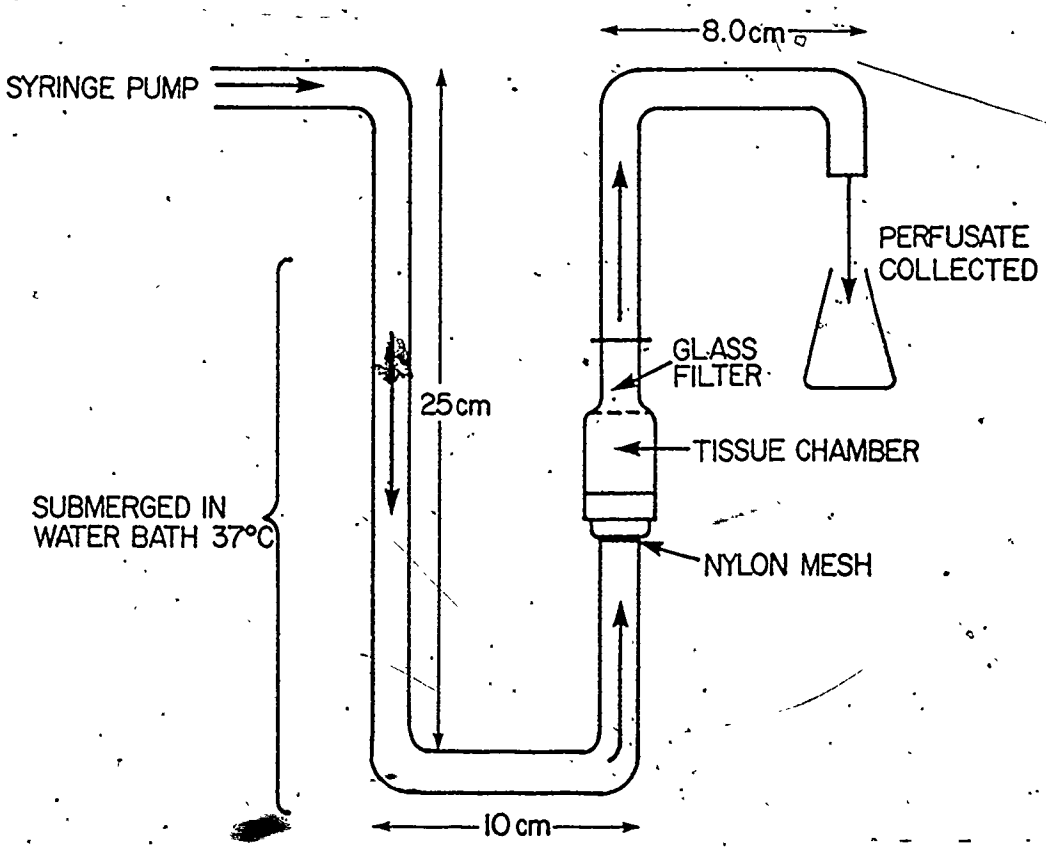

Figure 2. The perfusion apparatus employed for tissue superfusion.

(700-800 mg wet weight) prepared as described above and cut into finer pieces were placed in the tissue chamber which was submerged in a water bath at 37°C. Labeled steroids, ^{14}C-E_2, 696.0 ng/10 ml buffer or 2.58 nmol/10 ml buffer, and ^{3}H-$E_1$3S, 58 ng/10 ml buffer or 0.16 nmol/10 ml buffer were suspended in Krebs Ringer phosphate buffer (pH 7.4) containing glucose (2 mg/ml).

The buffer solution was transferred to a 50 ml syringe and the tissue was perfused as the buffer was forced through the apparatus by a syringe pump (Sage Instruments, Subsidiary of Orion Research Incorp., Model 352). Rate of perfusion was 15 ml/hour. Cold methanol was added to the tissue slices to terminate the incubation and to the perfusates which were collected at half hour intervals. These solutions were stored overnight at -15°C and filtered with careful washing of the filter paper with cold methanol.

(d) MICROSOMAL INCUBATIONS

Liver tissue (3.0 g) was homogenized employing a Heidolph homogenizer (Polyscience Corp., Niles, Ill.) in 15 ml of modified Bucher medium, pH 7.4-7.6. Microsomes were obtained by differential centrifugation as described in Fig. 3. The tissue homogenate was centrifuged at 20,000 xg for 20 min. and the resulting supernatant was centri-

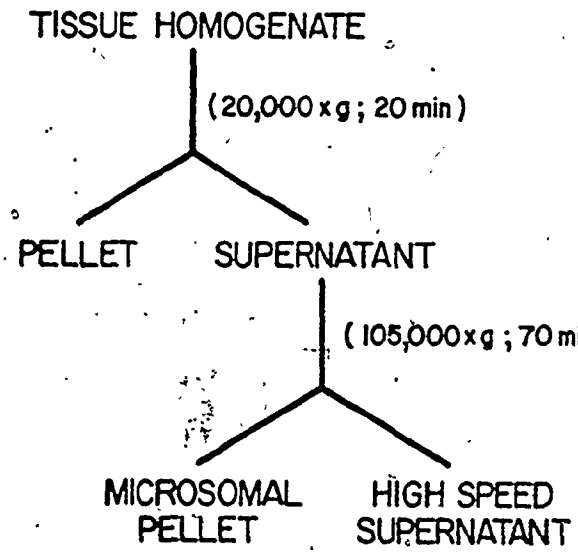

Figure 3. Microsomal preparation. The liver tissue (3.0 gm) was homogenized in Bucher medium, pH 7.4 to 7.6 (monobasic potassium phosphate, potassium hydroxide, magnesium chloride, nicotinamide).

fuged at 105,000 xg for 70 min. to obtain the microsomal pellet and high speed supernatant. The protein concentration of the microsomal fraction was determined by Biuret reaction (Gornall et al, 1949) using bovine serum albumin as standard.

Methanolic solutions of substrates (nanomolar to micromolar concentrations) were measured into test tubes and dried. Cofactors added to each tube were NADPH (2.3 µmol), ATP (3.6 µmol) and $MgSO_4$ (4.1 µmol). In later experiments which specifically involved the study of 16α-hydroxylation, only the cofactor NADPH (4.2 µmol) was added to each tube. All cofactors were added in 0.2 ml of Bucher medium. Bucher buffer was pipetted into each tube to give a total volume of 4.0 ml. One ml of a microsomal protein solution (6-10 mg protein/ml of solution, prepared by resuspending the microsomal pellet in Bucher buffer or in 105,000 xg supernatant, was added to the substrate solution to start the incubation. In all cases, the incubation system was of 5.0 ml total volume. Incubations were performed in air in a Dubnoff shaking incubator at 37°C for 5 to 120 min. Reactions were terminated by adding 50 ml of cold methanol and, after being mixed, the mixtures were kept at -15°C at least overnight.

4.3 INVESTIGATION OF PRODUCTS

(a) DEAE SEPHADEX CHROMATOGRAPHY

Following methanol extraction and filtration, as described in tissue incubations [section 4.2 (b)], the methanol was evaporated at reduced pressure (40-50°C). The extracted residue was dissolved in water (5 ml followed by 2 x 3.0 ml washes) with warming, and chromatographed on DEAE Sephadex columns (60 x 0.9 cm) with increasing linear concentration gradients of NaCl (Hobkirk, Musey and Nilsen, 1969; Hobkirk and Nilsen, 1970).

The Sephadex (steeped in water for at least 24 hours to swell and thoroughly washed to remove the fine particles prior to use) was allowed to settle under gravity in the columns to a height of about 58 cm. Each gram of original dry A_{25} occupied a packed volume of about 8 ml, making a total bed volume of 37 ml. The flow rate varied between 40 and 50 ml per hour from one column to another. Linear NaCl gradients were established employing two identical 1000 ml polyethylene bottles connected at their lower ends by polyethylene tubing.

The following gradient systems were employed:

1. Linear gradient 0-0.8M NaCl

The mixing container (with magnetic stirrer) contained 500ml of H_2O, while the donor vessel contained 500

ml of 0.8M NaCl. This gradient system separated unconjugated estrogens, glucuronides and monosulfates [see Fig. 4].

2. Linear gradient 0.8M-2.0M NaCl

The mixing vessel contained 300 ml of 0.8M NaCl and the donor vessel 300 ml of 2.0M NaCl. This gradient was employed, following the first gradient system, to elute any estrogen disulfates.

Ten ml fractions were collected with the aid of a LKB fraction collector and elution profiles determined by assaying 0.5 ml aliquots for radioactivity. Fractions (2 to 10) corresponding to unconjugated estrogens were extracted with ethylacetate (3 x 80 ml). The other radioactive peaks (glucuronides, monosulfates and disulfates) were each pooled and Amberlite XAD-2 resin columns were employed to recover the steroid conjugates.

(b) AMBERLITE XAD-2 RESIN EXTRACTION

The conjugate fractions were recovered from aqueous solution by Amberlite XAD-2 chromatography as described by Bradlow (1968).

Glass columns (25.0 x 3.0 cm packed with 50 g of resin or 50.0 x 3.0 cm packed with 100 g of resin) were employed for the procedure. Prior to use, the resin, washed

extensively with MeOH, was steeped in H_2O and any fine particulate material sucked off. The aqueous solution was applied (200 ml/100 g resin), and allowed to percolate through the bed. This was followed by washing with H_2O (twice the volume of the solution initially applied) which removed the inorganic materials. The column was then allowed to drain to remove the water as completely as possible. The absorbed steroid conjugates were then removed from the resin by eluting the column with methanol (500 ml/100 g resin). While eluting with methanol, air pockets were removed from the resin by inverting the columns. The flow rate throughout was kept at about 1500-2000 ml/hr. The methanol eluate containing the conjugates was evaporated to dryness under vacuum employing a rotary evaporator. The residue was redissolved in methanol (concentrated NH_4OH - 3 drops were added in the case of estrogen sulfates) and stored at -15°C until further processed. Columns could be reused and were left in water when not in use. It should be noted that recent batches of resin have been shown not to work under the above conditions (Bradlow, Common, Musey and Hobkirk, personal communications). The resin employed in the present study was purchased between 1969 and 1971.

(c) ENZYME HYDROLYSIS OR SOLVOLYSIS OF ESTROGEN CONJUGATES

Following Amberlite XAD-2 chromatography, as des-

cribed above, specific enzymatic incubations or solvolysis were performed on the methanolic extracts of the conjugated products. For glucuronides, Ketodase, a β-glucuronidase preparation, was employed, while for the monosulfates, mylase P, a phenolsulfatase preparation, was used. Solvolysis was performed on the estrogen disulfates.

(i) Hydrolysis of Glucuronides

Estrogen glucuronides were incubated with Ketodase in 0.1M acetate buffer, pH 5.0, for 24 hours at 37°C (Hobkirk, Nilsen and Blahey, 1969). Acetate buffer (1.0M; pH 5.0), prepared 10 times the required concentration, was stored at 4°C and diluted to the appropriate concentration when needed. The concentration of Ketodase used for hydrolysis was 500 Fishman units† /ml of incubation solution. The total incubation mixture in each case was 50 ml. Incubations were terminated by cooling (4°C) and extracting the released unconjugated estrogens with benzene or diethyl ether (3 x 50 ml). The combined extracts were washed with 10 ml of water, dried over anhydrous crystalline Na_2SO_4 and evaporated to dryness. The residue was redissolved in MeOH to await further processing.

†A Fishman unit of activity is defined as the amount of enzyme liberating 1 mg of phenolphthalein from a 0.001M solution of phenolphthalein glucuronide in 1.0 hour at 37°C and pH 4.5.

(ii) Hydrolysis of Monosulfates

Hydrolysis of estrogen-3-monosulfates was performed by employing mylase P (Emerman et al, 1965). The estrogen sulfates were dissolved in 50 ml of 0.1M acetate buffer (pH 6.0). This buffer was also prepared at a concentration 10 times that required (1.0M; pH 6.0), stored at $4^{\circ}C$ and diluted prior to each incubation. Mylase P concentration was 12-13 mg per 5.0 ml of buffer. Incubations were performed at $37^{\circ}C$ and terminated after 24 hours by cooling to $4^{\circ}C$ and extracting the unconjugated estrogens with ethyl acetate. The combined extracts were dried over anhydrous Na_2SO_4, evaporated and redissolved in MeOH.

(iii) Solvolysis of Disulfates

Disulfates were solvolyzed (Segal et al, 1960) in the presence of ascorbic acid (Elce et al, 1973) to avoid autoxidation of any possible estrogen catechols†. To ethyl acetate (50 ml) that was dried over Na_2SO_4 overnight, was added ascorbic acid (2.5 gm). The estrogen disulfates were dissolved in this mixture and H_2SO_4 was added to give a final concentration of $2 \times 10^{-4}M$. This was achieved by adding 0.05 ml of a solution of 1 ml of concentrated H_2SO_4

†At the outset of the study, formation of catechol estrogens was considered to be a distinct possibility. While none was found, it is likely that the ascorbic acid may provide a protective environment during solvolysis of the ring Dα-ketol disulfates which are now known to be formed.

in 100 ml of ethyl acetate. The whole was then incubated at 37°-40°C for 3 days††. After this period, the reaction mixture was cooled (4°C) and the ethyl acetate was washed with 1/10 volume (5.0 ml) of 1M $NaHCO_3$ and then 1/20 volume (2.5 ml) of H_2O. The washed ethyl acetate was dried over Na_2SO_4, evaporated to dryness under reduced pressure and the residue redissolved in MeOH.

(d) ANALYSIS OF FREE AND DECONJUGATED ESTROGENS

(i) Girard Separation

The Girard reaction (Girard and Sandulesco, 1936), as modified by (Givner et al, 1960), was employed to separate the ketonic from the nonketonic steroids. The free and deconjugated estrogens, as prepared above, were dissolved in 1.25 ml of specially purified ethanol (redistilled twice under anhydrous conditions and kept in a desiccator over anhydrous $CaSO_4$) to which was added Girard T reagent (125 mg) plus glacial acetic acid (0.15 ml). The mixture was left overnight (16-20 hours) in a stoppered vessel at room temperature in the dark. Following this period, the mixture was diluted with 15 ml of H_2O at 0°C and 1.45 ml of cold 10% NaOH was added dropwise

††A three day incubation period has lately been shown to be insufficient for complete deconjugation (Dr. Hobkirk, personal communication).

to give a pH of approximately 6.0. The total nonketonic fraction was then extracted with 3 x 20 ml of ethyl acetate. The combined extracts were washed with 5 ml of cold H_2O and the wash was added to the aqueous (ketonic-containing) phase.

The aqueous ketonic fraction was acidified with 4.2 ml of 7N H_2SO_4 and put aside for 2.0 hours at room temperature in the dark to cleave the Girard derivative. The extract containing the nonketonic fraction was washed with 2.5 ml of 1M $NaHCO_3$ followed by water washes of 5, 5 and 20 ml. The organic extract was evaporated to dryness and the residue was dissolved in MeOH.

The ketonic fraction was extracted from the acidified aqueous solution with ethyl acetate (3 x 30 ml). The combined extracts were shaken with 4.0 ml of 1M $NaHCO_3$ and allowed to stand 5-10 min, followed by a second and third shaking with the same bicarbonate. The extract was then washed successively with 5, 5, 20 and 10 ml of H_2O. The ethyl acetate was dried over anhydrous Na_2SO_4, evaporated to dryness and the residue dissolved in methanol.

(ii) Chromatography of Ketonic and Nonketonic Steroids

1. Sephadex LH_{20} Chromatography

Sephadex LH_{20} chromatography was then performed on

the ketonic and nonketonic material employing the solvent system n-Hexane/$CHCl_3$/EtOH (50:50:10).

The Sephadex was allowed to swell in the solvent system overnight. Glass columns (1 x 25 cm) equipped with Teflon plug and a reservoir at the top were packed to a height of 17 cm allowing the Sephadex to settle under gravity. An aliquot of sample dissolved in MeOH was transferred to a 3.0 ml conical tube, the MeOH evaporated and the residue redissolved in 0.2 ml of the eluting solvent to be applied to the column (followed by one 0.2 ml and one 0.4 ml washes).

One ml fractions were collected and elution profiles [see results, Figs. 9 and 10] determined by assaying 0.1 ml aliquots for radioactivity. Eluted peaks of radioactivity were crystallized with appropriate carrier steroids from methanol or methanol-H_2O with or without further purification by Celite partition chromatography [see below].

2. Celite Column Chromatography

Labeled metabolites tentatively considered to be 16αOHE_1, 16β-hydroxyestrone (16βOHE_1) and 16epiE_3 were further purified by Celite partition chromatography employing the system benzene:hexane/methanol: H_2O (55:45/70:30) and benzene/methanol:H_2O (100/70:30) as

described by Givner et al (1960).

The solvent systems were equilibrated overnight. The separated aqueous phase was added to dry Celite (5.0 ml/ 10 g) and mixed thoroughly ensuring that all the stationary phase was absorbed by the Celite. An excess of mobile phase was added to form a uniform slurry. A glass column (1 cm i.d.) with a porous glass filter at the bottom was packed to the required height (20 cm) by means of a perforated plunger. The surface of the finished column was covered with an excess of mobile phase to prevent the Celite from drying.

The sample (dissolved in 0.5 ml of MeOH) to be applied to the column was thoroughly mixed with Celite (0.5 g) and the mixture dried overnight in a vacuum desiccator. The Celite-containing sample was applied to the column with a minimum amount of mobile phase and packed on top. The column was eluted with an excess of mobile phase and aliquots of fractions collected were counted to determine the elution profile.

(iii) Crystallization of Estrogens and Acetylation

Final verification of the identity of the labeled estrogens was obtained by crystallization to constant specific activity of the isolated steroids, by reverse isotope

dilution with authentic unlabeled estrogens, and of the acetate derivatives of the steroids (Hobkirk, Nilsen and Blahey, 1969).

Crystallizations from MeOH were performed by adding a known weight of unlabeled pure carrier (20-30 mg) to a known isotopic activity of the labeled estrogen. The specific activity (dpm/mg) of the crystals and of the mother liquor was determined and crystallization was repeated until constant specific activity was obtained for both crystals and mother liquor.

Acetate derivatives were obtained by dissolving the crystals in dry pyridine (0.2 ml) followed by the addition of dry acetic anhydride (0.1 ml). The mixture was stoppered and allowed to stand overnight at room temperature. Methanol (1.0 ml) was added to the reaction mixture and the contents dried under air. This process was repeated until the pyridine was removed. The residue was crystallized from MeOH as described above.

Acetonides were prepared by the procedure of Nocke et al (1961). The dried residue from Celite columns plus carrier was dissolved in a dry solution of 1% HCl in acetone (10 ml). The solution was shaken in a stoppered vessel at room temperature for 30 min and then made alka-

line by the addition of 4 ml 1N NaOH. The acetone was removed in vacuo and the residue was taken up in 25 ml of 0.4N NaOH. The acetonide extracted with 3 x 25 ml $CHCl_3$ (extracts washed with 2 x 5 ml H_2O and dried over Na_2SO_4) was crystallized to constant specific activity.

4.4 MEASUREMENT OF RADIOACTIVITY

All radioactive counting was performed on a three channel, Unilux IIA Nuclear Chicago liquid scintillation spectrometer in 20 ml screw cap polyethylene vials or minivials of 6 ml capacity (Canadian Scientific Products Ltd., London, Ontario).

Aquasol (New England Nuclear Corp., Boston, Mass.), a xylene-based scintillation solution, was employed as the scintillation fluid in earlier studies. In later studies, Aqueous Counting Scintillant (ACS) was employed (Amersham/Searle Corp., Oakville, Ontario).

Calculations of ^{3}H dpm and ^{14}C dpm in dual labeled samples were performed by the discriminator-ratio method of Okita et al (1957). This method uses the ratio of channel B cpm (^{14}C widow) to channel A cpm (^{3}H widow) which can be obtained from standard samples of ^{3}H-hexadecane and ^{14}C-hexadecane. An equation that utilizes this discriminator ratio for each isotope is then employed in the calculation. These equations for ^{3}H dpm and ^{14}C dpm determinations are as follows:

1. For ^{3}H dpm:

$$^{3}H_A = \frac{bA-B}{b-a}$$

$$\text{and } {}^{3}\text{H dpm} = \frac{{}^{3}H_A}{\text{channel A } {}^{3}\text{H efficiency factor}}$$

2. For ^{14}C dpm:

$$^{14}C_B = \frac{b(B-aA)}{b-a}$$

$$\text{and } {}^{14}\text{C dpm} = \frac{{}^{14}C_B}{\text{channel B } {}^{14}\text{C efficiency factor}}$$

where:

$^{3}H_A$ = net cpm of ^{3}H in channel A

$^{14}C_B$ = net cpm of ^{14}C in channel B

A = net cpm of channel A ($H_A + C_A$)

B = net cpm of channel B ($H_B + C_B$)

a = discriminator ratio for ^{3}H

= net ^{3}H cpm in channel B to net ^{3}H cpm in channel A

b = discriminator ratio for ^{14}C

= net ^{14}C cpm in channel B to net ^{14}C cpm in channel A

The spectrometer was set to give ^{3}H efficiency of 30-35% and a ^{14}C efficiency of 55-60% in samples containing labeled hexadecane and scintillation fluid only.

Since a major fraction of the energy spectrum of ^{14}C appears above the maximum energy spectrum of the lower energy isotope of ^{3}H, channel settings for the high energy isotope, ^{14}C (channel B), were selected that lowered the

count of ^{3}H in that channel to an insignificant level. Less than 1% of the ^{3}H counts appeared in the ^{14}C channel. (Approximately 15% of the ^{14}C counts appeared in the ^{3}H channel or channel A).

This arrangement simplified the above calculations as no correction for tritium counts in channel B was required. The equations for dpm calculations reduce to the following form since "a" becomes zero.

For ^{3}H dpm:

$$^{3}H_{A} = A - \frac{B}{b}$$

For ^{14}C dpm:

$$^{14}C_{B} = B$$

Thus with a negligible ^{3}H counting rate in the ^{14}C channel, the ^{14}C dpm could be calculated directly from the recorded count rate in the ^{14}C channel and its known efficiency. The tritium disintegration rate was determined by net counts in the ^{3}H channel minus net counts in ^{14}C channel divided by the discriminator ratio for ^{14}C all divided by the known efficiency of ^{3}H.

During later studies in which only tritium labeled samples were counted, counting efficiency was determined by the channels ratio method. Two counting channels were

chosen so that together they included most of the energy spectrum of ^{3}H observed for the least quenched sample. Standard samples were counted and their efficiencies were plotted as a function of their channel's ratios. The efficiencies of unknown samples were determined from this calibration curve by their measured ratios.

Sufficient counts were accumulated for all important samples to ensure a counting error of no more than ±5%.

CHAPTER 5

RESULTS

5.1 STATEMENT ON EXPERIMENTAL RECOVERIES

Recoveries from the variety of analytical techniques employed in this investigation usually exceeded 85%. Filtration through Whatman No. 40 paper following incubations of ^{14}C-E_1 or ^{14}C-E_2 with microsomes alone was the only case that suggested the loss of a quantitatively significant metabolite. Recovery in this case was only 60-70%. The remaining 30-40% was to be found in the methanol precipitates. "Binding" or loss of activity on the methanol precipitate was shown to require a supply of NADPH and adequate incubation time (recovery decreased with time of incubation).

If the other experimental techniques gave rise to lower than expected recoveries, which seldom occurred, the procedure was repeated or rechecked. Table I indicates the average recoveries (plus range) from 10 randomly selected experiments for the various procedures employed. Final results were corrected for experimental losses.

TABLE I

Recoveries from experimental procedures (% of original isotope)

Procedure	Average[a]	Range
Filtration on Whatman No. 40 paper	95.9	91.3-100.1
Sephadex A_{25} Chromatography	92.8	83.2-102.0
Amberlite XAD-2 Resin Extraction	91.3	85.3-97.4
Hydrolysis: Ketodase	97.7	91.0-106.8
Mylase P	93.4	81.0-105.8
Solvolysis	72.2	65.3- 79.0
Girard Reaction (ketonic + nonketonic)	88.8	78.9- 93.3
Sephadex LH_{20} Chromatography	95.9	90.9-105.3
Celite Column Chromatography	94.7	91.0-104.3

[a]Average of 10 experimental procedures

5.2 LIVER TISSUE SLICE EXPERIMENT

(a) UPTAKE OF ^{3}H-ESTRONE SULFATE AND ^{14}C-ESTRADIOL BY LIVER SLICES

Table II summarizes the results of the uptake of ^{3}H-$E_1$3S and of ^{14}C-E_2 by liver tissue slices from the incubation medium. The effect of albumin, guinea pig plasma and γ-globulin in the incubation medium on the uptake of these steroids is also shown. E_2 (nmols) in the buffer medium in the absence of plasma proteins is taken up by the liver tissue of guinea pigs more rapidly than $E_1$3S (nmols) at short incubation periods, as shown by the greater percent of E_2 associated with the tissue as compared to $E_1$3S. At longer incubation times of 90-120 min, however, the percent radioactivity of ^{3}H-$E_1$3S associated with the tissue becomes comparable to that of ^{14}C-E_2. This relationship failed to hold when tissue slices were incubated in the presence of albumin or guinea pig plasma. The percent of ^{14}C-E_2 associated with the tissue at longer incubation times was still greater than that of the percent association of ^{3}H-$E_1$3S (eg. albumin, 120 min incubation: ^{14}C - 23.3%, 13.9% compared to ^{3}H - 9.8%, 8.8% respectively for the two incubations).

Table III compares the uptake of ^{14}C-E_2 and ^{3}H-$E_1$3S

TABLE II

Uptake of $^{3}HE_{1}3S$[a] and $^{14}CE_{2}$ by Guinea Pig Liver Tissue Slices in Presence of Albumin, Plasma, and γ-Globulin.

Incubation Time			Buffer (nmol/mg Tissue) x 10^6		Albumin (nmol/mg Tissue) x 10^6		Plasma (nmol/mg Tissue) x 10^6		γ-Globulin (nmol/mg Tissue) x 10^6	
10 Min	$^{14}CE_2$ Exp	1	24.7[b]	2381[c]	7.4[b]	712[c]	13.7[b]	1320[c]	-	-
		2	10.3	992	5.1	492	4.9	472	10.2[b]	984[c]
	$^{3}HE_{1}3S$	1	13.6	11	2.7	2	5.3	4	-	-
		2	8.0	7	3.1	3	3.6	3	8.4	7
20 Min	$^{14}CE_2$	1	28.6	2757	18.4	1774	-	-	-	-
	$^{3}HE_{1}3S$	1	14.1	12	5.9	5	-	-	-	-
30 Min	$^{14}CE_2$	1	29.9	2882	15.3	1475	18.7	1803	-	-
		2	13.2	1272	9.7	935	6.6	636	17.6	1697
	$^{3}HE_{1}3S$	1	14.9	13	5.0	4	7.3	6	-	-
		2	12.3	10	6.2	5	4.8	4	14.6	12
60 Min	$^{14}CE_2$	1	22.9	2208	15.2	1465	17.2	1658	-	-
		2	14.1	1359	8.4	810	11.7	1128	13.8	1330
	$^{3}HE_{1}3S$	1	14.4	12	5.2	4	7.6	6	-	-
		2	12.4	10	6.5	5	8.0	7	12.6	11
90 Min	$^{14}CE_2$	1	29.6	2853	23.8	2294	14.3	1379	-	-
	$^{3}HE_{1}3S$	1	20.2	16	8.3	7	7.2	6	-	-
120 Min	$^{14}CE_2$	1	35.0	3374	23.3	2246	30.1	2902	-	-
		2	21.1	2034	13.9	1340	14.7	1417	21.6	2082
	$^{3}HE_{1}3S$	1	30.0	25	9.8	8	14.0	11	-	-
		2	20.7	17	8.8	7	12.3	10	18.2	15

[a] Incubations performed in presence of $E_{1}3S$ - 210 x 10^{-4} nmol and $^{14}CE_2$ - 24,100 x 10^{-4} with liver tissue slices weighing approximately 250 mg (incubation time 10-120 min. at 37°C).

[b] % Associated with Tissue

[c] nmol of Substrate/mg Tissue

TABLE III

Comparison of ^{14}C-E_2 and $^{3}HE_1S$ Uptake by Guinea Pig Liver Tissue in Presence of Albumin, Plasma and γ-Globulin.

(Averaged Percent Uptake of that Found in Buffer Alone for Incubations 10-120 min., 37°C).

Incubation	% Uptake Compared to Incubation with Buffer Alone	
	^{14}C-Estradiol	^{3}H-Estrone Sulfate
Albumin	59	38
Plasma	57	43
γ-Globulin	102	110

[a] Incubations performed in presence of E_1S - 210×10^{-4} nmol and ^{14}C-E_2 - $24,100 \times 10^{-4}$ nmol with liver tissue slices weighing approximately 250 mgm.

by guinea pig liver tissue in the presence of albumin, plasma and γ-globulin to that of the uptake which occurred in the buffer medium in the absence of plasma proteins. Uptake of both steroids, $E_1$3S and E_2, was reduced when the incubation was performed in the presence of albumin or plasma. $E_1$3S uptake was reduced to a greater extent than E_2 uptake in these media. In the presence of albumin, the average uptake of $E_1$3S for the two experiments (10-120 min incubations) was 38% and in the presence of plasma, 43% of the uptake that occurred in the buffer medium in the absence of plasma proteins. For E_2, the corresponding values were, in the presence of albumin, 59% and plasma, 57% of the uptake in buffer medium alone. γ-globulin had no effect on the uptake of the steroids.

(b) METABOLISM BY SLICES

Figure 4 shows the chromatographic pattern of labeled metabolites contained in the tissue and medium following simultaneous incubation of ^{14}C-E_2 and ^{3}H-$E_1$3S with a liver slice from a pregnant guinea pig.

Table IV summarizes the results of the metabolic patterns found in tissue plus medium after 2-hour incubations from liver slices obtained from female (pregnant and non-

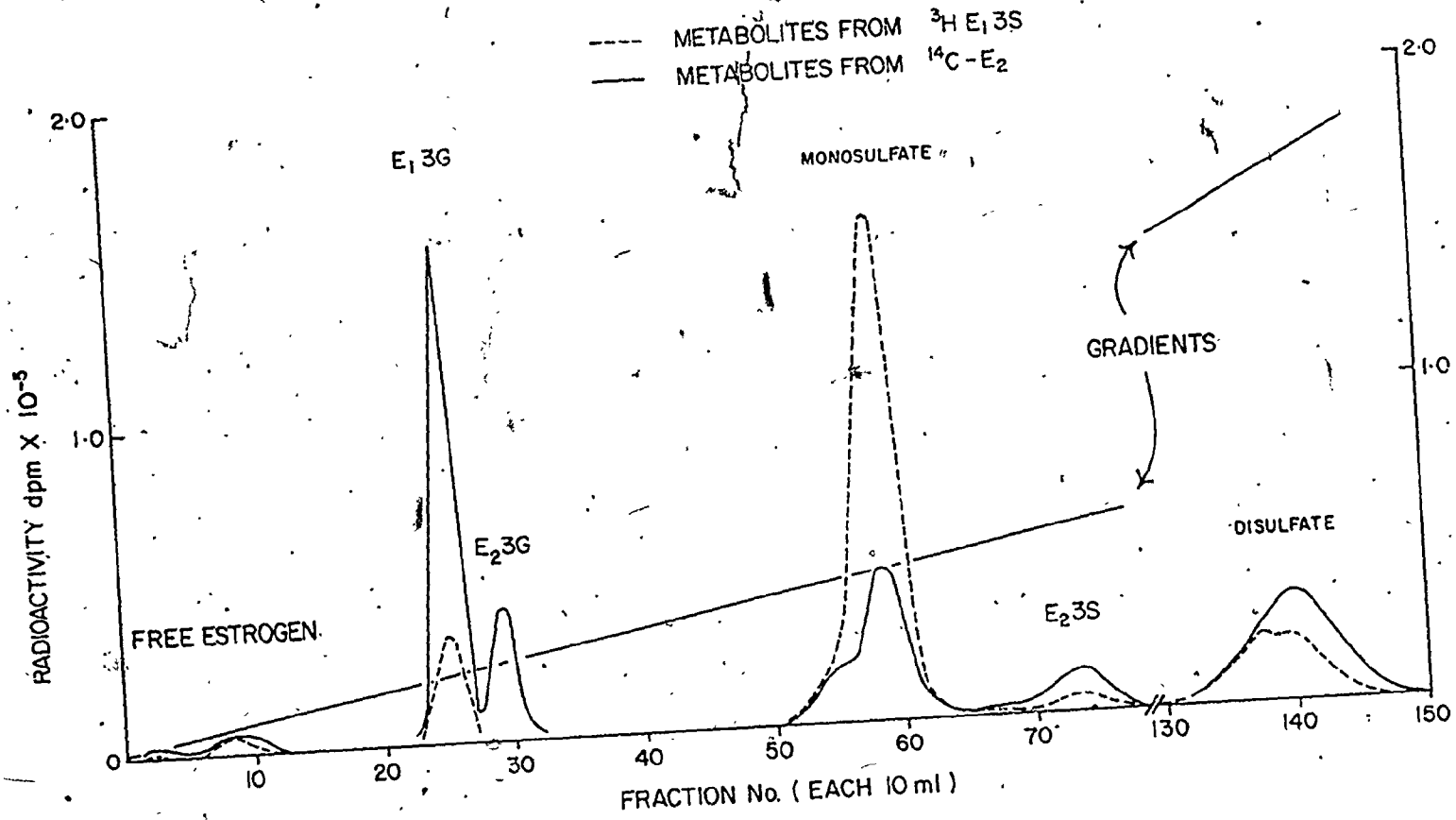

Figure 4. Elution pattern from a DEAE Sephadex column (60 x 0.9 cm, 0-0.8M followed by 0.8M-2.0M NaCl) of labeled metabolites contained in tissue and medium from pregnant guineæ pig liver slice after a 120 min. incubation.

TABLE IV

Metabolite Pattern in Tissue Plus Medium After 2-Hour Incubation of ^{3}H-$E_1$3S and ^{14}C-E_2 with Guinea Pig Liver Slices.

Type	N	No. of Incub.	Substrate[a]	Product % of Isotope in Tissue + Medium					
				Free	$E_1$3G	$E_2$3G	$E_1$3S[b]	$E_2$3S	Disulfate
Non-Pregnant	2	3	$E_1$3S	1.6[c] (0.3-2.7)	7.0 (6.7-7.4)	0.4 (0.3-0.8)	59.2 (38.3-73.0)	3.2 (1.5-4.6)	26.7 (12.9-47.1)
Non-Pregnant	2	3	E_2	3.0 (tr-6.1)	39.8 (34.9-44.8)	10.9 (5.1-22.1)	19.6 (12.3-25.3)	6.1 (1.8-9.2)	15.5 (17.8-20.4)
Pregnant	7	7	$E_1$3S	2.7 (0.4-5.2)	15.0 (8.9-36.8)	1.3 (tr-4.4)	57.7 (48.7-67.5)	2.9 (1.3-5.4)	16.0 (tr-33.8)
Pregnant	5	5	E_2	3.4 (tr-8.7)	39.8 (28.8-52.6)	18.2 (4.0-40.5)	16.1 (6.4-30.3)	3.9 (2.3-4.8)	16.3 (2.1-24.9)
Male	5	5	$E_1$3S	3.5 (2.0-5.2)	10.0 (6.2-14.7)	3.3 (1.3-5.3)	76.8 (67.0-84.7)	5.4 (3.6-8.9)	n.d.[d] n.d.
Male	5	5	E_2	8.1 (2.0-15.3)	35.3 (29.5-45.0)	35.0 (29.3-38.8)	15.3 (8.8-21.8)	6.3 (5.0-7.9)	n.d. n.d.

[a] Concentration E_2=241nM; $E_1$3S=2.1nM [metabolite pattern independent of this range (Hobkirk et al, 1977)].

[b] $E_1$3S peak contains variable amounts of 16αOH$E_1$3S and very small amounts of $E_3$3S (Hobkirk et al, 1977).

[c] Mean values, ranges in parentheses; tr, trace

[d] n.d. = not detectable

pregnant) and male guinea pigs. Thorough investigation of metabolites formed from liver tissue incubations has been published (Hobkirk, Nilsen and Jennings, 1975; Hobkirk et al, 1977). The liver slices formed monoglucuronides, monosulfates and a disulfate fraction. The metabolism of ^{3}H-$E_1$3S by liver tissue slices seems to be qualitatively similar to the metabolism of ^{14}C-E_2. Quantitatively, however, more glucuronide was produced from E_2 than from $E_1$3S.

Disulfate ranged widely from one animal to another and seemed to be absent in male animals. Other studies with young male animals in the lab, however, were shown to produce disulfates (Hobkirk et al, 1977). Pretreatment of males with E_2 had little influence on the metabolic picture. Investigation of the tissue and medium separately showed that, of the total percent associated with tissue, a greater fraction of this was disulfate compared to the percent disulfate associated with the medium.

5.3 LIVER SUPERFUSION

Liver tissue perfusion was employed to determine a correlation of metabolites formed with time by collecting perfusates at different time intervals. The percent uptake by the tissue in the perfusion study was somewhat lower than the percent uptake by the tissue incubated in flasks (^{14}C-E_2 uptake - 15%; ^{3}H-$E_1$3S uptake - 6%). The total radioactivity, however, associated with the tissue was greater in the perfusion study.

Figure 5 shows the metabolic pattern of metabolites contained in the tissue slice perfused with ^{14}C-E_2 and ^{3}H-$E_1$3S simultaneously. DEAE Chromatography indicated the presence of ^{3}H and ^{14}C labeled free estrogens (^{3}H - 5.4%; ^{14}C - 8.5%), $E_1$3G (^{3}H - 10.7%; ^{14}C - 31.3%), $E_2$3G (^{3}H - 2.7%; ^{14}C - 7.3%), $E_1$3S (^{3}H - 41.1%; ^{14}C - 16.2%), $E_2$3S (^{3}H - 6.7%; ^{14}C - 2.3%) and a disulfate fraction (^{3}H - 28.7%; ^{14}C - 25.0%).

Perfusates were collected in half hourly intervals and radioactivities associated with a particular metabolite were expressed as a percentage of the total activity found in that interval. Results expressed in histograms produced patterns for metabolites as shown in Figures 6 and 7.

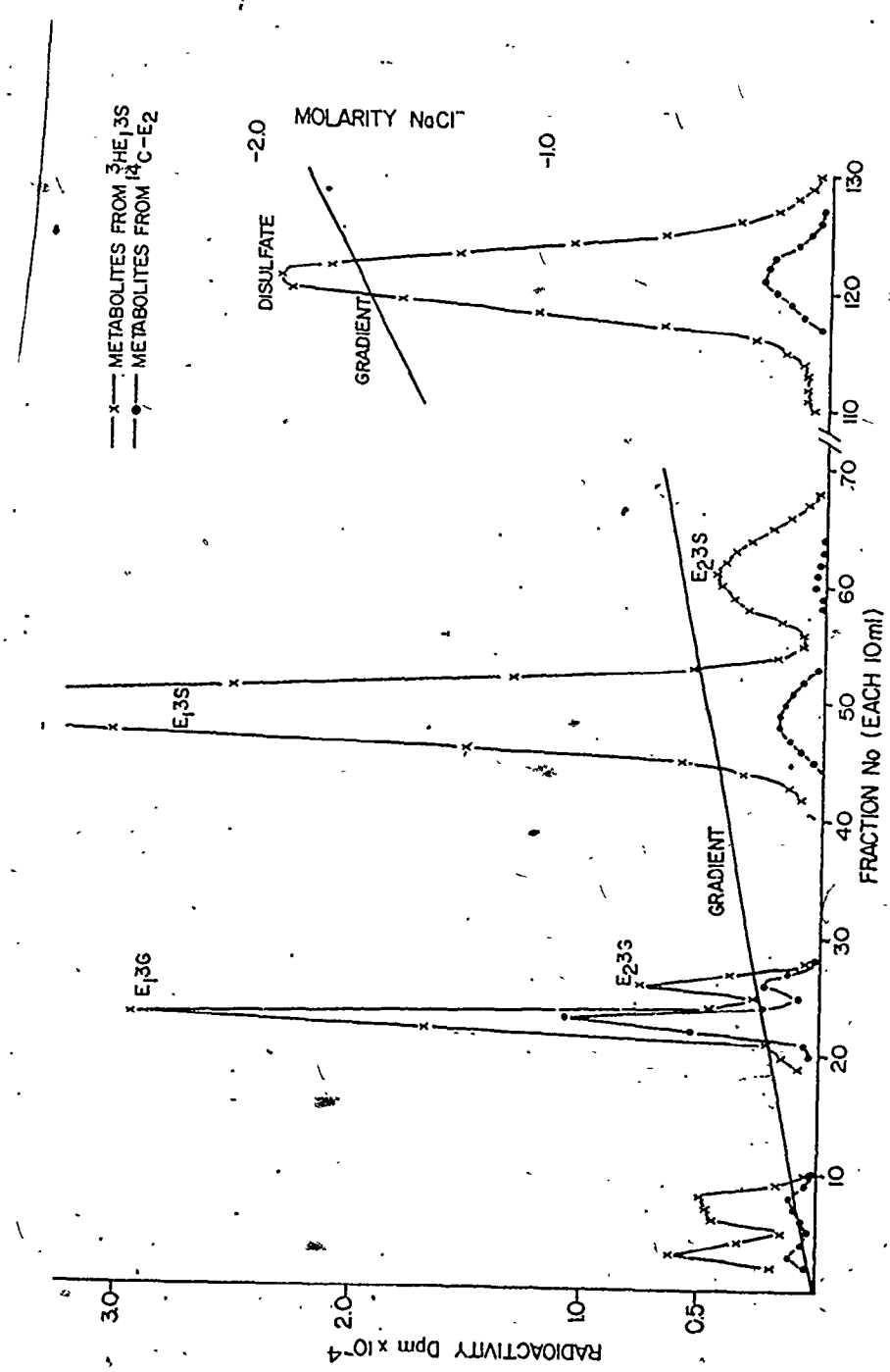

Figure 5. DEAE Sephadex chromatograph of metabolites contained in female guinea pig liver slice perfused simultaneously with $^{3}H-E_{1}3S$ (nmol concentration) and $^{14}C-E_2$ (nmol concentration) for 2.5 hours.

For the metabolites derived from ^{3}H-$E_1$3S (Fig. 6), free estrogen, $E_1$3G and the disulfate increased with time while $E_2$3S fell with time. For the metabolites derived from ^{14}C-E_2 (Fig. 7), $E_1$3G and the disulfate increased with time while $E_2$3S and $E_2$3G decreased with time. Formation of $E_1$3S from ^{14}C-E_2 reached 3% of total radioactivity off Sephadex A_{25} columns in the first half hour perfusate and remained relatively constant thereafter.

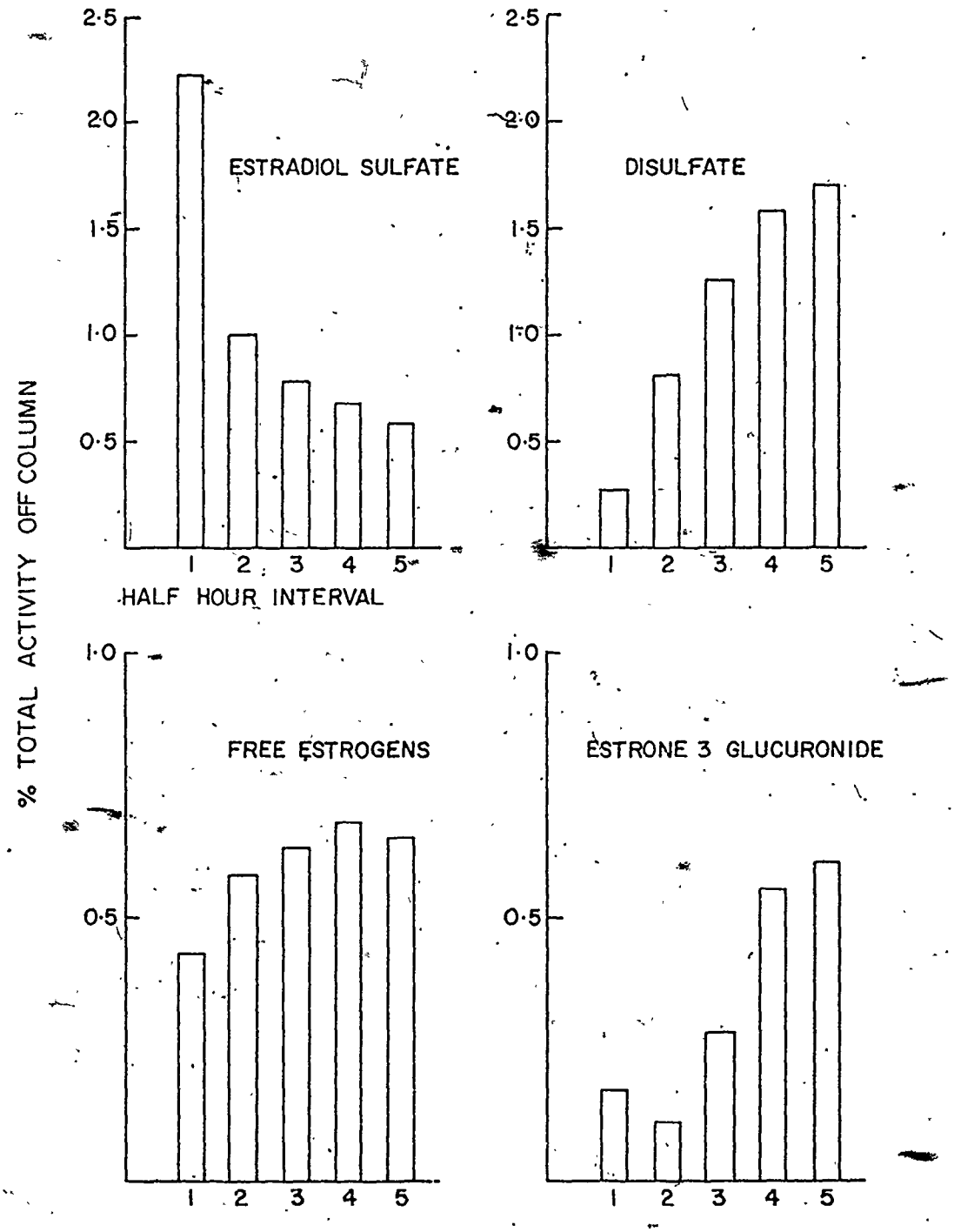

Figure 6. Metabolites contained in half hour perfusates derived from ^{3}H-$E_1$3S.

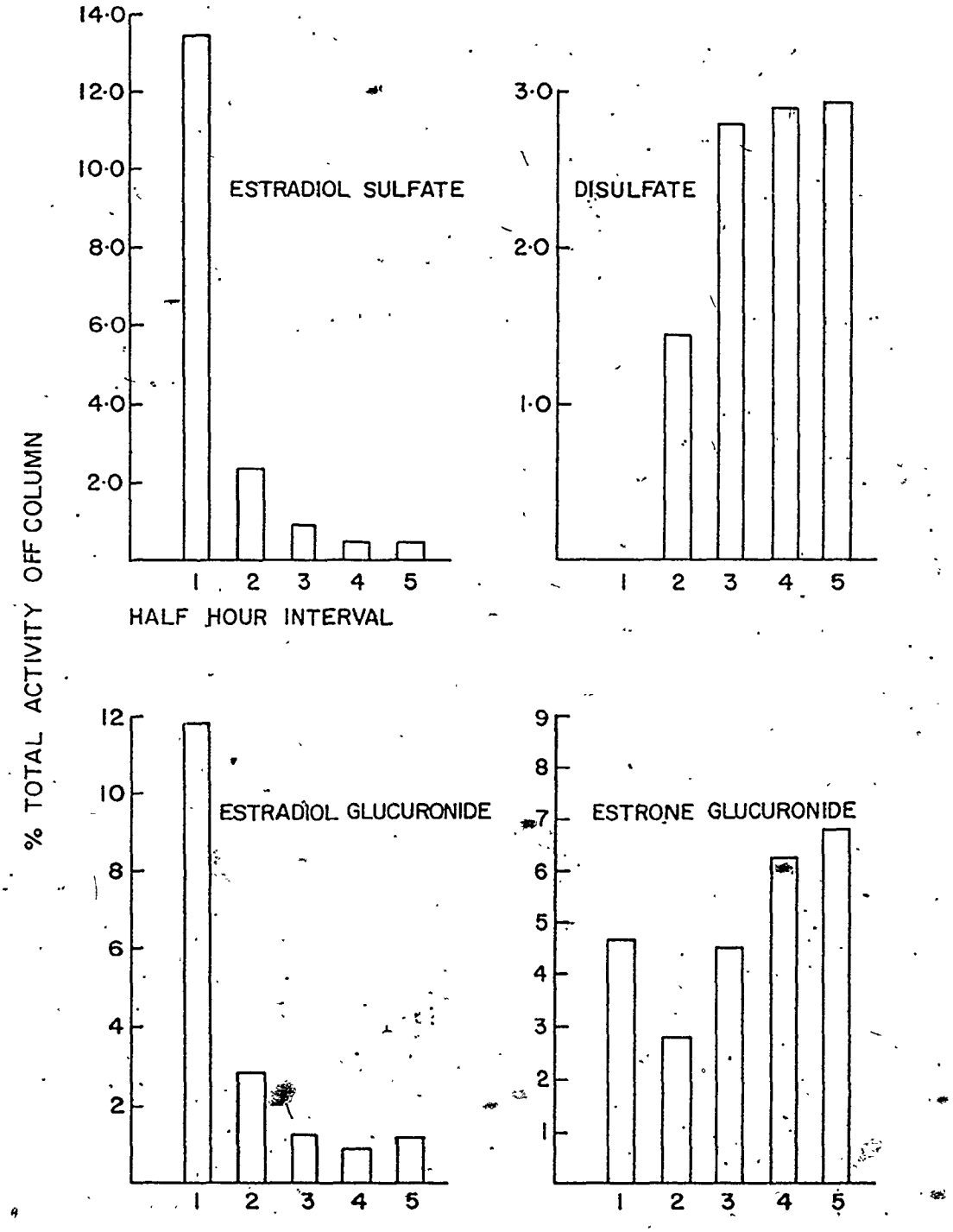

Figure 7. Metabolites contained in half hour perfusates derived from ^{14}C-E_2.

5.4 MICROSOMAL INCUBATIONS

Little difference could be detected between the metabolism of E_2 and $E_1$3S employing guinea pig liver slices. In early 1975, being familiar with the work of Gustafsson et al (Gustafsson and Ingelman-Sundberg, 1974, 1975; Ingelman-Sundberg, Rane and Gustafsson, 1975), it was decided to investigate the metabolism of these two substrates by guinea pig liver microsomes.

A DEAE Sephadex chromatographic pattern obtained after incubating ^{3}H-$E_1$3S and ^{14}C-E_2 simultaneously with microsomes (105,000 xg pellet) is shown in Fig. 8. Free steroids and steroid glucuronides, each possessing the ^{3}H and ^{14}C labels, were detected. Monosulfates were labeled with ^{3}H but contained little or no ^{14}C. This first incubation employed ^{14}C-E_2 as substrate since this is generally thought of as the active circulating form of estrogen. In order, however, to compare more directly the effect of a 3-sulfate group upon the metabolism by microsomes, further experiments involved simultaneous incubation of ^{3}H-$E_1$3S and ^{14}C-E_1. DEAE Sephadex chromatographic patterns obtained were similar to that shown in Fig. 8. Detailed investigation of the metabolites formed from ^{3}H-$E_1$3S and ^{14}C-E_1 [see below] revealed that E_1 was metabolized differently from $E_1$3S. Hydroxylation of the unconjugated estro-

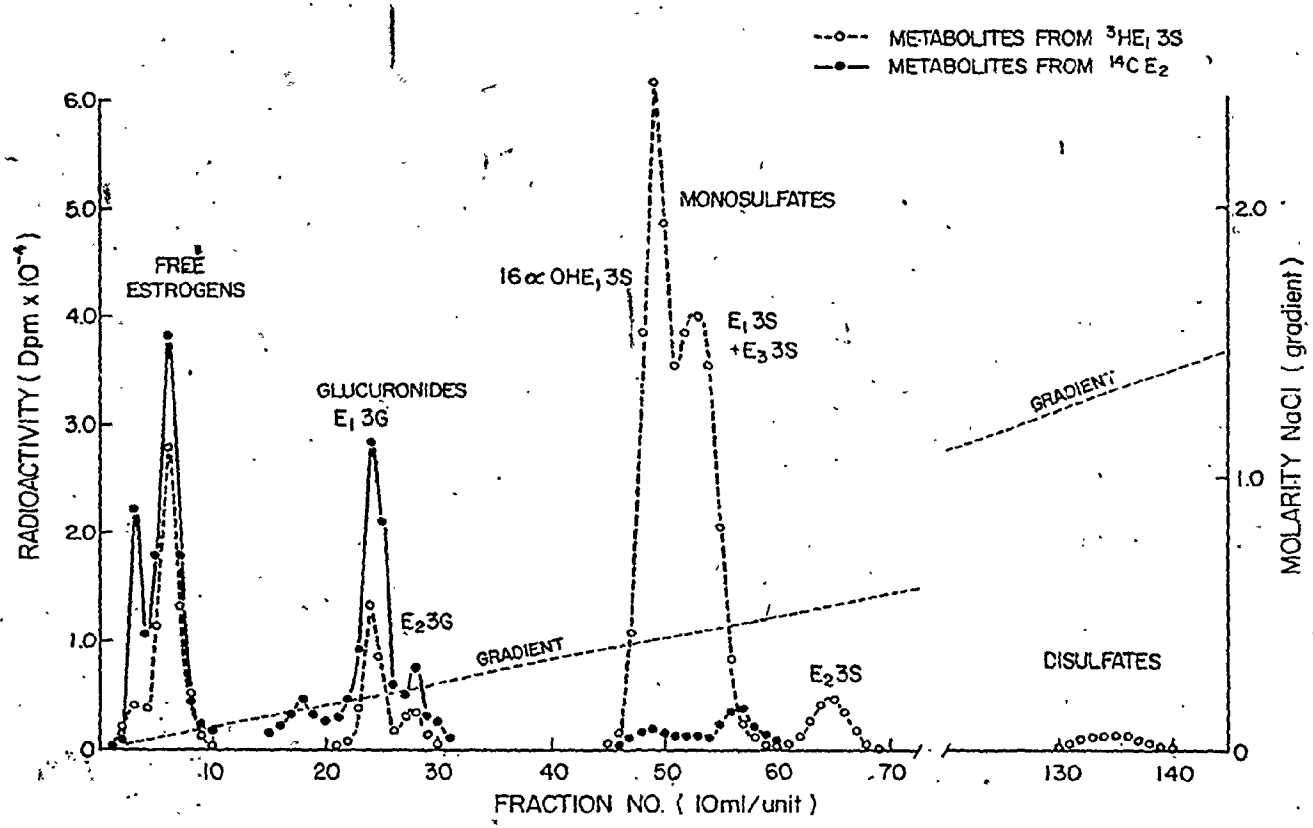

Figure 8. DEAE Sephadex chromatograph (60 x 0.9 cm, 0-0.8M followed by 0.8M-2.0M NaCl) of metabolites of ^{3}H-$E_1 3S$ and ^{14}C-E_2 from a microsomal incubation of 120 min. at 37°C, pH 7.4.

gen occurred predominantly at the β configuration of carbon-16 while the 3-sulfated estrogen formed mainly 16α-hydroxylated products.

Girard separation of the free steroids in these studies (fractions 2-10 from DEAE Sephadex chromatography) yielded ^{3}H, which was about 40% ketonic (average of six incubations with substrates ^{3}H-$E_1$3S and ^{14}C-E_1; range 34%-43%) and 60% nonketonic (range 57%-66%). Radioactive ^{14}C-labeled steroids in the free fraction were 32% ketonic (range 28%-35%) and 68% nonketonic (range 65%-72%).

Sephadex LH_{20} chromatography of a ketonic free steroid fraction from a typical incubation with ^{3}H-$E_1$3S and ^{14}C-E_1 [see Fig. 9] revealed three peaks primarily labeled with ^{14}C. The least polar fraction eluted from the Sephadex LH_{20} columns (first eluted fraction in tubes 6-10) was not further purified and shown to be E_1 by crystallization to constant specific activity. The other two peaks that were not completely separated on Sephadex LH_{20} (fractions 15-23) were pooled from a number of incubations and further resolved by Celite partition column chromatography followed by reduction with sodium borohydride. Results indicated that the major component of the pool was 16βOHE_1 (approx. 70%). 16αOHE_1 was identified as the other component (approx. 30%).

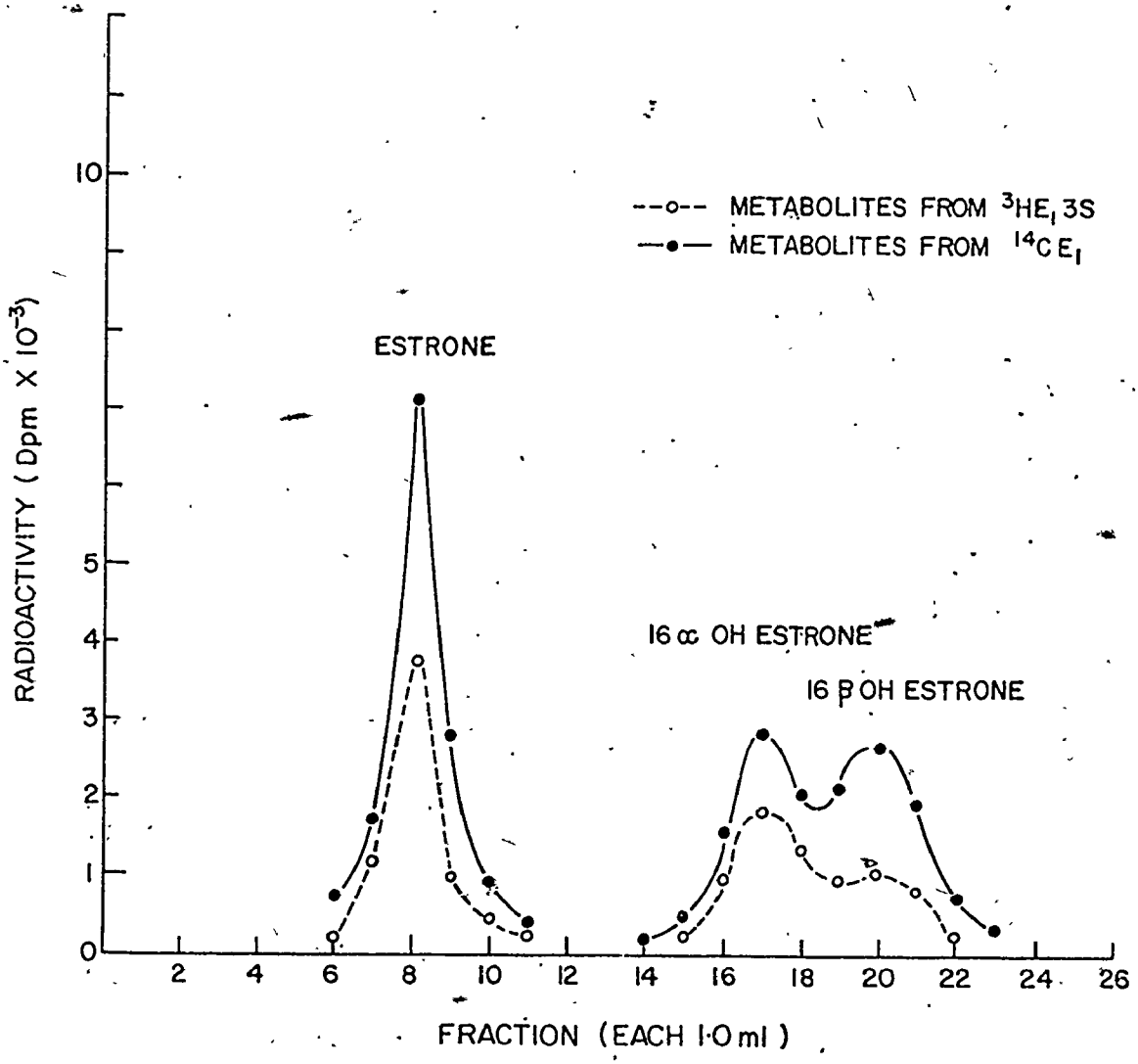

Figure 9. Sephadex LH_{20} chromatograph of ketonic fraction of free metabolites from a microsomal incubation (60 min. at 37°C, pH 7.4) with ^{3}H-$E_1$3S and ^{14}C-E_1.

A typical Sephadex LH_{20} chromatographic pattern of the nonketonic fraction of unconjugated estrogens (fraction 2-10 from DEAE Sephadex chromatography) from a microsomal incubation with ^{3}H-$E_1$3S and ^{14}C-E_1 is shown in Fig. 10. The results revealed two peaks. The more polar fraction (eluted in fractions 29-37) was predominantly labeled with ^{14}C and was identified as 16epiE_3 following Celite partition chromatography and crystallization with authentic 16EpiE_3 (Table V). The other fraction (16-21) was identified as E_2.

Pooled glucuronides (fractions 20-30 from DEAE Sephadex A_{25} chromatography - Fig. 8) were shown to be primarily $E_1$3G and $E_2$3G following ketodase hydrolysis, Sephadex LH_{20} chromatography and crystallization. Crystallizations of labeled E_1 and E_2 following deconjugation of the glucuronides are shown in Table VI. $E_2$17G, if formed, would have been separated from the other glucuronides following DEAE Sephadex chromatography. The absence of an $E_2$17G peak in the chromatogram excluded the possibility of this glucuronide as being a metabolite.

Tritium labeled fractions 45-60 from DEAE Sephadex chromatograms (Fig. 8 - monosulfates excluding $E_2$3S) were completely hydrolyzed by mylase P. Analysis by LH_{20}

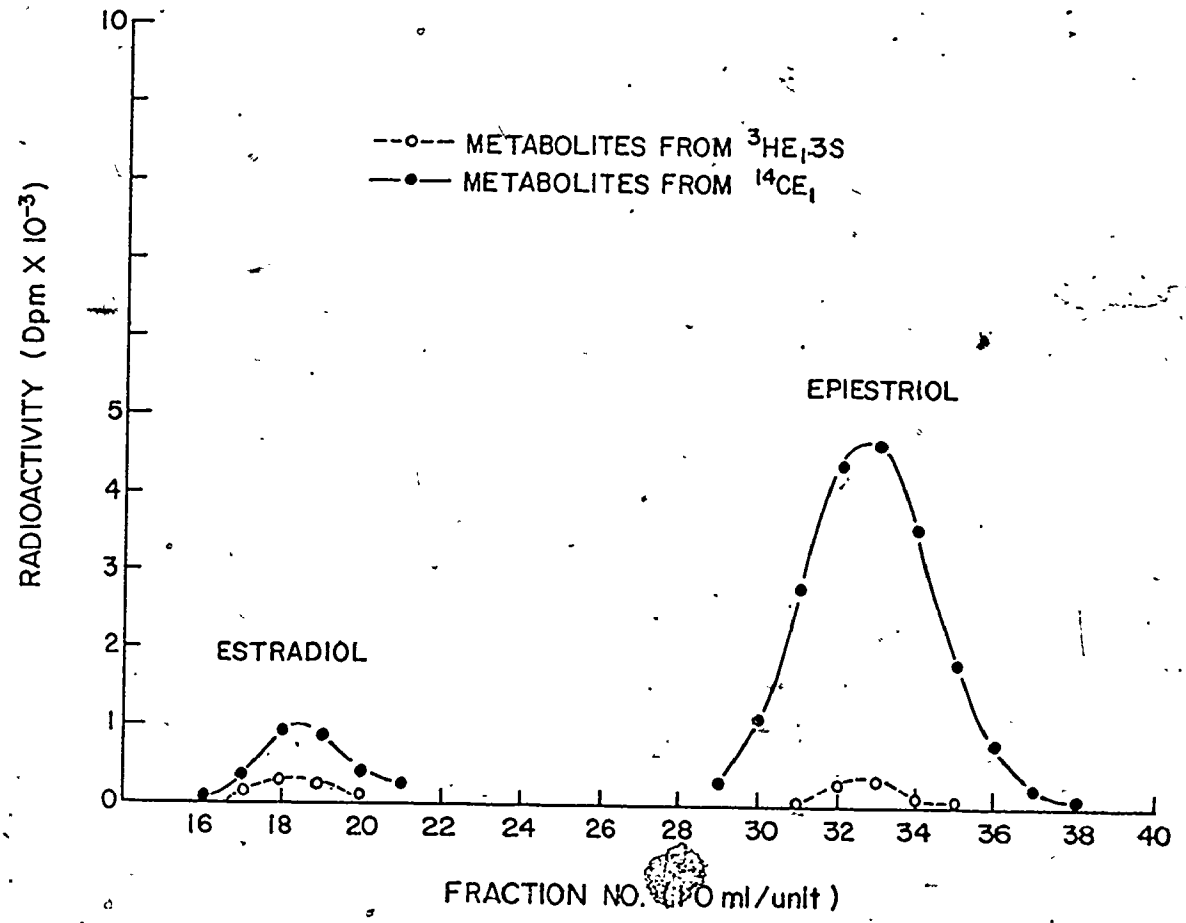

Figure 10. Sephadex LH_{20} chromatograph of nonketonic fraction of free metabolites from a microsomal incubation (60 min. at 37°C, pH 7.4) with ^{3}H-$E_{1}3S$ and ^{14}C-E_{1}.

TABLE V

Identification of ^{14}C-16epiE_3 by Crystallization

Derivative or Form	Calculated S.A.	Specific Activity (dpm/mg) Crystal.	Crystals.	Mother Liquor
Free	1350[a]	1	1370	1460
		2	1390	1380
Triacetate	956[b]	1	940	930
		2	900	910
Acetonide	1182[b]	1	1150	1120
		2	1170	1240

[a] Represents crystallization of 27,480 dpm of ^{14}C with 20.0 mg of carrier 16epiE_3.

[b] Represents calculated values for triacetate and acetonide respectively, as prepared from original carrier diluted ^{14}C-fraction.

TABLE VI

Crystallization of Labeled E_1 and E_2 Isolated from the Glucuronide Fraction

		Specific Activity (dpm/mg)					
		Crystals		M.L.		Calc.	
		^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
E_1 from $E_1$3G						57[a]	1420[a]
Crystallization	1	76	1280	101	1440		
	2	89	1300	82	1320		
	3	79	1290	87	1280		
Acetylation	1	61	1110	147	1140	70[c]	1120[c]
	2	53	1100	45	1140		
E_2 from $E_2$3G							
Crystallization	1	149	1090	174	1300	156[b]	1270[b]
	2	159	1070	176	1100		
	3	129	1100	133	1100		
Acetylation	1	110	880	108	880	112[c]	833[c]
	2	73	895	85	868		

[a] Calculation based on crystallization of ^3H-1,380 and ^{14}C-34,690 dpm with 24.38 mg of carrier E_1.

[b] Calculation based on crystallization of ^3H-3,840 and ^{14}C-31,170 dpm with 24.64 mg of carrier E_2.

[c] Values corrected for presence of acetyl groups and based on determined specific activity of free steroid.

chromatography after Girard separation† of the released free steroids from mylase P hydrolysis yielded two ketonic peaks (fraction 6-10 and fraction 14-19) and one non-ketonic peak (more polar than 16epiE_3 and eluted between fraction 40-50). Crystallization with carrier steroids followed by acetate formation of the crystals is shown in Table VII. The ketonic fraction 6-10 was shown to be E_1, while the nonketonic fraction was E_3. Crystallization of the ketonic fraction 14-19 from Sephadex LH_{20} chromatography revealed only 54% of it to be 16αOHE_1 (Table VII). Thus, the presence of an additional compound was indicated. Further investigation by Celite chromatography followed by $NaBH_4$ reduction indicated that 51.6% of the peak was 16αOHE_1 and 28.3% was 16ketoE_2. Whether 16ketoE_2 is a tissue metabolite or arises from 16αOHE_1 due to methodological manipulations is now known. It has been assumed, however, through the remainder of this study, that 16ketoE_2 has arisen from 16αOHE_1. The virtual absence of 16βOHE_1 in the monosulfate form supports this assumption.

A fraction eluted in tubes 60-70 (Fig. 8) was shown

† Girard separation was not required during later studies since LH_{20} chromatography separated E_1, 16αOHE_1 and E_3 adequately.

TABLE VII

Crystallization of ^3H-Labeled Deconjugated Steroids Isolated from the Monosulfate Fraction.

Derivative or Form		Specific Activity (dpm/mg)		
		Crystals	Mother Liquor	Calculated
E_1 from $E_1$3S				
Crystallization	1	858	1520	1090[a]
	2	943	962	
Acetylation	1	790	802	816[e]
	2	743	807	
16αOHE_1 from 16αOH$E_1$3S				
Crystallization	1	991	2910	1380[b]
	2	899	1900	
	3	746	949	
Acetylation	1	648	1470	574[e]
	2	615	804	
E_3 from $E_3$3S				
Crystallization	1	1020	1140	1190[c]
	2	1040	1110	
Acetylation	1	779	729	709[e]
	2	746	725	
E_2 from $E_2$3S				
Crystallization	1	1350	1780	1550[d]
	2	1400	1420	
Acetylation	1	1170	967	1040[e]
	2	1150	910	

[a] Calculation based on crystallization of 26,280 dpm of ^3H with 24.04 mg of carrier E_1.

[b] Calculation based on crystallization of 26,430 dpm of ^3H with 19.61 mg of carrier 16αOHE_1.

[c] Calculation based on crystallization of 29,100 dpm of ^3H with 24.54 mg of carrier E_3.

[d] Calculation based on crystallization of 40,490 dpm of ^3H with 26.06 mg of carrier E_2.

[e] Represents calculated values for acetate derivatives as prepared from original carrier diluted ^3H-fraction.

through mylase P hydrolysis, LH_{20} chromatography and subsequent crystallization (Table VII) to be E_2 3S.

Table VIII summarizes the results obtained from incubating microsomes with ^{3}H-E_1 3S and ^{14}C-E_2. These results correspond to the DEAE Sephadex A_{25} chromatographic pattern shown earlier (Fig. 8). Conversion of E_1 3S to its 16α-hydroxylated monosulfate forms (16αOHE_1 3S plus E_3 3S) was 45.8% with no detectable 16β-hydroxylation. Conversion of E_2 to free 16α- and 16β-hydroxylated metabolites was 5.0 and 20.1% respectively.

Results of incubating ^{3}H-E_1 3S simultaneously with ^{14}C-E_1 with microsomes are shown in Table IX. The data indicate that from a microsomal incubation with substrate ^{3}H-E_1 3S, 33-50% of the total radioactivity recovered after DEAE Sephadex chromatography was in the form of 16αOHE_1 3S plus E_3 3S. 16α-hydroxylation of the substrate ^{14}C-E_1 represented an average of 5.6% in the free form. 16β-Hydroxy products from ^{3}H-E_1 3S were detected in trace amounts only in the free fraction, if at all, while from the substrate ^{14}C-E_1, they represented a 42% conversion.

A time study employing the substrates ^{14}C-E_2 and ^{3}H-E_2 3S indicated that these substrates are oxidized

TABLE VIII

Products of Incubation of ^{3}H-$E_1$3S and ^{14}C-E_2[d] with Female Guinea Pig Liver Microsomes (% of Recovered Isotopes).

Products		Substrate	
		^{3}H-$E_1$3S	^{14}C-E_2
Unconjugated:	E_1	12.4[a]	9.7
	16αOHE_1		5.0
	16βOHE_1		5.4
	E_2		2.2
	16epiE_3		14.7
Glucuronides:[b]	E_1	5.4	25.5
	E_2	2.4	9.6
3-Sulfates:	E_1	19.1	9.5[c]
	16αOHE_1	22.6	
	E_3	23.2	
	E_2	4.2	

[a] Not further resolved.

[b] Only minor amounts of other glucuronides detected.

[c] Not further resolved; formation may represent contamination by high speed supernatant.

[d] Substrate concentrations E_2 - 480nM; $E_1$3S - 570 nM.

TABLE IX

Products of Incubation of ^{3}H-$E_1$3S and ^{14}C-E_1[e] with Female Guinea Pig Liver Microsomes (% of Recovered Isotope).

Products		Substrate ^{3}H-$E_1$3S		Substrate ^{14}C-E_1	
		Average[a]	Range	Average[a]	Range
Unconjugated:	E_1	6.7[b]	4.1-8.3	10.6	8.8-13.2
	16αOHE_1			2.2[c]	-
	16βOHE_1			6.4[c]	-
	E_2			3.9	2.4- 5.6
	16epiE_3			35.2	33.4-36.9
	E_3			3.4	3.0- 3.9
Glucuronides (total)[d]		1.1	0.9-1.3	12.8	11.8-13.7
3-Sulfates:	E_1	26.9	18.8-30.8	7.6[b]	6.3- 9.7
	16αOHE_1	22.9	18.2-26.5		
	E_3	19.7	14.0-23.9		
	E_2	17.3	8.6-23.2		

[a] Average of 6 incubations with tissue from 3 animals.

[b] Not further resolved.

[c] Determined on a pool from the 6 incubations (range not appropriate).

[d] Major glucuronides were of estradiol-17β and estrone.

[e] Substrate concentration E_1 - 480 nM; $E_1$3S - 570 nM.

readily to ^{14}C-E_1 and ^{3}H-$E_1$3S respectively and do not form distinct hydroxylated products from those obtained by incubating ^{14}C-E_1 and ^{3}H-$E_1$3S. For example, Figure 11 shows the conversion of ^{3}H-$E_2$3S to other monosulfates with increasing incubation time. These monosulfates were shown to be composed of $E_1$3S, $E_3$3S and 16αOH$E_1$3S.

Experiments employing simultaneous incubations of ^{3}H-$E_1$3S plus ^{14}C-$E_1$3S or ^{3}H-E_1 plus ^{14}C-E_1 revealed the absence of any isotope effect upon the hydroxylations.

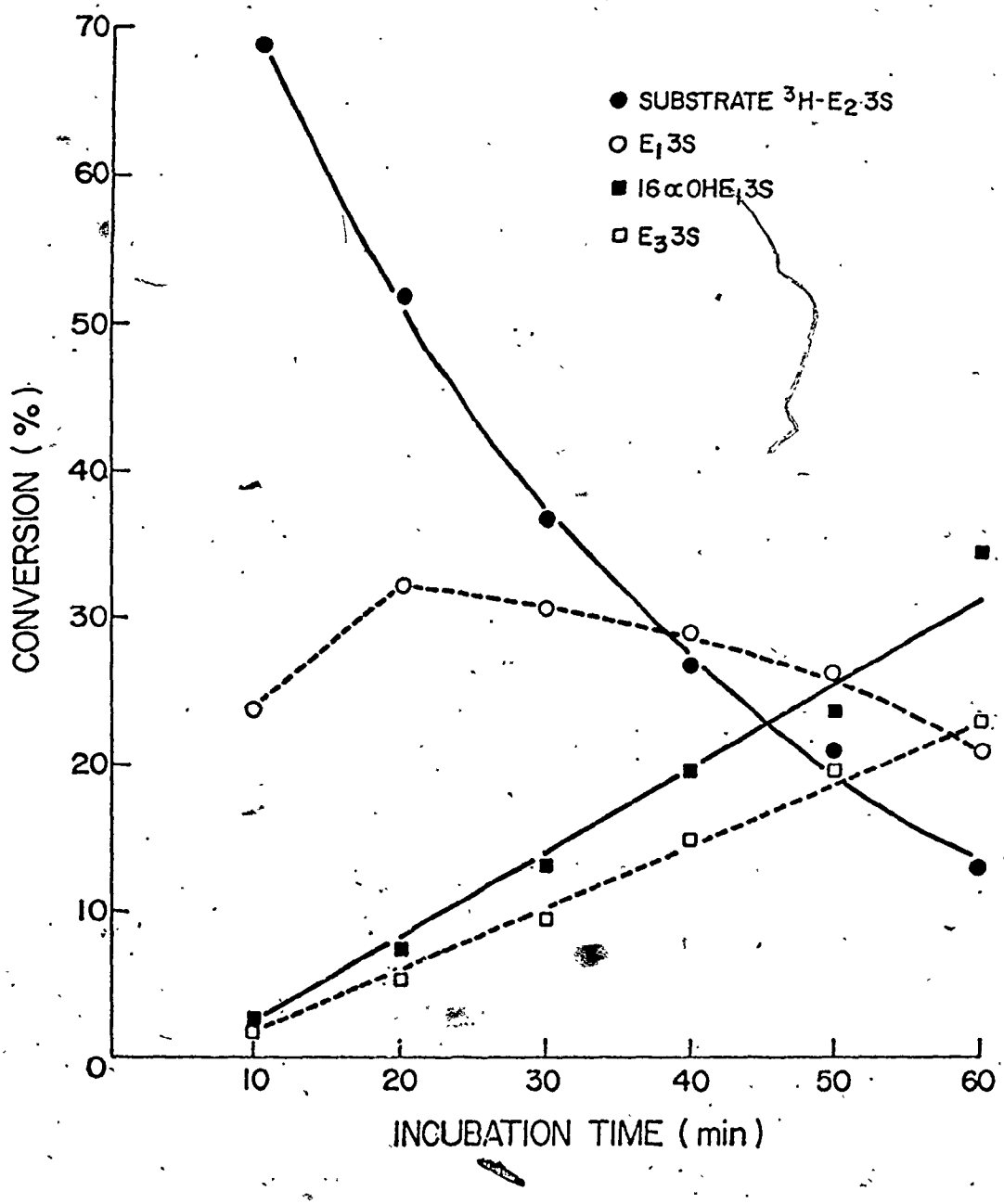

Figure 11. Conversion of ^{3}H-E$_2$3S to other monosulfates with increasing incubation time at 37°C, pH 7.4.

5.5 SIMULTANEOUS INCUBATIONS OF MICROSOMES AND HIGH SPEED SUPERNATANT

Incubation of high speed supernatant (105,000 xg) plus microsomes dramatically changed the DEAE Sephadex chromatographic pattern shown in Figure 8 to that shown in Figure 12. Addition of the 105,000 xg supernatant to the microsomes followed by incubation with ^{14}C-E_1 or ^{14}C-E_2 increased ^{14}C recovery to 90-100% following filtration on Whatman No. 40 paper compared to the recovery of 60-70% from incubations with microsomes alone. Recovery of ^{3}H from the substrate ^{3}H-$E_1$3S or ^{3}H-$E_2$3S was similar (90-100%) from both microsomal and microsomal plus 105,000 xg supernatant incubations.

Table X summarizes the results obtained for microsomal plus high speed supernatant incubations. Sulfurylation of ^{14}C-E_2 and its metabolites occurred and a large amount of ^{3}H and ^{14}C-labeled disulfate (the radioactive material eluted over 1.25-1.75M NaCl) was produced.

Metabolites of glucuronides and monosulfates were identified as above [see microsomal incubations section 5.4] except extensive crystallizations were not performed. The identity of the disulfates was established by solvolysis followed by Girard separation and Sephadex LH_{20} chromatography of the ketonic and nonketonic fractions. Meta-

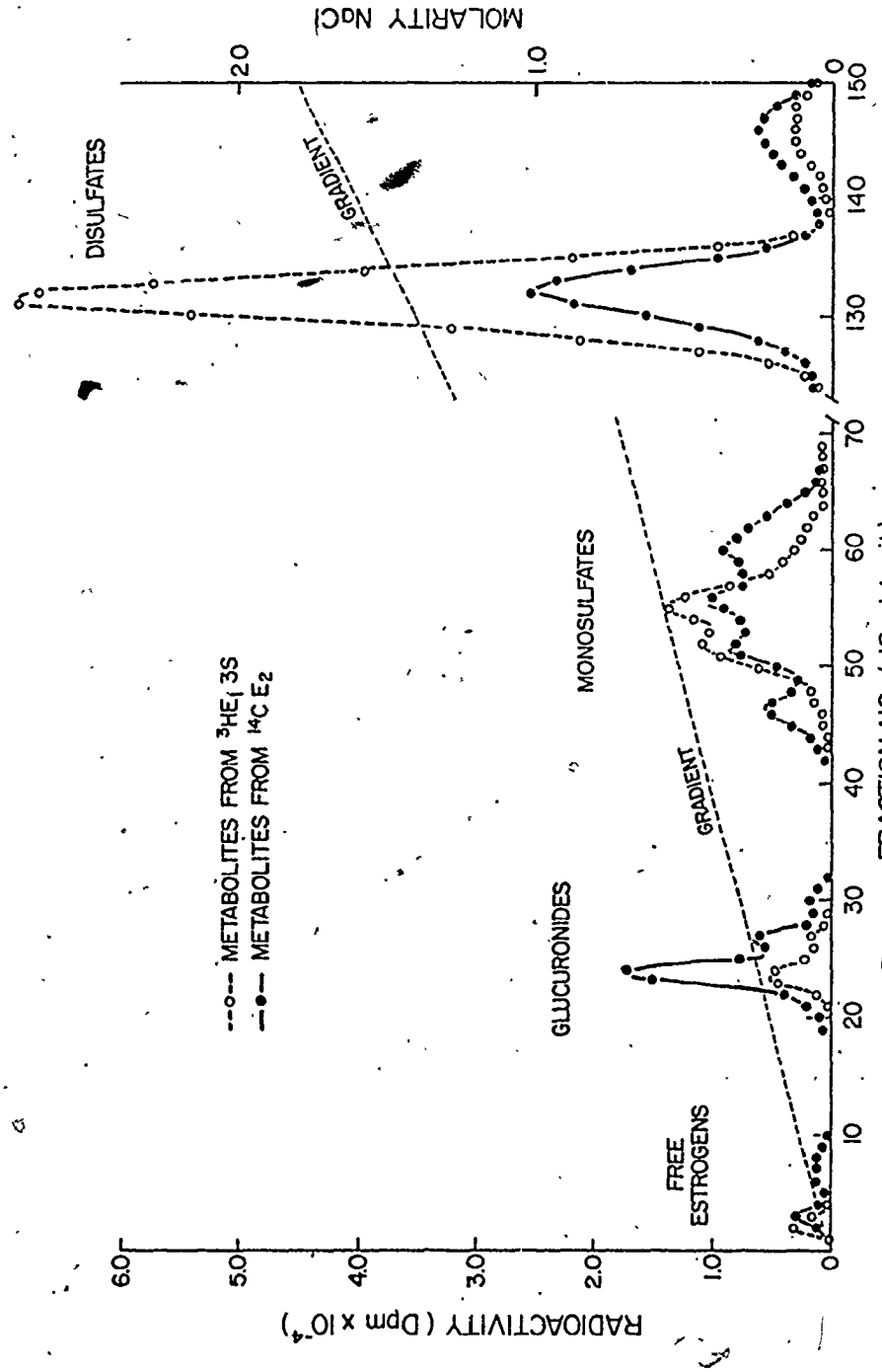

Figure 12. DEAE Sephadex A_{25} chromatograph (60 x 0.9 cm, 0-0.8M followed by 0.8-2.0M NaCl) of metabolites of ^{3}H-$E_1$3S and ^{14}C-E_2 from a microsomal plus high speed supernatant incubation of 120 min. at 37°C, pH 7.4.

TABLE X

Metabolic Pattern of Metabolism of ^{3}H-$E_1$3S and ^{14}C-E_2[c] by Female Guinea Pig Liver Microsomes Plus 105,000 xg Supernatant (% of Total Radioactivity Recovered from A_{25} Chromatography.

Metabolites	Substrate			
	^{3}H-$E_1$3S		^{14}C-E_2	
	Pregnant	Non Pregnant	Pregnant	Non Pregnant
Free Estrogens	1.9[a]	1.6[b] (tr-2.8)	2.3[a]	1.3[b] (1.1-2.2)
Glucuronides				
E_1	2.4	6.2 (1.6-8.9)	13.0	31.0 (18.3-39.2)
E_2	0.6	1.2 (tr-1.8)	2.9	4.1 (1.3-5.6)
Monosulfates				
E_1	10.7	30.6 (18.7-37.1)	9.6	21.1 (14.2-28.3)
16αOHE_1	3.0	1.0 (tr-1.6)	3.5	0.9 (tr-1.7)
16epiE_3	2.0	0.5 (0.2-1.0)	11.5	2.1 (0.4-3.1)
E_3	-	4.0 (0.6-6.9)	-	2.4 (0.6-3.9)
E_2	-	4.3 (2.7-5.6)	-	2.7 (2.2-3.3)
Disulfates				
16αOHE_1	3.7	3.5 (0.7-9.1)	2.0	2.6 (0.3-7.2)
16epiE_3	5.5	0.7 (tr-1.1)	3.1	0.4 (tr-0.7)
E_3	55.4	31.0 (23.5-35.7)	26.9	18.2 (10.7-25.0)
E_2	4.1	5.6 (4.1-7.5)	10.2	5.9 (4.1-9.9)

[a] Mean value of 2 incubations, data corresponds to chromatogram shown in Fig. 12.

[b] Mean value of 6 incubations, 3 guinea pigs, ranges in parentheses; tr, trace.

[c] Substrate concentration E_2 - 480 nM; $E_1$3S - 570 nM.

bolites could be adequately identified by their mobility on LH_{20} chromatographic columns.

Monoglucuronides were identified as $E_1$3G and $E_2$3G. More glucuronide was produced from ^{14}C-E_2 than from ^{3}H-$E_1$3S. The monosulfate region contained both ^{3}H and ^{14}C-labeled $E_1$3S, 16αOH$E_1$3S, 16epi$E_3$3S, $E_3$3S and $E_2$3S. The disulfate was shown to be composed mainly of $E_3$3,16diS and tentatively identified 16-epiestriol disulfate. A second disulfate peak eluted at 1.5M-1.75M NaCl was $E_2$3,17diS. The unconjugated radioactivity remaining after an incubation of microsomes plus supernatant was small in amount.

(a) FACTORS INFLUENCING DISULFATE FORMATION FROM MICROSOMAL PLUS 105,000 xg SUPERNATANT INCUBATIONS

It was planned to employ disulfate formation as an indication of hydroxylation of $E_1$3S and therefore cofactor requirements, as well as other parameters of disulfate formation, were investigated. Figure 13 shows the effect of NADPH, ATP and $MgSO_4$ concentration on disulfate formation (excluding $E_2$3,17diS) from the substrate $E_1$3S. Disulfate formation with increasing incubation time is also shown.

This method of investigation of hydroxylation seemed

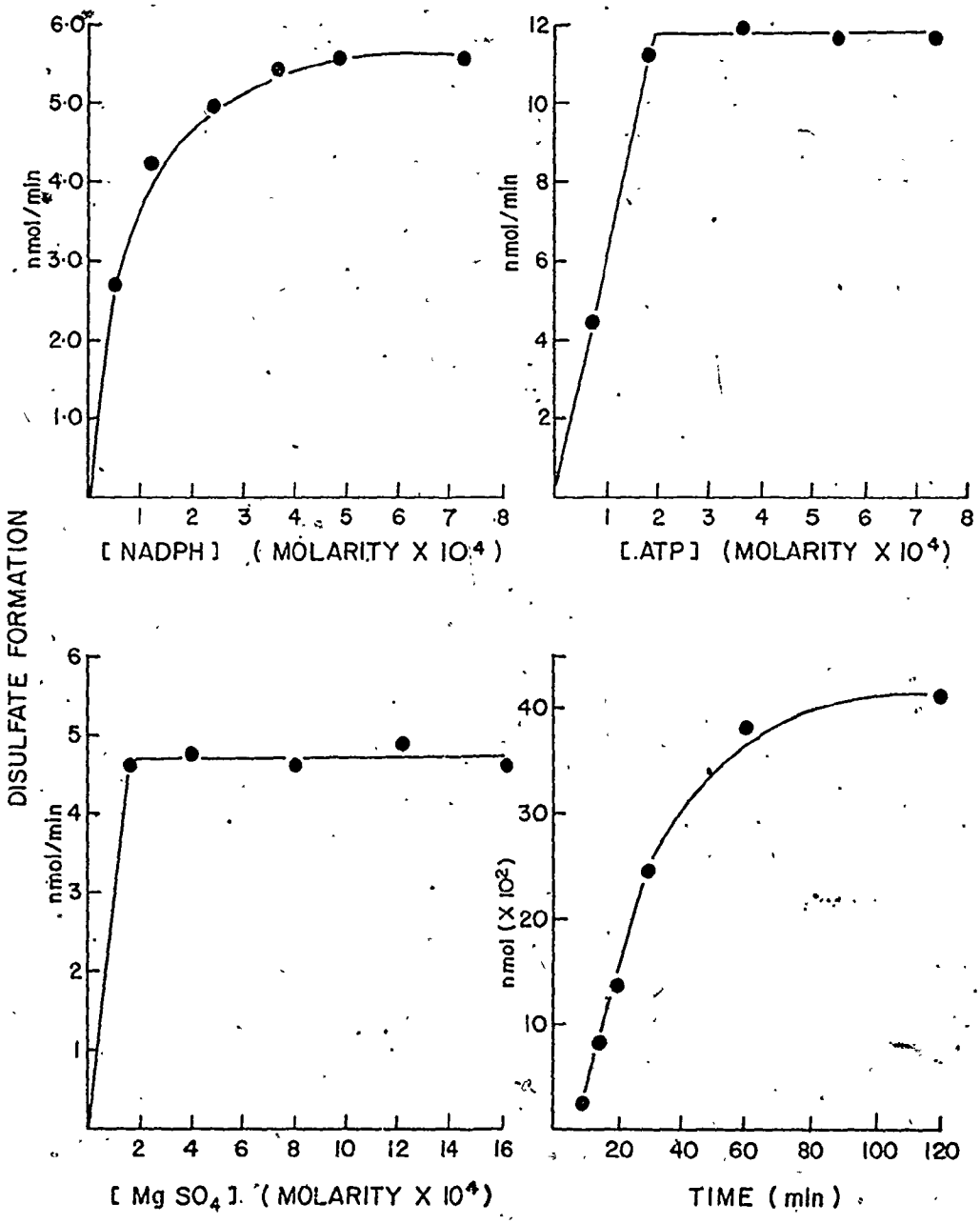

Figure 13. The effect of NADPH, ATP, $MgSO_4$ concentrations (incubation of 120 min. at 37°C, pH 7.4) and time on disulfate formation from ^3H-$E_1$3S (nmol concentration) incubated with microsomes plus high speed supernatant.

to be inadequate [see discussion section 6.3]. Microsomal plus high speed supernatant incubations were therefore abandoned. A more favorable approach to study hydroxylation, particularly 16α-hydroxylation, was employed as described in the next section.

5.6 16α-HYDROXYLATION OF ESTRONE SULFATE BY LIVER MICROSOMES

Results from a microsomal incubation without the high speed supernatant employing $E_1$3S as substrate indicated that two major pathways of metabolism of $E_1$3S exist. These were reduction of $E_1$3S to $E_2$3S [described in next section 5.7] and hydroxylation at C-16 in the α-configuration. The 16α-hydroxylation of $E_1$3S by guinea pig liver microsomes offered a useful model to study this enzymatic activity apparently under circumstances uncomplicated by the presence of additional hydroxylases which act upon the substrate. Following a microsomal incubation, the monosulfates were investigated for the presence of 16αOH$E_1$3S and $E_3$3S. Formation of 16αOH$E_1$3S plus $E_3$3S gave the total 16α-hydroxylation of $E_1$3S. Employing this method of investigation of 16α-hydroxylation, properties of this enzymatic system or optimum conditions for 16α-hydroxylated product formation were established.

(a) EFFECT OF MICROSOMAL PROTEIN CONCENTRATION

Figure 14 shows that 16α-hydroxyproduct formation was linear in the presence of up to 10 mg of microsomal protein. Preliminary conditions of incubation time 30 min, NADPH weight of 2.4 μmol and a $E_1$3S substrate weight of

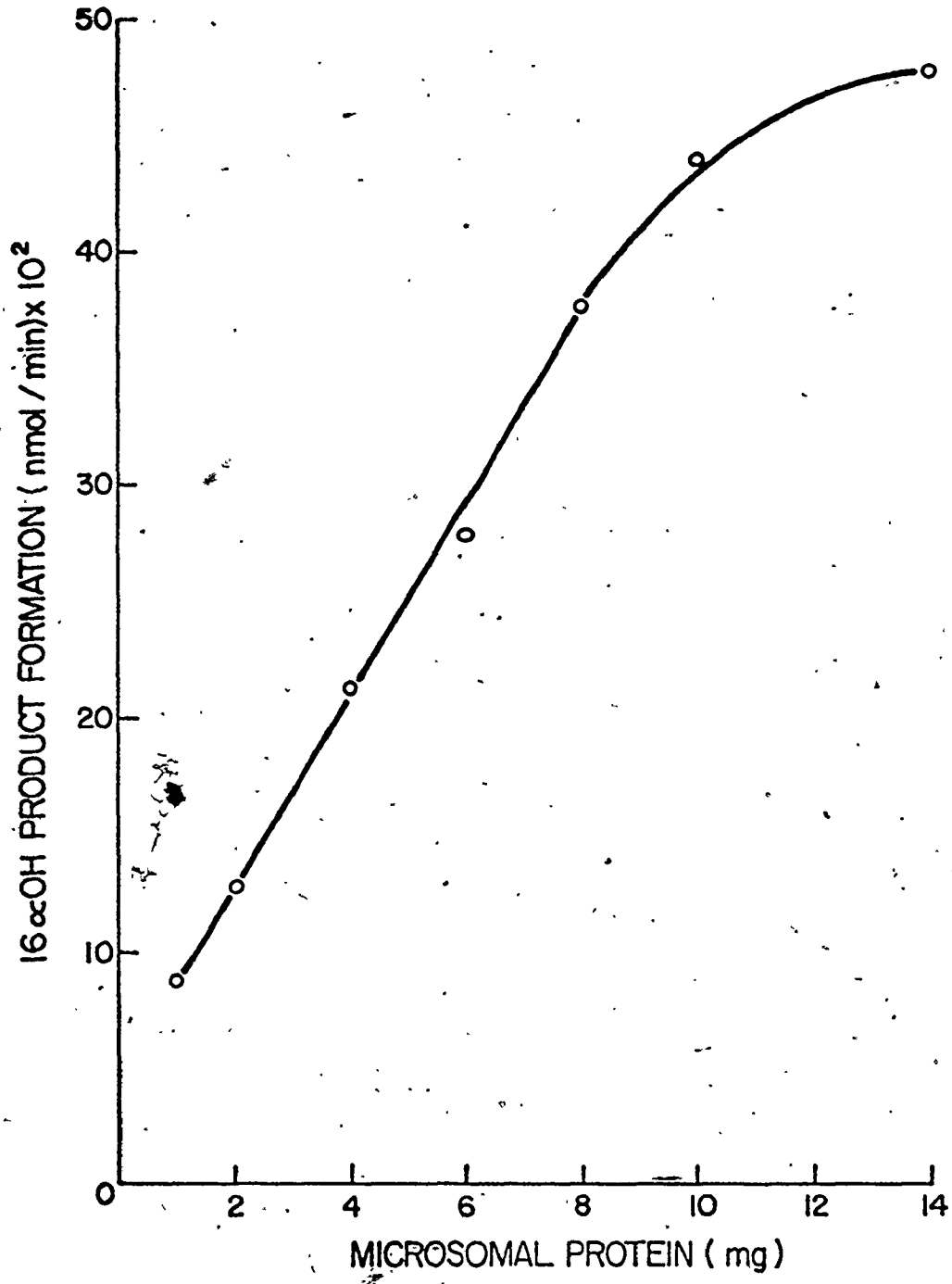

Figure 14. Effect of protein concentration on the formation of 16α-hydroxylated products (i.e. 16αOH$E_1$3S plus $E_3$3S) from $E_1$3S. Incubations were performed at 37°C for 30 min., pH 7.5. $E_1$3S was 85.5 nmol and NADPH 2.4 μmol per 5.0 ml of incubation mixture.

30 ug (85.5 nmol) were employed in this experiment. From these results, a microsomal protein concentration of 6-8 mg protein per incubation was chosen for future experiments.

(b) THE EFFECT OF INCUBATION TIME

The effect of incubation time on 16α-hydroxylation of E_1 3S was determined in a preliminary experiment employing the initial conditions of substrate, 2.85 nmol and NADPH, 2.4 μmol. Once standard conditions were established for E_1 3S concentration; 2.85×10^{-5} M or 142.5 nmol/incubation of 5.0 ml [see section 5.6 (e)] and NADPH concentration 8.4×10^{-4} M or 4.2 μmol/incubation of 5.0 ml [see section 5.6 (c)], this experiment was repeated in duplicate. Data obtained from these latter two experiments which were performed on the same guinea pig are given in Table XI. The average results obtained from these experiments are shown in Figure 15. 16α-Hydroxyproduct formation was linear up to 60 min. This result was similar to that obtained employing the initial conditions of lower substrate and NADPH concentrations. A standard incubation time of 30-35 min was employed in later experiments.

TABLE XI

The Effect of Incubation Time on 16α-Hydroxylation of $E_1$3S by Guinea Pig Liver Microsomes. ($E_1$3S - 142.5 nmol; NADPH - 4.2 µmol per 5.0 ml of Incubation Mixture).

Time (min)	Experiment 1		Experiment 2		Average[c]
	% of $A_{2.5}$[a]	(nmol·mg protein^{-1})[b]x10	% of $A_{2.5}$	(nmol·mg protein^{-1})[b]x10	(nmol·mg protein^{-1})x10
10	6.3	11.9	6.7	12.7	12.3
20	9.3	17.6	11.7	22.2	19.9
30	13.5	25.6	14.4	27.3	26.4
40	18.8	35.7	16.2	30.7	33.2
50	21.7	41.2	23.3	44.2	42.7
60	23.8	45.2	26.5	50.4	47.8

[a] Percent 16αOH products. (16αOH$E_1$3S plus $E_3$3S) of total radioactivity recovered from DEAE Sephadex $A_{2.5}$ chromatography.

[b] nmol of 16αOH products formed per mg protein.

[c] Average results from Experiment 1 and Experiment 2 nmol of 16αOH products formed per mg protein.

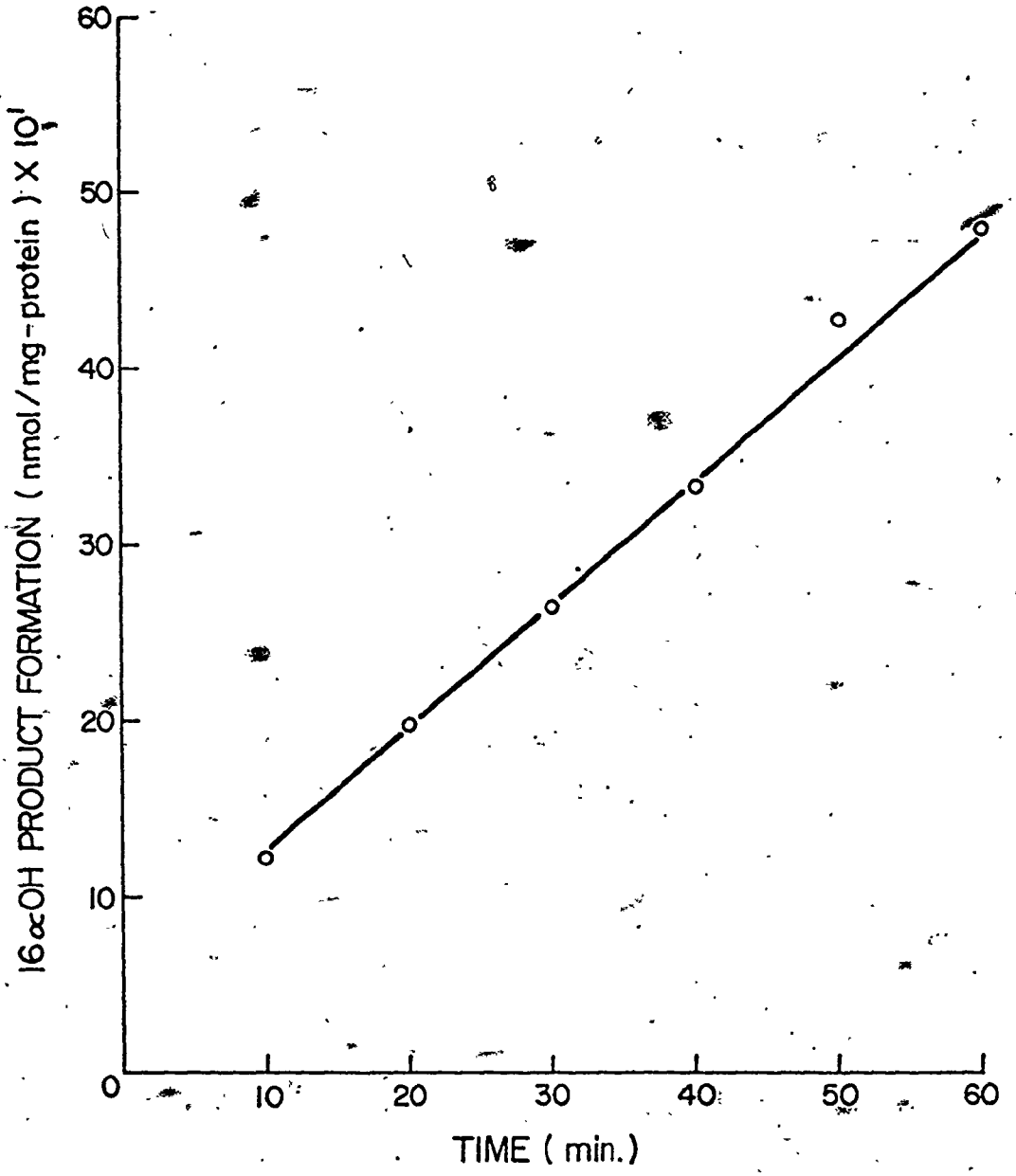

Figure 15. Effect of incubation time on 16α-hydroxylation of $E_1$3S by guinea pig liver microsomes (7.5 mg of microsomal protein) incubated at 37°C, pH 7.5. $E_1$3S was 142.5 nmol and NADPH 4.2 μmol per 5.0 ml of incubation mixture.

(c) COFACTOR REQUIREMENT FOR 16α-HYDROXYLATION

Addition of Mg^{+2} as $MgCl_2$ salt (in excess of that already present in buffer) had no effect on hydroxylase activity (Figure 16A). Studies with increasing concentrations of EDTA in the incubating medium indicated that no metal cofactor was required for 16α-hydroxylation (Figure 16B).

Data indicating the cofactor requirement of NADPH for 16α-hydroxylase are given in Table XII. In the initial study (experiment 1) the substrate weight employed was only 1 µg (2.85 nmol). Before proceeding to study the effect of substrate concentration on 16α-hydroxylation, the experiment was repeated (Table XII - Exp 2) employing a concentration of E_1S 100 times that used above (5.7×10^{-5}M or 285 nmol per 5.0 ml of incubation mixture). An increased substrate concentration was desired so that it would not be limiting in determining the cofactor requirement for NADPH. A Michaelis-Menten plot (rate of 16-OH product formation vs [NADPH]) from experiment 2 is shown in Figure 17. The Michaelis-Menten constant (Km) for the cofactor NADPH estimated from ½ Vmax of this plot is 2.28×10^{-4}M (1.14 µmol or 0.95 mg per 5.0 ml incubation mixture). A Lineweaver-Burk plot (Fig. 18) of these results gives a Km value of 4.18×10^{-4}M. To ensure that

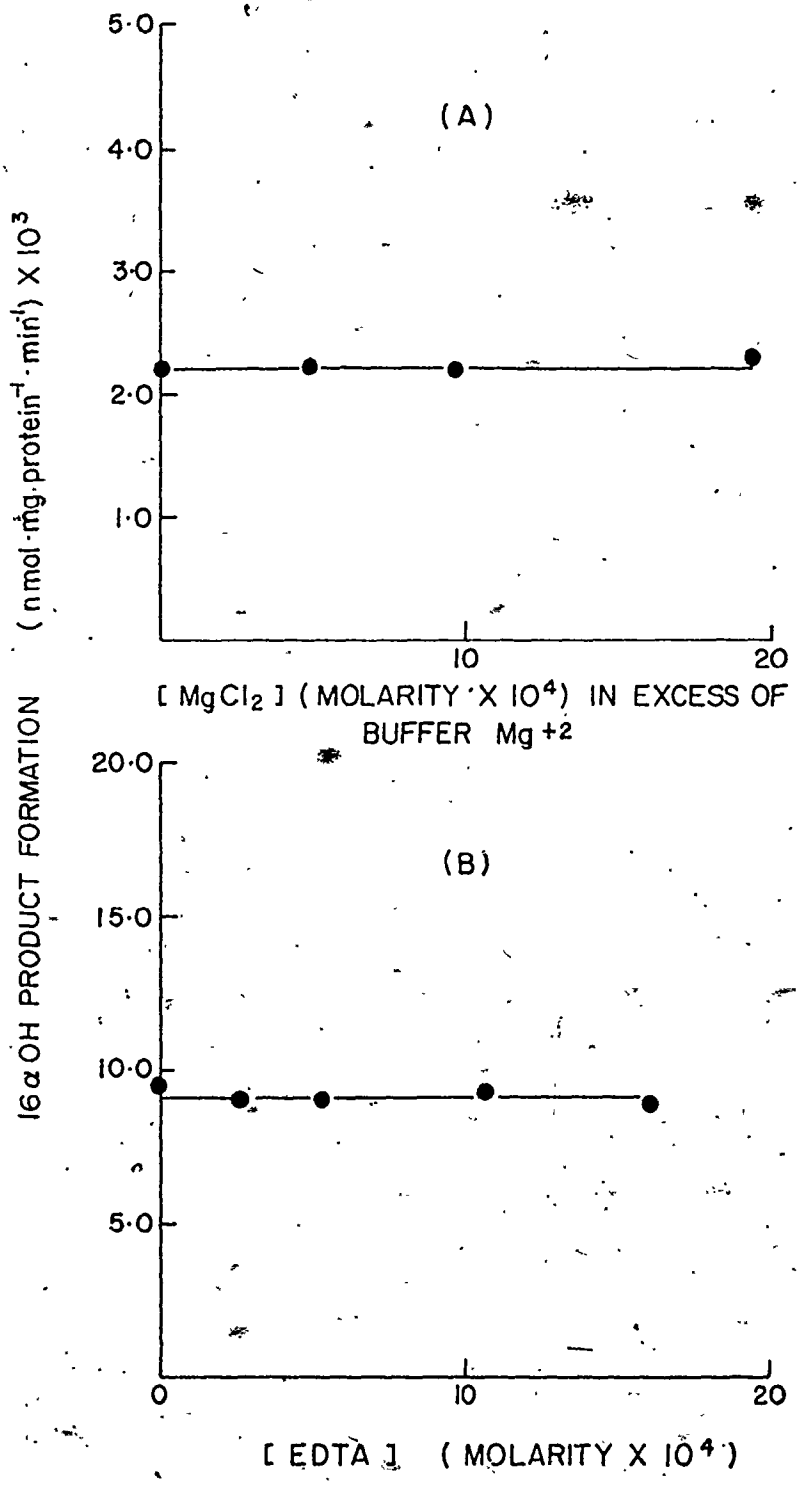

Figure 16. Cofactor requirement for 16α-hydroxylation. (A) Mg^{+2} concentration in excess of that present in buffer, (B) Effect of increasing concentration of EDTA. Estrone sulfate was 2.85 nmol and NADPH 2.4 μmol per 5.0 ml of incubation mixture for both experiments. Incubations were performed at 37°C for 30 min. pH 7.5 in the presence of 7.0 mg of microsomal protein.

TABLE XII

NADPH Requirement for 16α-Hydroxylation of $E_1$3S

[NADPH] Molarity (X 10^4)	[NADPH] 1/[NADPH] ($\times 10^{-2}$)	Experiment 1 ($E_1$3S - 2.85 nmol) % of A_{25} [a]	Experiment 1 Rate v (nmol·mg protein^{-1}·min^{-1}) [b] x 10^3	Experiment 2 ($E_1$3S - 285 nmol) % of A_{25} [a]	Experiment 2 Rate v (nmol·mg protein^{-1}·min^{-1}) [b] x 10^2	1/v
0	-	-	-	-	-	-
0.48	-	4.51	0.61	2.24	2.28	-
1.20	-	9.83	1.33	7.91	8.05	-
2.4	41.7	12.13	1.65	14.52	14.78	6.77
3.6	-	15.62	2.12	n.d.	n.d.	n.d.
4.8	20.8	16.52	2.24	21.35	21.73	4.60
7.2	13.9	14.93	2.03	27.27	27.77	3.60
9.6	10.4	n.d. [c]	n.d.	26.04	26.50	3.77

[a] Percent 16αOH products (16αOH$E_1$3S plus $E_3$3S) of total radioactivity recovered from DEAE Sephadex A_{25} chromatography minus blank [NADPH]-0 (respectively: 0.53% and 1.14% for $E_1$3S 2.85 nmol and 285 nmol).

[b] nmol of 16αOH products formed per mg protein·min or rate of reaction 'v'.

[c] not determined.

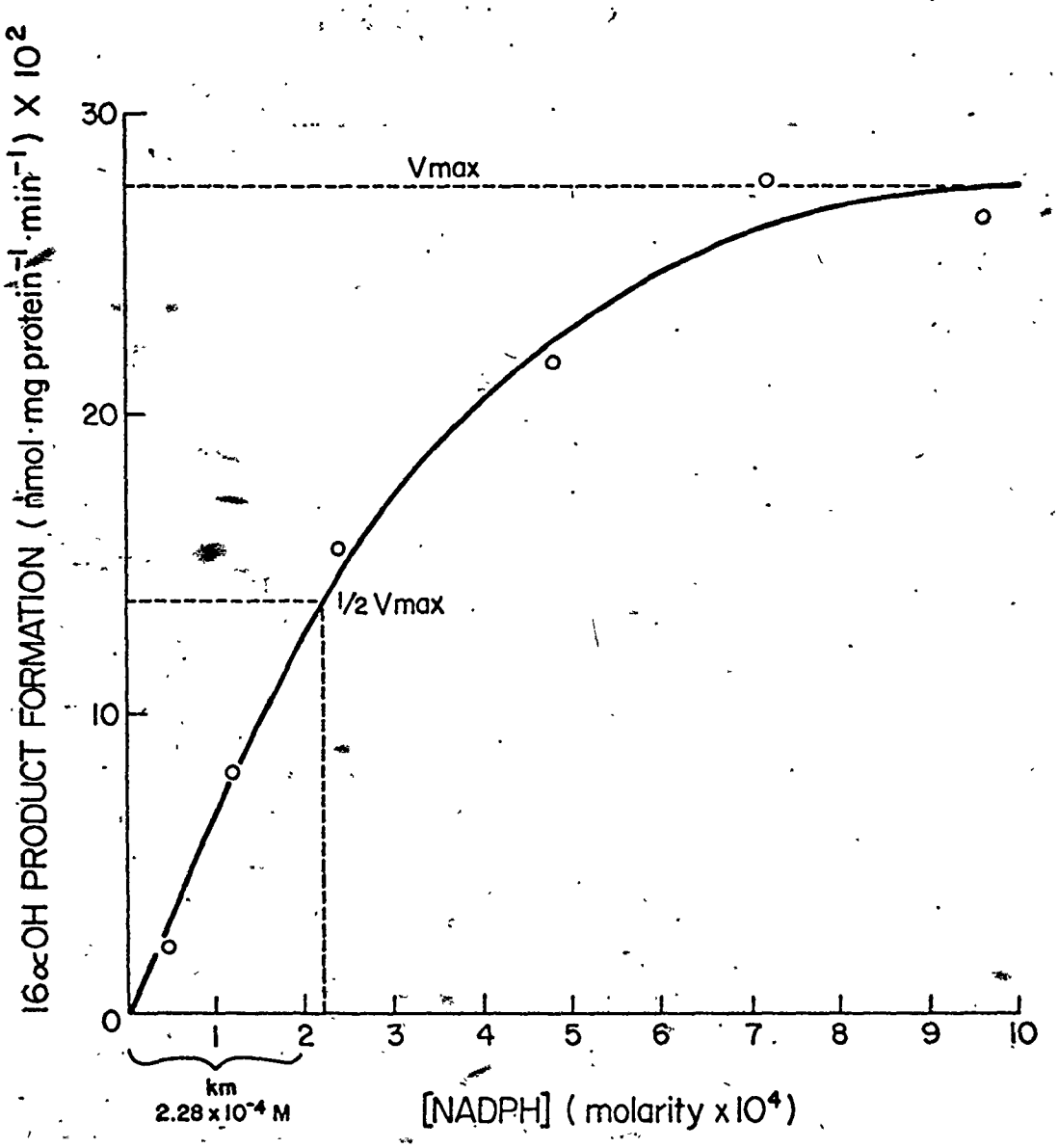

Figure 17. Effect of cofactor NADPH concentration on the rate of formation of 16α-hydroxylated products from $E_1$3S (Michaelis-Menten plot). Incubations were performed for 30 min. at 37°C, pH 7.5, in the presence of 8.0 mg of microsomal protein. $E_1$3S was 285 nmol per 5.0 ml of incubation mixture.

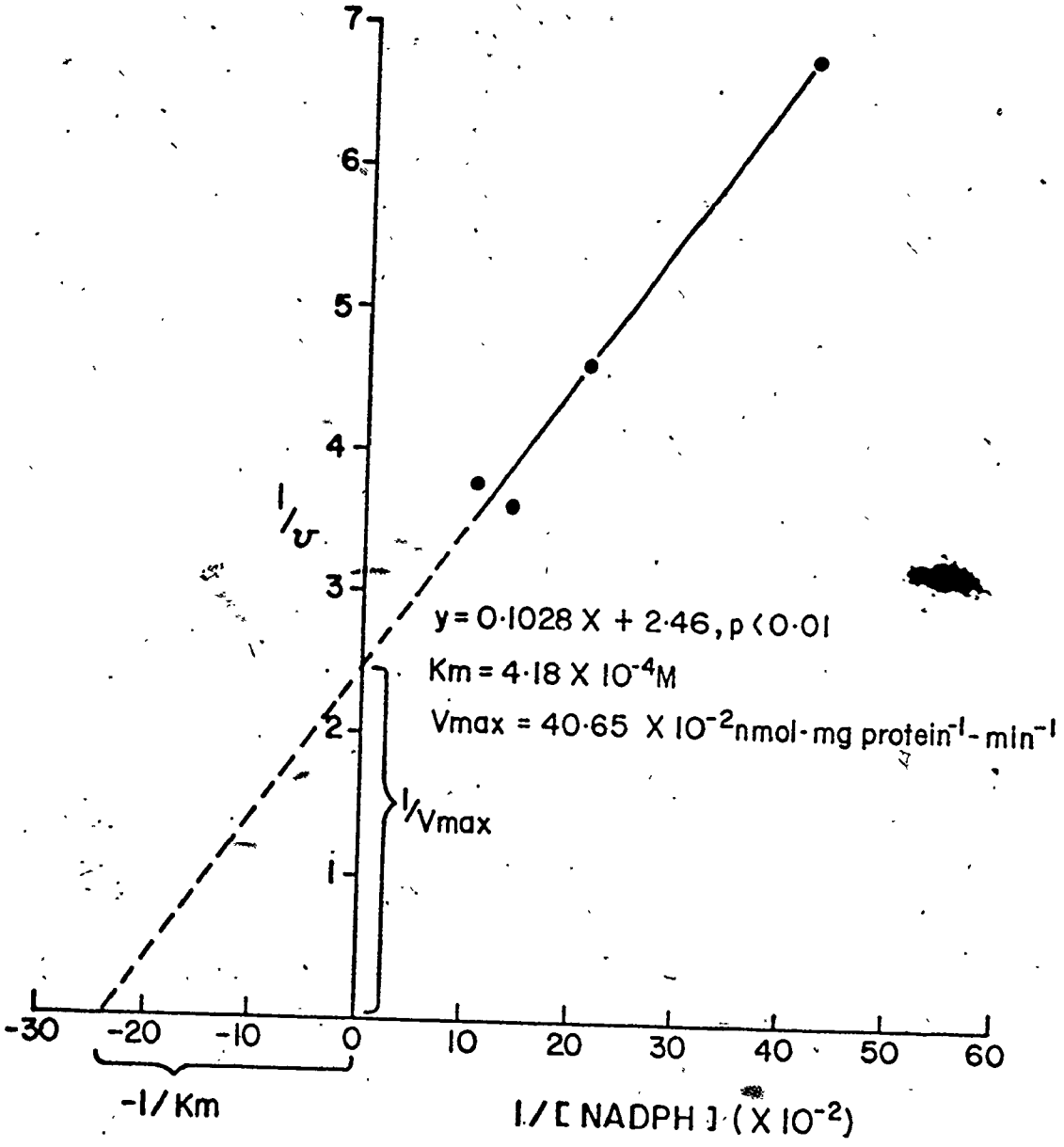

Figure 18. Effect of cofactor NADPH concentration on the rate of formation of 16α-hydroxylated products from $E_1$3S (Lineweaver-Burk plot). Experimental conditions as described in Figure 17.

the cofactor was not limiting, a NADPH concentration of 8.4×10^{-4}M or 4.2 µmol/incubation was employed for most of the remaining studies.

Figure 19 shows the effect of increasing concentration of the oxidized form of the cofactor (i.e. $NADP^+$) on 16α-hydroxylation of $E_1$3S. This experiment was performed following the kinetic studies [section 5.6 (e)] so that the substrate concentration employed was equal to the Km value for $E_1$3S. Addition of $NADP^+$ to a microsomal incubation to give a concentration twice that of the NADPH concentration inhibited 16α-hydroxylase activity to the extent of only 10%.

(d) THE pH OPTIMUM FOR 16α-HYDROXYLATION OF ESTRONE SULFATE

The pH optimum for 16α-hydroxylation was shown to be about 7.5 (Figures 20 and 21). Increasing the substrate weight from 2.85 nmol (Fig. 20) to 142.5 nmol, the Km value for $E_1$3S (Fig. 21), had no effect on this value. The results of the latter study (Fig. 21) are the average of two experiments performed on liver microsomes obtained from the same guinea pig.

(e) KINETIC STUDIES WITH SUBSTRATE ESTRONE SULFATE

Data obtained from three experiments with microsomes

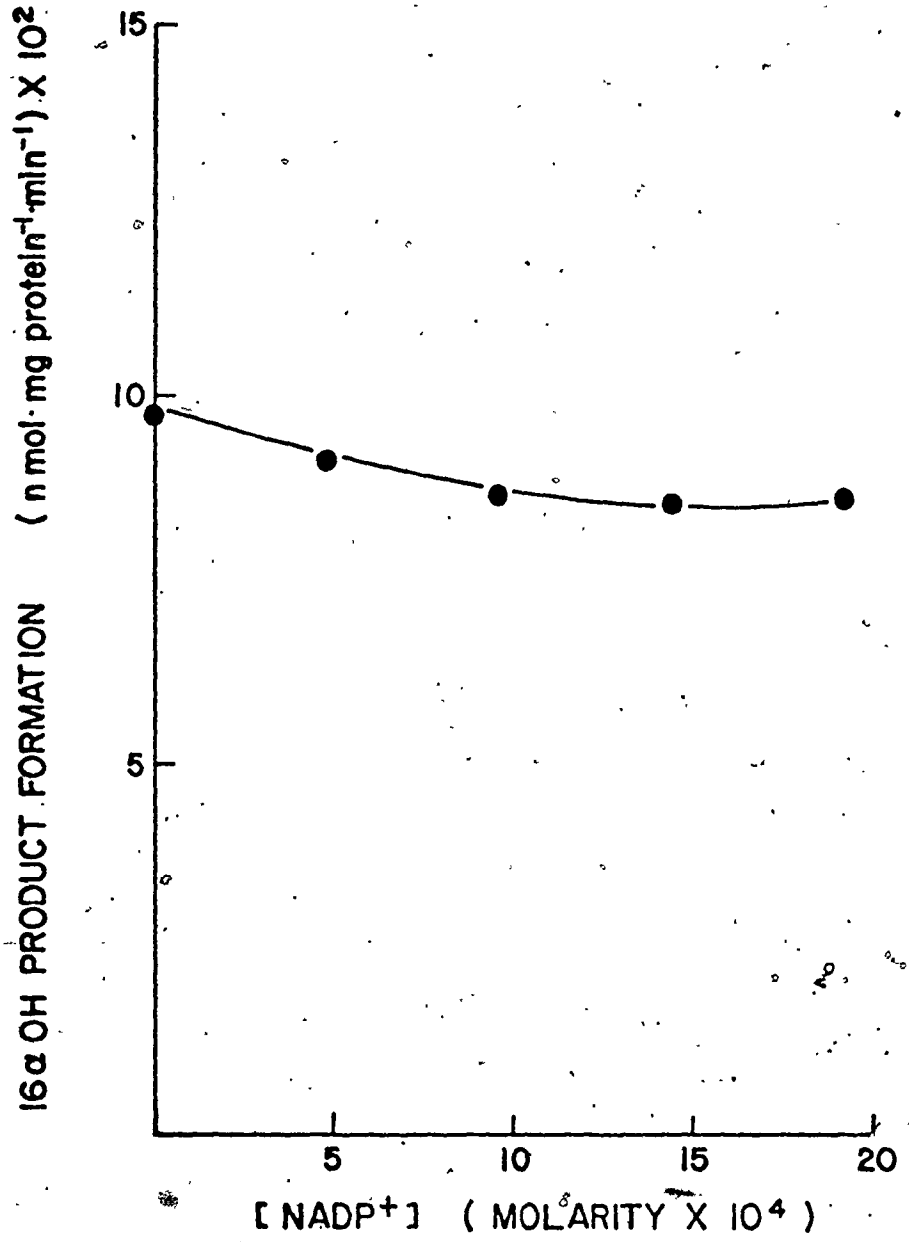

Figure 19. Effect of oxidized form of cofactor NADPH ($NADP^+$) on 16α-hydroxylation of $E_1$3S. Incubations were performed at 37°C for 35 min., pH 7.5, in the presence of 9.0 mg of microsomal protein. $E_1$3S was 142.5 nmol and NADPH 4.2 µmol per 5.0 ml of incubation mixture.

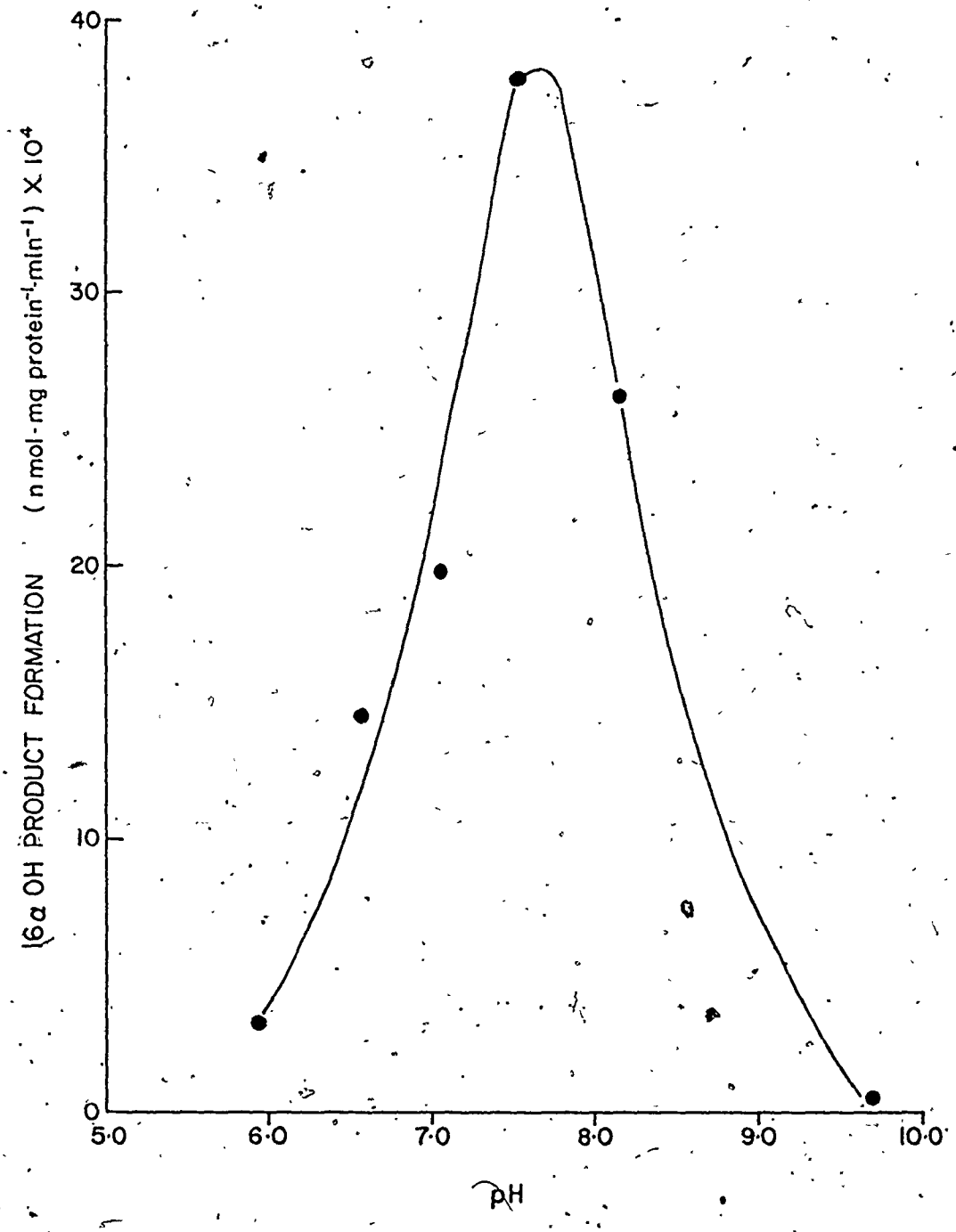

Figure 20. Effect of pH on 16α-hydroxylation of $E_1$3S (2.85 nmol per 5.0 ml of incubation mixture). Incubations were performed for 30 min. at 37°C in the presence of 7.0 mg of microsomal protein. NADPH was 2.4 µmol per 5.0 ml of incubation mixture.

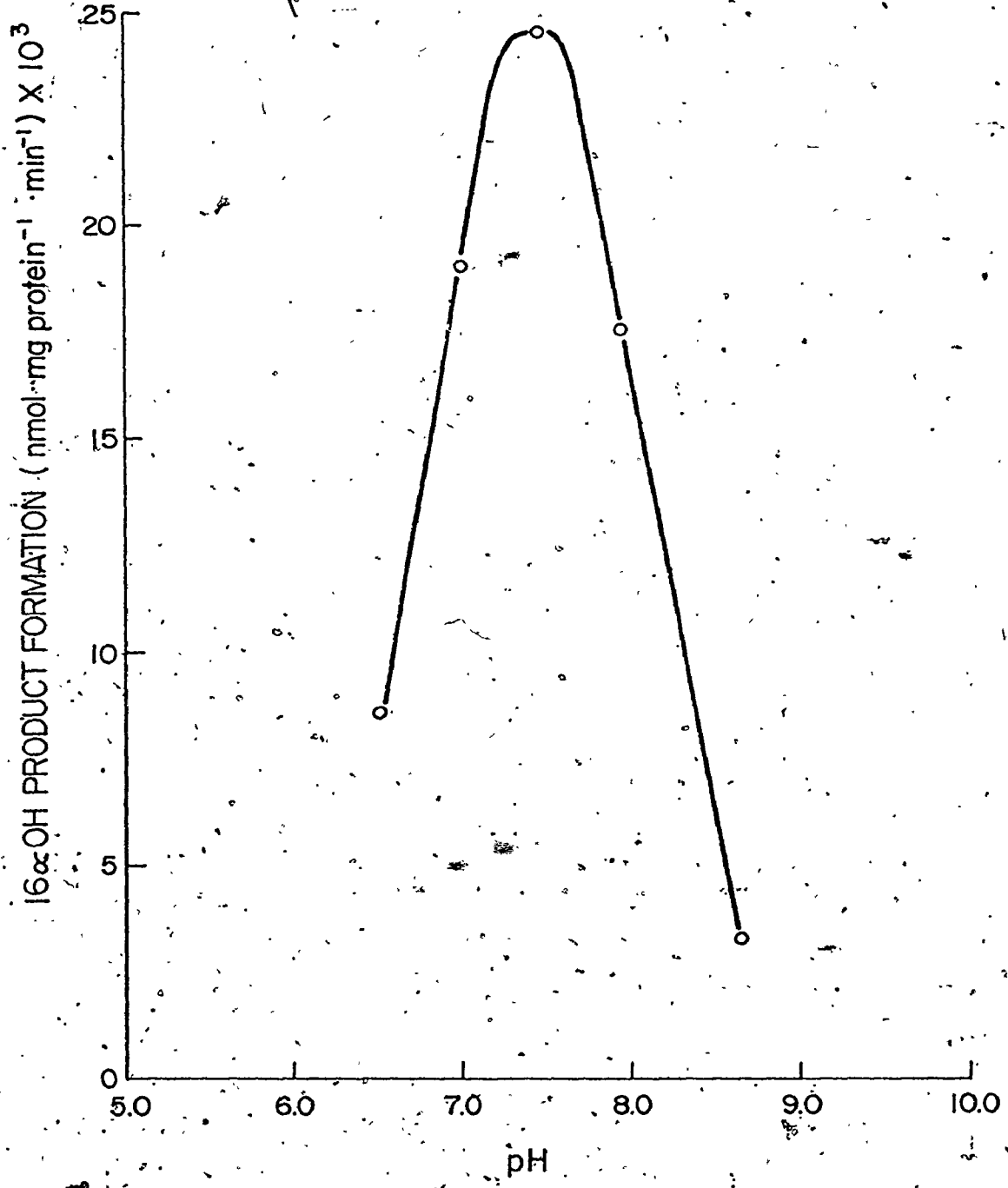

Figure 21. Effect of pH on 16α-hydroxylation with increased substrate weight of E$_1$3S (142.5 nmol per 5.0 ml of incubation mixture). Incubations were performed at 37°C for 30 min. in the presence of 8.3 mg of microsomal protein. NADPH was 4.2 µmol per 5.0 ml of incubation mixture.

from two different guinea pigs showing the effect of $E_1$3S concentration on the rate of the enzymatic 16α-hydroxylation are given in Table XIII. A Michaelis-Menten plot of the average results from these three experiments is shown in Figure 22. The Michaelis-Menten constant, Km, as determined from the substrate concentration at which the velocity or rate of the reaction is half maximal, was 2.85×10^{-5} moles/liter. The maximum velocity, Vmax, from the Michaelis-Menten plot was about 20.5×10^{-2} nmol·mg protein^{-1}·min^{-1}. Values for Km and Vmax, as determined from a Lineweaver-Burk plot (Figure 23), are 3.55×10^{-5} moles/liter and 24.88×10^{-2} nmol·mg protein^{-1}·min^{-1} respectively. For the enzyme inhibition studies [section 5.6 (f)], substrate concentration employed was that of the approximate km value, 2.85×10^{-5} (50 µg or 142.5 nmol/incubation).

(f) INHIBITION STUDIES

The effect of a few synthetic steroids, such as those contained in oral contraceptive preparations, were investigated for their possible inhibition of 16α-hydroxylation of $E_1$3S. The synthetic estrogens tested were ethinyl estradiol and mestranol. The synthetic progestins investigated for possible inhibition were norethynodrel and medroxyprogesterone acetate. Two other steroids,

TABLE XIII

Effect of Substrate ($E_1$3S) Concentration on the Formation of 16α-Hydroxylated Products from Microsomal Incubations (30 min at 37°C).

[$E_1$3S] Molarity (x 10^5)	$1/[E_1 3S]$ (x 10^{-3})	16αOH Product Formation (nmol·mg protein^{-1}·min^{-1})x 10^2 Guinea Pig 1[a] Experiment 1 v	Experiment 2 v	Guinea Pig 2 Experiment 1 v	Average[b] v	Average 1/v
1.14		5.7	5.8	4.5	5.3	
2.28		10.1	9.2	7.2	8.8	
4.56	21.93	15.7	14.4	13.1	14.4	6.94
6.84	14.62	18.0	17.5	13.1	16.2	6.17
9.12	10.96	19.6	20.3	15.6	18.5	5.41
11.40	8.77	20.1	18.4	14.5	17.7	5.66
13.68	7.31	21.1	21.5	16.8	19.8	5.05
17.09	5.85	22.5	21.6	18.4	20.8	4.80

[a] Experiment duplicated (Experiment 1 and 2) with tissue obtained from guinea pig 1

[b] Average rate (v) of 16αOH product formation (nmol·mg protein^{-1}·min^{-1})x 10^2 for the three experiments.

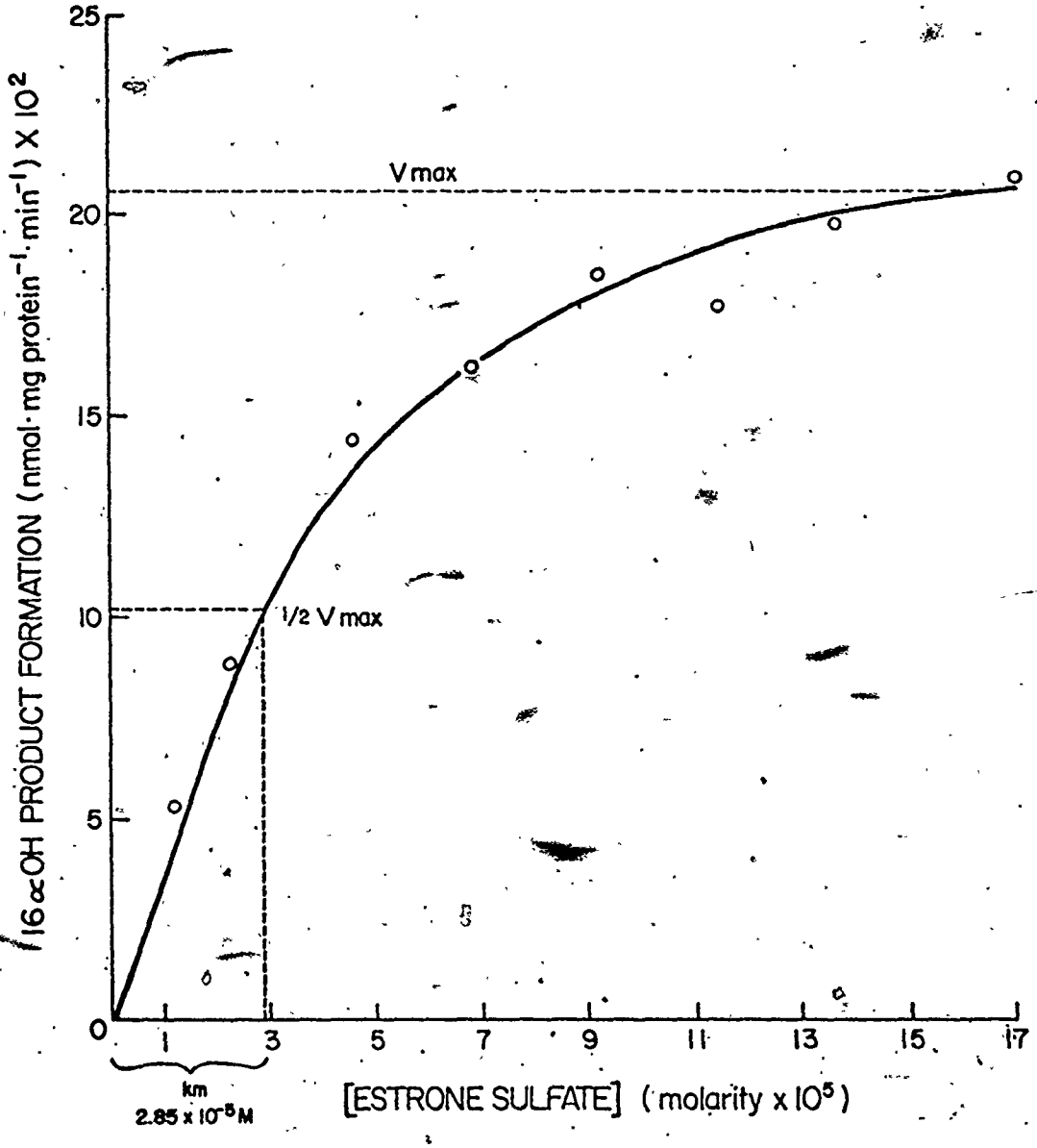

Figure 22. Effect of E_1S concentration on the rate of formation of 16α-hydroxylated products (Michaelis-Menten plot). Incubations were performed at 37°C for 30 min., pH 7.5 in the presence of 7.5 mg of microsomal protein. NADPH was 4.2 μmol per 5.0 ml of incubation mixture.

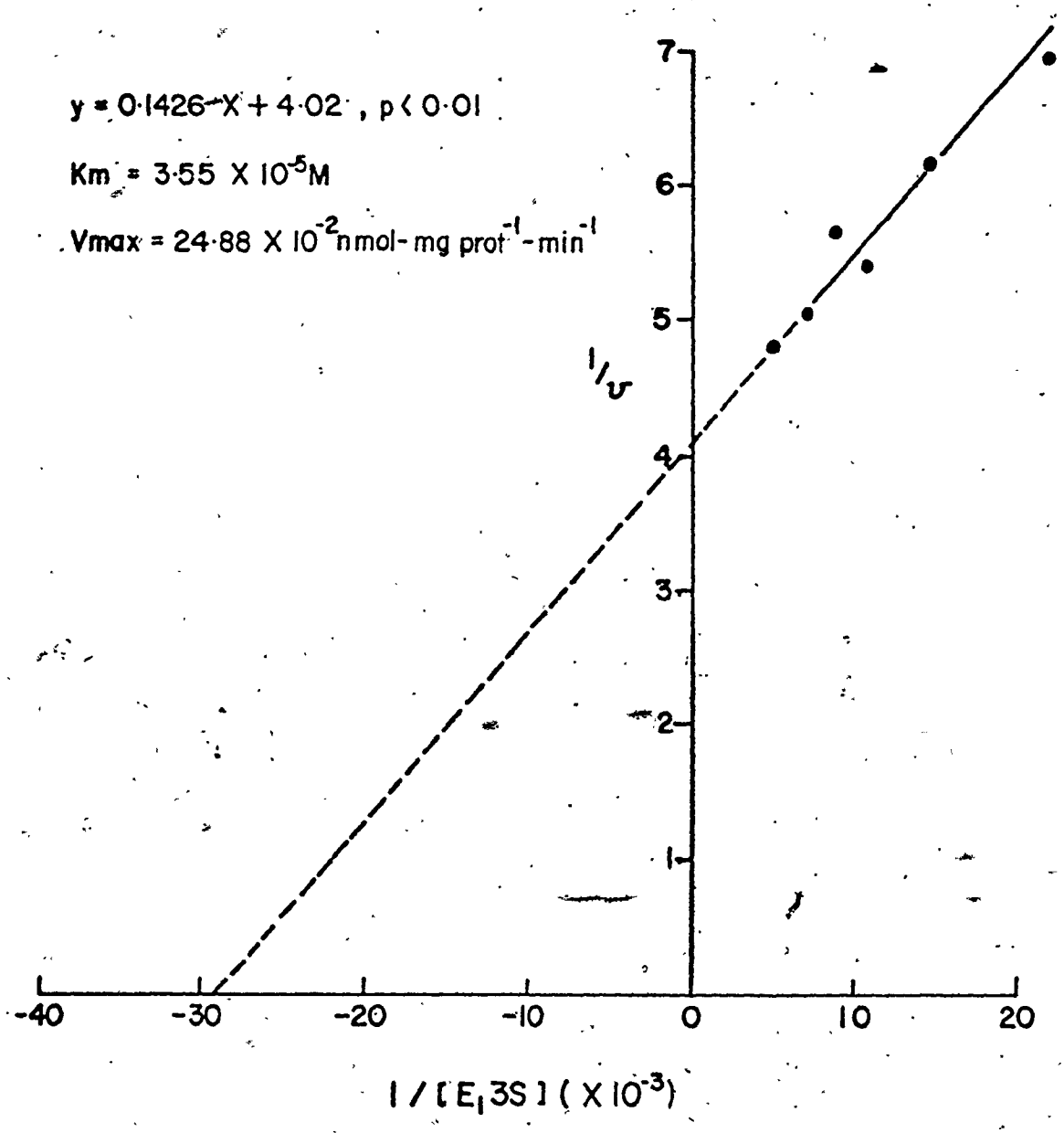

Figure 23. Effect of $E_1$3S concentration on the rate of formation of 16α-hydroxylated products (Lineweaver-Burk plot). Experimental conditions as described in Figure 22.

dehydroepiandrosterone (DHA) and dehydroepiandrosterone sulfate (DHAS), were also studied.

A standard microsomal incubation, as determined from previous results, of: incubation time, 35 min; microsomal protein concentration of 6-8 mg/5 ml of incubation mixture; pH 7.6; substrate concentration, E_13S, of 2.85×10^{-5} M and a cofactor NADPH concentration of 8.4×10^{-4} M, was employed for these inhibition studies. The steroids tested were added to microsomal incubations in increasing concentrations to give a dose-response curve. The weight (μg) of steroid added to a microsomal incubation was plotted against 16α-hydroxyproduct formation (nmol·mg protein^{-1}·min^{-1}).

Although the extent of 16α-hydroxylation varied from one animal to another (Table XIV), similar patterns of inhibition were obtained from experiments performed on microsomes prepared from different animals. For example, the results obtained from the two different guinea pigs for the progestins are shown in Figure 24. Results from duplicated experiments were, therefore, averaged to give a general pattern of inhibition if it existed. Patterns of inhibition for the progestins are shown in Figure 25, for the estrogens in Figure 26 and for the other steroids (DHA and DHAS) in Figure 27.

TABLE XIV

The Effect of Other Steroids on 16α-Hydroxylation of E_1S

Steroid		16α-Hydroxylation Activity ($nmol \cdot mg\ protein^{-1} \cdot min^{-1}$) x 10^2 Amount of Steroid Added (μg)					
		0	25	50	75	100	150
Progestins:							
Norethynodrel	1[a]	20.7	19.3	18.1	15.8	16.0	13.2
	2	11.3	10.7	9.4	8.4	8.5	7.8
Average[b]		16.0	15.0	13.8	12.1	12.3	10.5
Medroxyprogesterone Acetate	1	20.6	18.4	20.3	17.5	17.9	18.0
	2	11.6	10.6	10.2	10.3	10.3	10.3
Average		16.1	14.5	15.3	13.9	14.1	14.2
Estrogens:							
Ethinyl Estradiol	3	10.1	8.8	8.4	8.0	7.1	6.7
	4	25.2	21.9	17.5	16.3	14.8	14.6
Average		17.7	15.3	13.0	12.2	11.0	10.7
Mestranol	3	11.0	10.3	9.3	8.2	9.0	9.7
	4	25.3	25.0	24.0	25.0	24.2	23.9
Average		18.2	17.7	16.7	16.6	16.6	16.8
Other:	5	12.8	13.4	12.7	12.0	11.3	11.9
DHA	6	5.7	5.0	5.3	4.8	4.4	4.4
	7	20.4	19.4	17.7	16.8	17.9	16.9
Average		13.0	12.6	11.9	11.2	11.2	11.1
DHAS	5	10.1	10.1	10.9	10.0	10.0	10.2
	6	6.4	6.5	7.0	7.2	9.0	6.8
Average		8.3	8.3	9.0	8.6	9.5	8.5

[a] Result with tissue from guinea pig 1.
[b] Average results from the different guinea pigs.

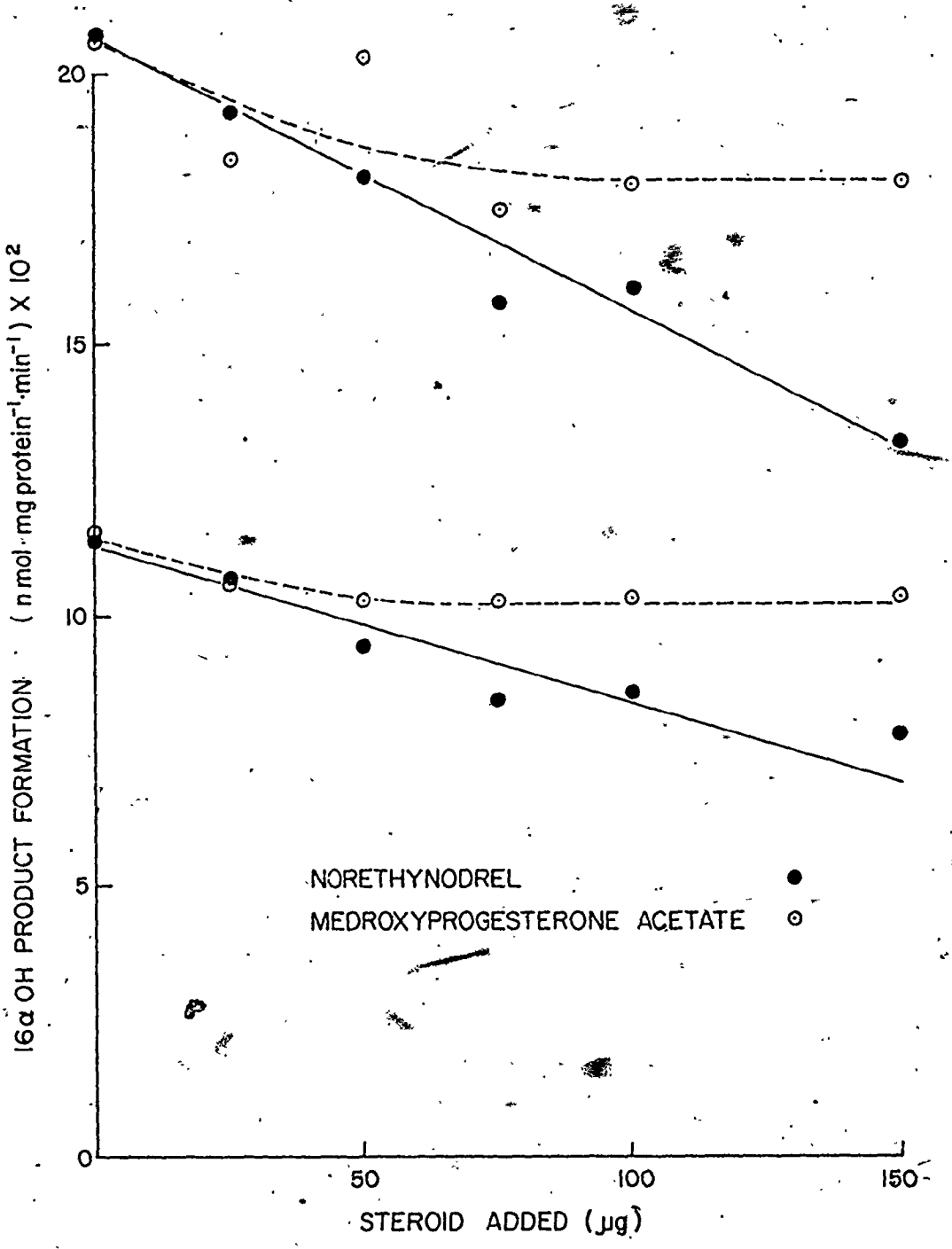

Figure 24. Effect of progestins (norethynodrel and medroxyprogesterone acetate) on 16α-hydroxylation of E_1S incubated with microsomes obtained from two different guinea pigs. Incubation conditions described in text, section 5.6 (f).

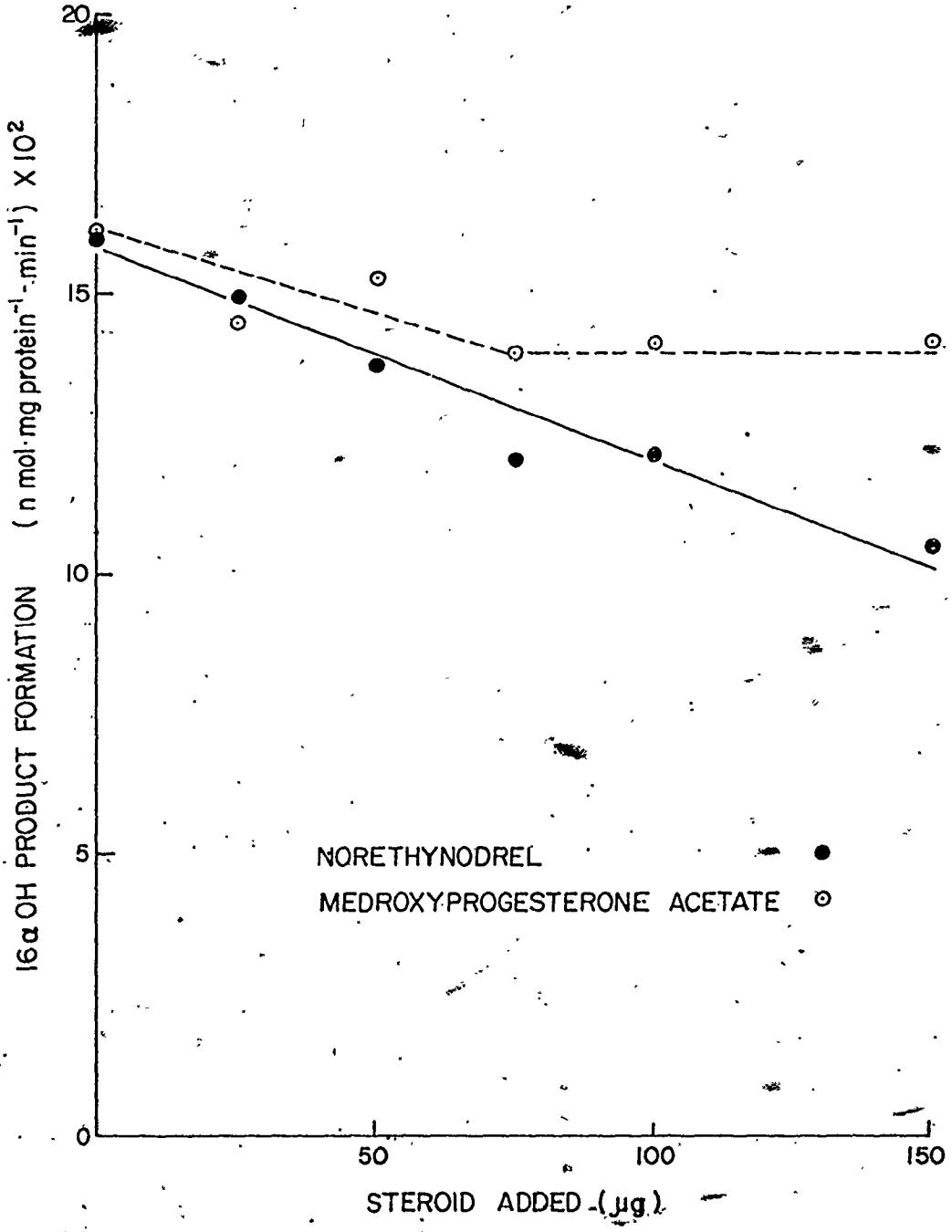

Figure 25. Effect of progestins (norethynodrel and medroxyprogesterone acetate) on 16α-hydroxylation of E_1S. Incubation conditions described in text, section 5.6 (f).

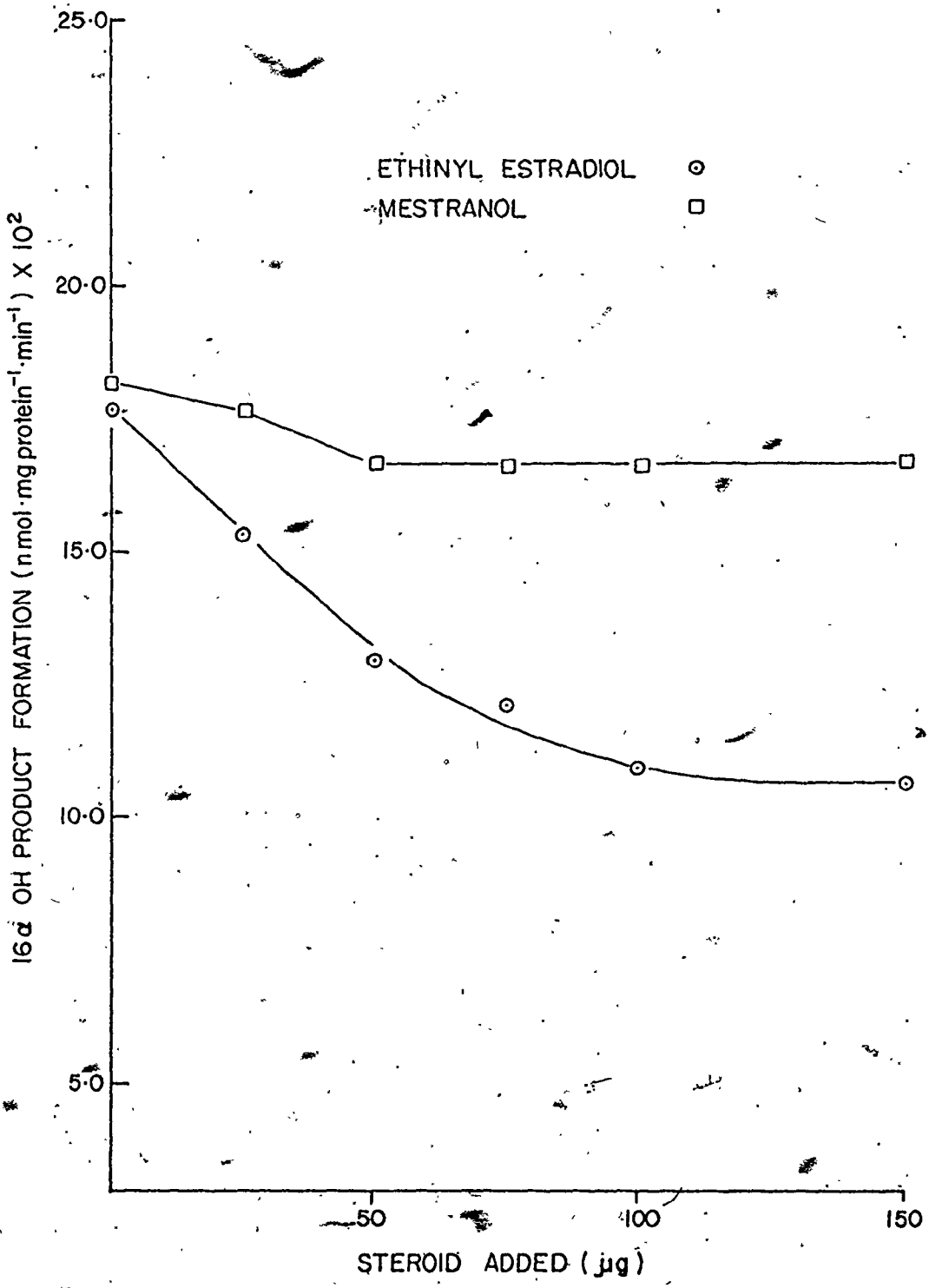

Figure 26. Effect of estrogens (ethinyl estradiol and mestranol) on 16α-hydroxylation of $E_1$3S. Incubation conditions described in text, section 5.6 (f).

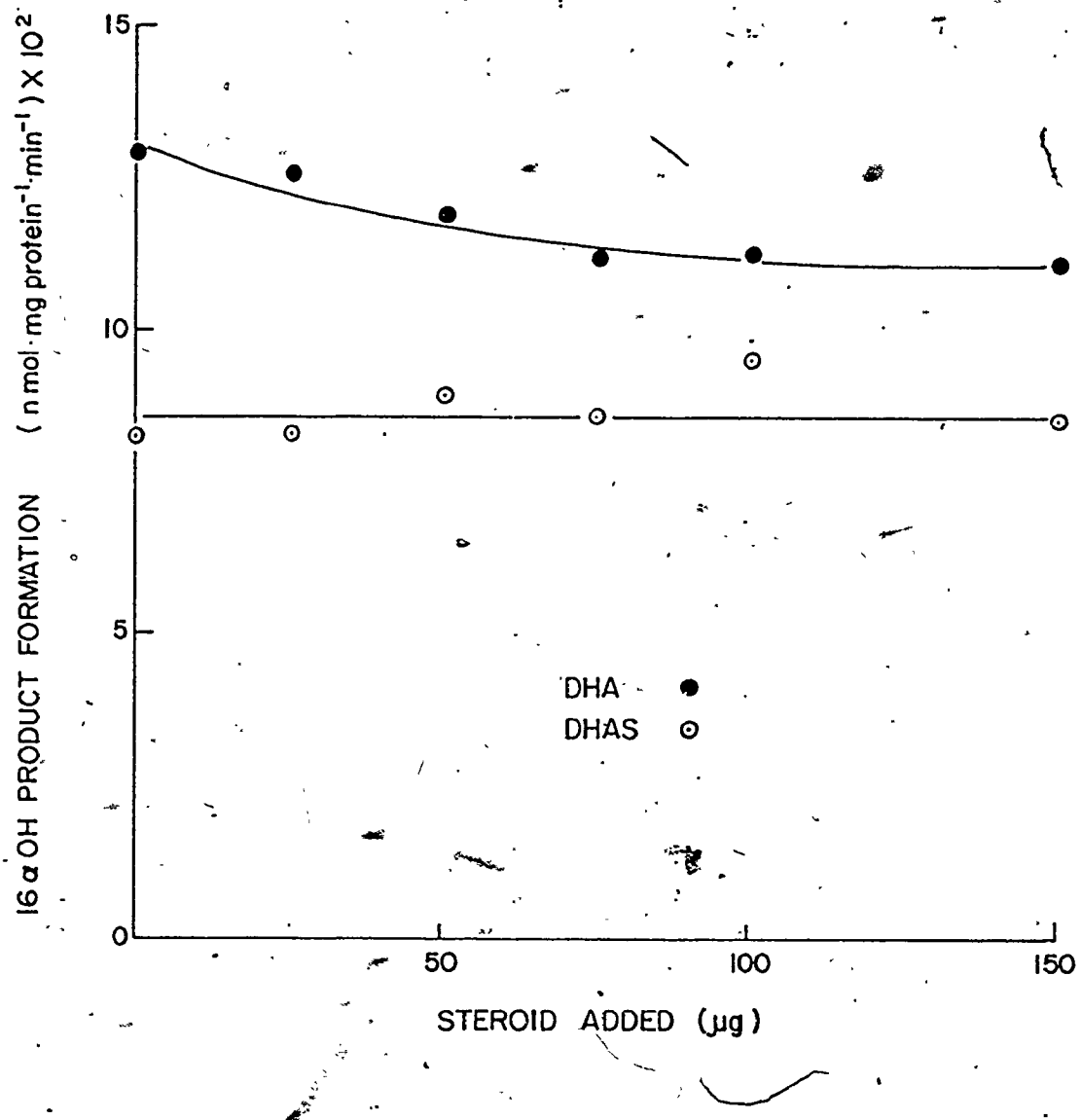

Figure 27. Effect of DHA and DHAS on 16α-hydroxylation of $E_1$3S. Incubation conditions described in text, section 5.6 (f).

Of the progestins, norethynodrel, and of the estrogens, ethinyl estradiol inhibited 16α-hydroxyproduct formation. Percent inhibition curves indicated that at a weight three times that of the substrate, norethynodrel inhibits 16α-hydroxylation of $E_1$3S to the extent of 34% (Fig. 28) and ethinyl estradiol inhibits the reaction to 38% (Fig. 29). The other steroids had little, if any, effect. Mestranol, medroxyprogesterone acetate and DHA inhibited 16α-hydroxyproduct formation slightly (10-15%). DHAS had no effect on the reaction.

The type of inhibition produced by ethinyl estradiol was investigated. Results (Table XV), although not conclusive, suggest that ethinyl estradiol is a competitive inhibitor. A Lineweaver-Burk plot of the results is shown in Figure 30. The approximate common intercept on the 1/v axis with lines of differing slope suggests competitive inhibition. The apparent K_I, as determined from the intercepts of the $1/[E_1S]$ axis was 5.54×10^{-5}M. This value is the average of those determined from the two intercepts of Lineweaver-Burk plots in the presence of inhibitor. In the presence of 50 µg of ethinyl estradiol, K_I was 5.25×10^{-5}M, while the value of K_I was 5.82×10^{-5}M for the inhibitor weight of 100 µg. The km of the reac-

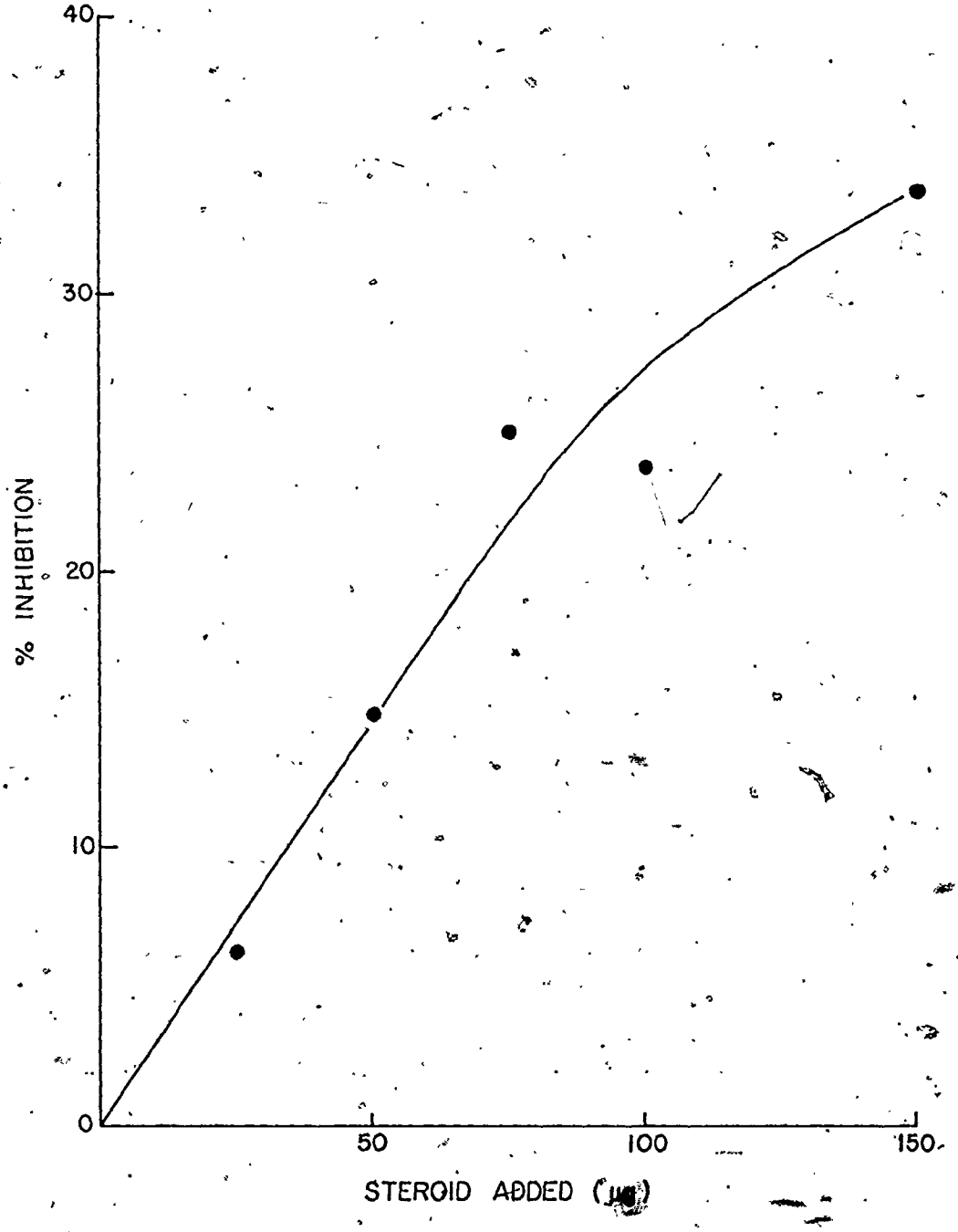

Figure 28. Percent inhibition by norethynodrel on 16α-hydroxylation of $E_1$3S.

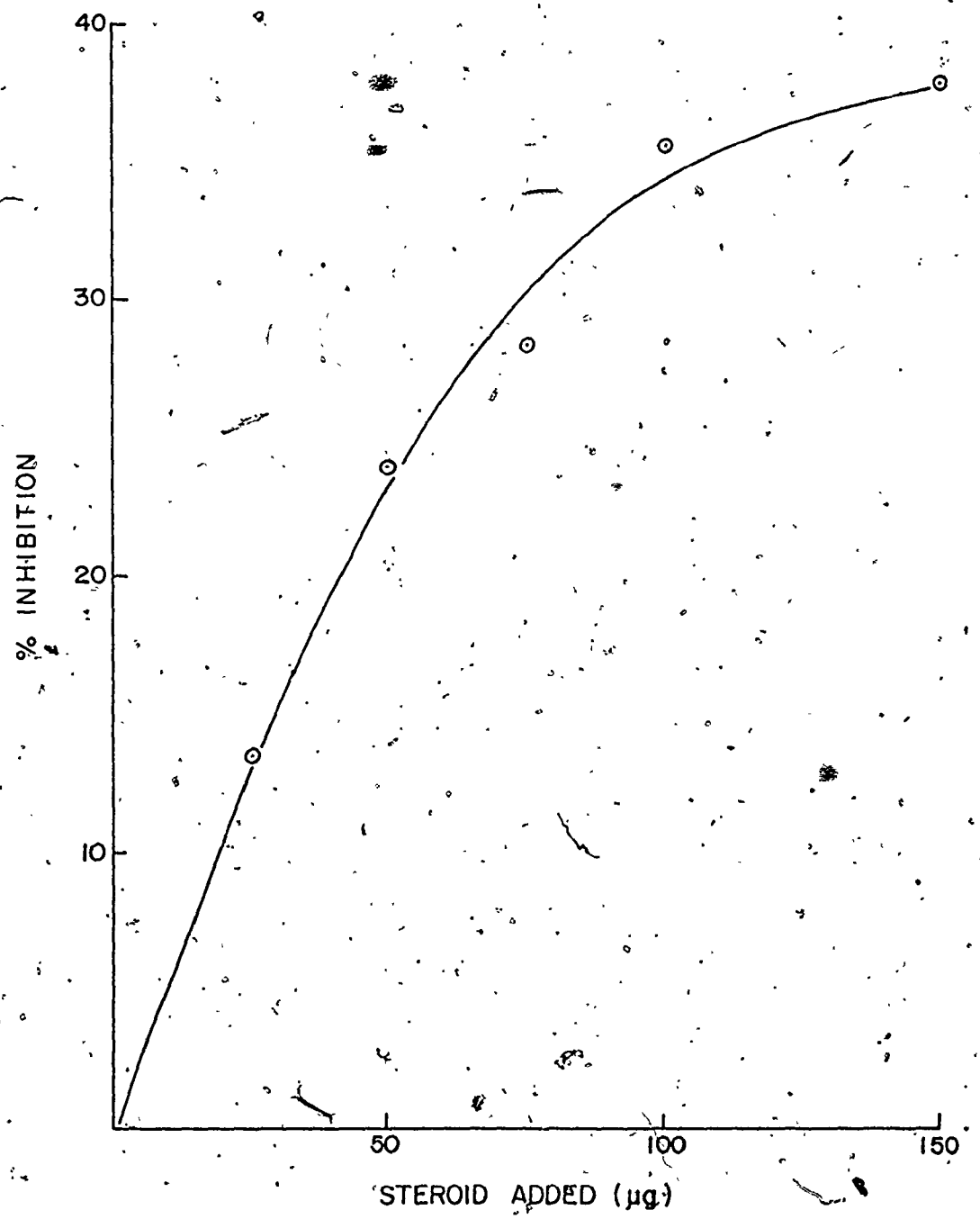

Figure 29. Percent inhibition by ethinyl estradiol on 16α-hydroxylation of $E_1$3S.

TABLE XV

Inhibition of 16α-Hydroxylation of $E_1$3S by Ethinyl Estradiol.

Substrate Conc. [$E_1$3S] x10^5	16α-Hydroxylase Activity (nmol·mg protein^{-1}·min^{-1}) x 10^2 Ethinyl Estradiol Added (μg)		
	0	50	100
1.42	13.32	10.39	8.52
2.85	23.08	18.39	17.55
5.70	32.98	28.75	25.07
8.55	31.85	31.84	27.19

Data for Lineweaver-Burk Plot

1/[$E_1$3S] x10^{-3}	1/(nmol·mg protein^{-1}·min^{-1}) or 1/v Ethinyl Estradiol Added		
	0	50	100
70.4	7.5	9.6	11.7
35.1	4.3	5.4	5.7
17.5	3.0	3.5	4.0
11.7	3.1	3.1	3.7

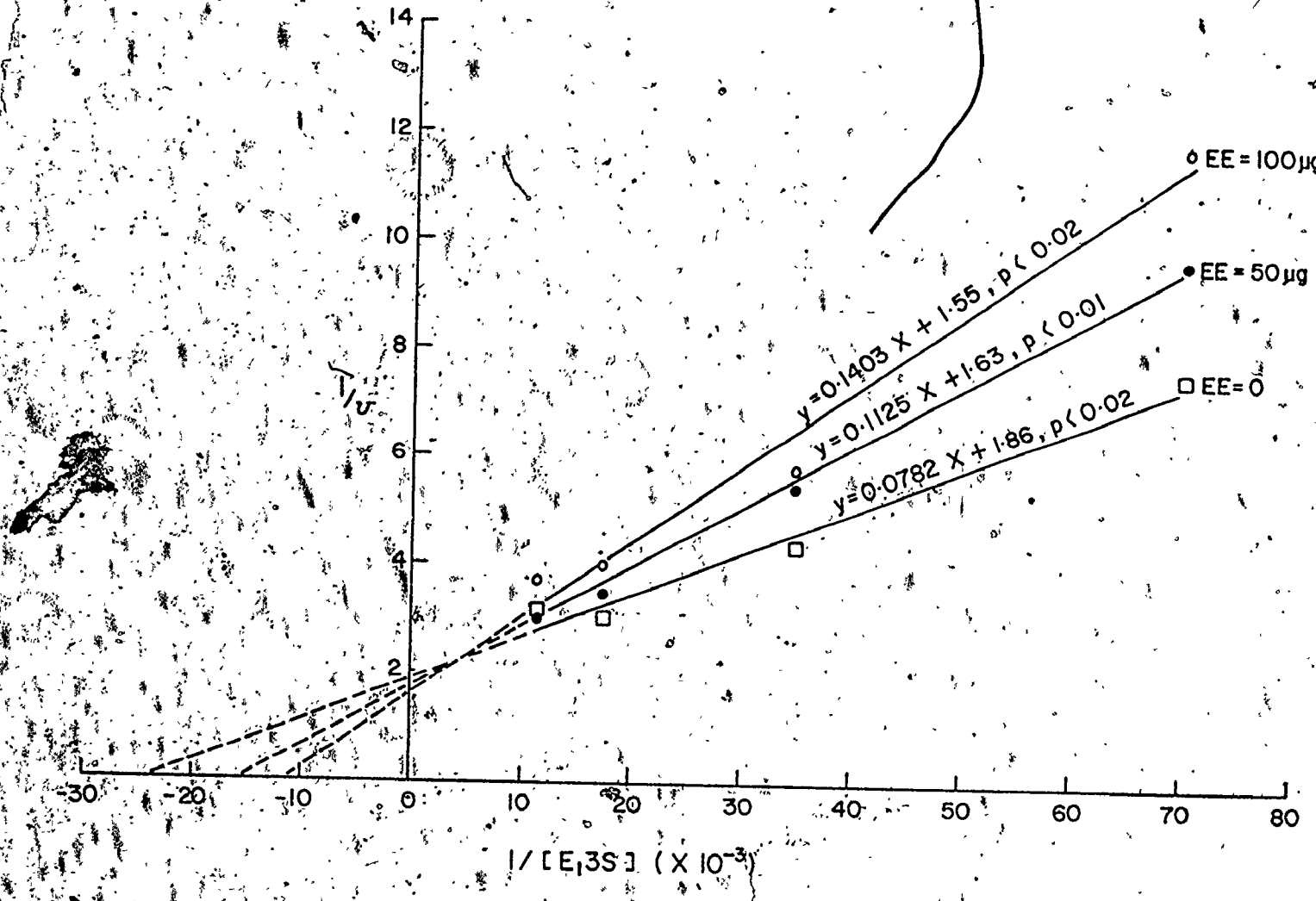

Figure 30. Lineweaver-Burk plots of inhibition of 16α-hydroxylase activity by ethinyl estradiol (EE represents the amount of ethinyl estradiol added to the incubation).

tion in the absence of inhibitor was 4.20×10^{-5} M compared to previous determined value of 3.55×10^{-5} M [section 5.6 (e)].

5.7 REDUCTION OF ESTRONE SULFATE TO ESTRADIOL SULFATE BY GUINEA PIG LIVER MICROSOMES

As mentioned earlier, an additional pathway of $E_1$3S metabolism by guinea pig liver microsomes is via reduction to $E_2$3S. Although this pathway was not of primary interest in this investigation, the fact that it might compete with the hydroxylase for the substrate $E_1$3S suggested the necessity for gaining some knowledge of the system. DEAE Sephadex chromatography clearly separated $E_2$3S from the rest of the monosulfates. Reductase activity could therefore be determined directly from the $E_2$3S formed as indicated by the chromatographic pattern.

(a) PROPERTIES OF THE REDUCTASE

Increasing microsomal protein concentration beyond 4 mg (Fig. 31) and increasing incubation time (Fig. 32) decreased $E_2$3S formation. These figures correspond to data obtained from the same microsomal incubations employed to investigate these parameters on 16α-hydroxylase activity† (i.e. Fig. 14 and Fig. 15 respectively).

† Other properties reported for reductase activity correspond to the same microsomal incubations employed to investigate 16α-hydroxylase activity.

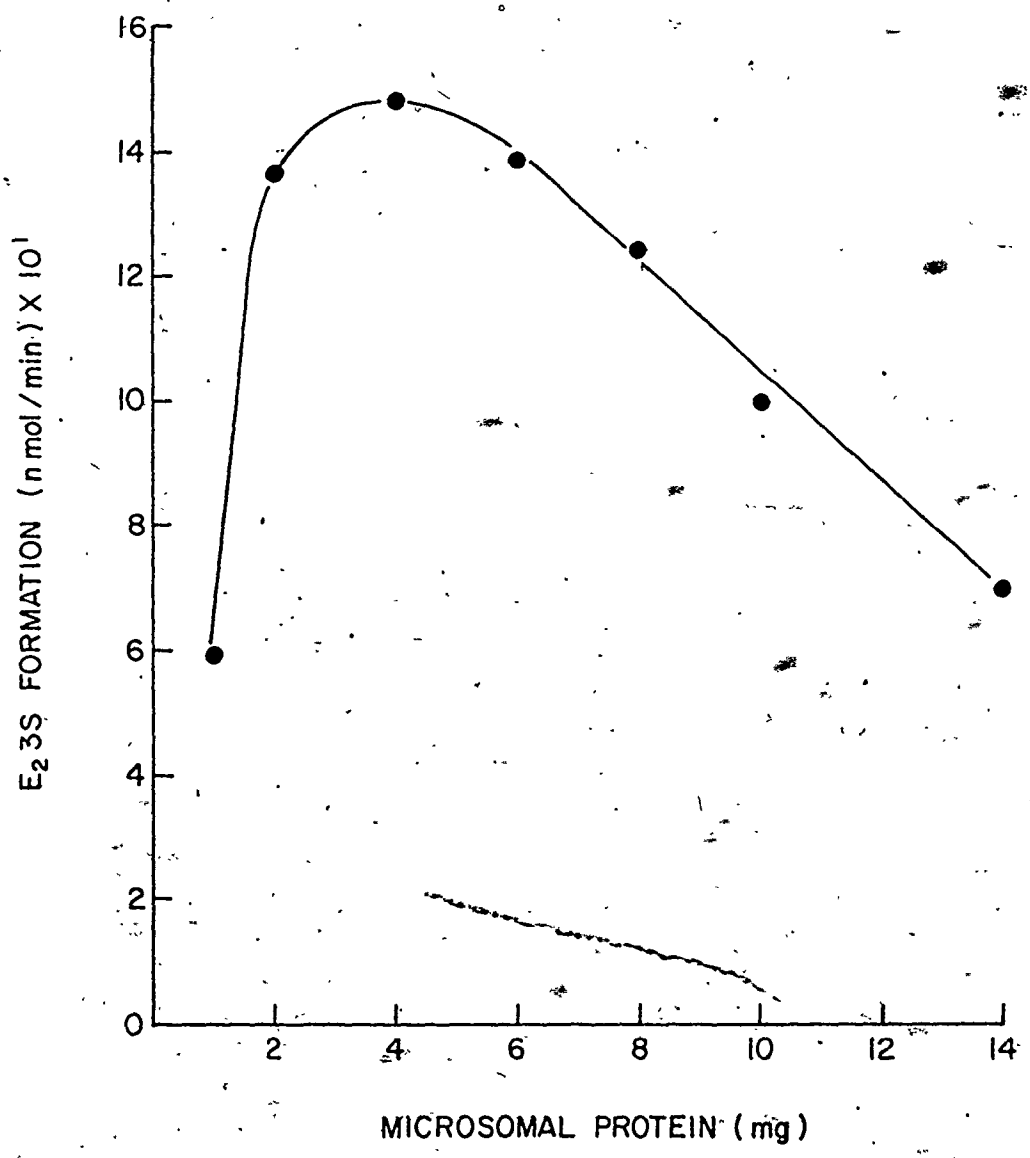

Figure 31. Effect of protein concentration on the reduction of $E_1$3S to $E_2$3S. Estrone sulfate was 85.5 nmol and NADPH 2.4 µmol per 5.0 ml of incubation mixture. Incubations were performed at 37°C for 30 min., pH 7.5.

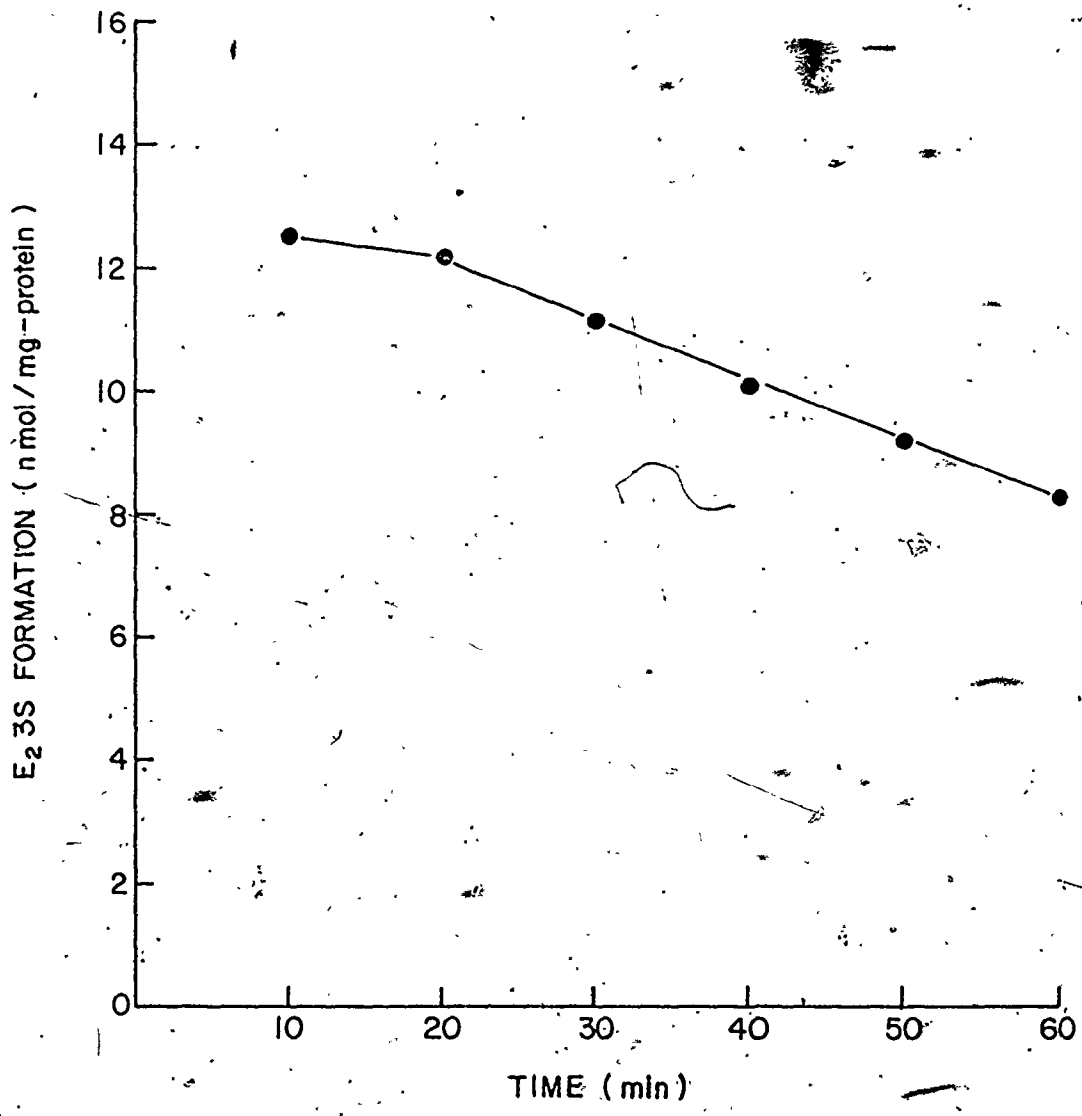

Figure 32. Effect of incubation time on the reduction of $E_1$3S to $E_2$3S. Incubations were performed at 37°C and pH 7.5 in the presence of 7.5 mg of microsomal protein. Estrone sulfate was 142.5 nmol and NADPH 4.2 µmol per 5.0 ml of incubation mixture.

The cofactor NADPH is also required for the reduction of $E_1$3S to $E_2$3S (Fig. 33). Under the experimental conditions employed (substrate $E_1$3S 285 nmol; pH 7.5, incubated 30 min. at 37°C), a Lineweaver-Burk plot (Fig. 34) gave a Km value for NADPH of 14.06×10^{-4}M. This value is 3.4 times greater than the km value of the 16α-hydroxylase reaction as determined under the same experimental conditions.

Increasing the concentration of the oxidized form of the cofactor (i.e. $NADP^+$) had a greater effect on $E_2$3S formation (Fig. 35) than on 16α-hydroxyproduct formation (see Fig. 19). Addition of $NADP^+$ to a microsomal incubation to give a concentration twice that of the concentration of NADPH inhibited reductase activity by 40%.

The pH optimum for the conversion of $E_1$3S to $E_2$3S was also different from that of 16α-hydroxylation. The pH optimum for reductase activity was between 5.0 and 5.5 (Fig. 36). Increasing the substrate weight ($E_1$3S, 2.85 nmol to 142.5 nmol) resulted in increased percent formation of $E_2$3S at higher pH's. For example, at pH 7.0 with substrate $E_1$3S of 2.85 nmol, the percent conversion to $E_2$3S was 20% compared to 43% conversion when the substrate was increased to 142.5 nmol. To dis-

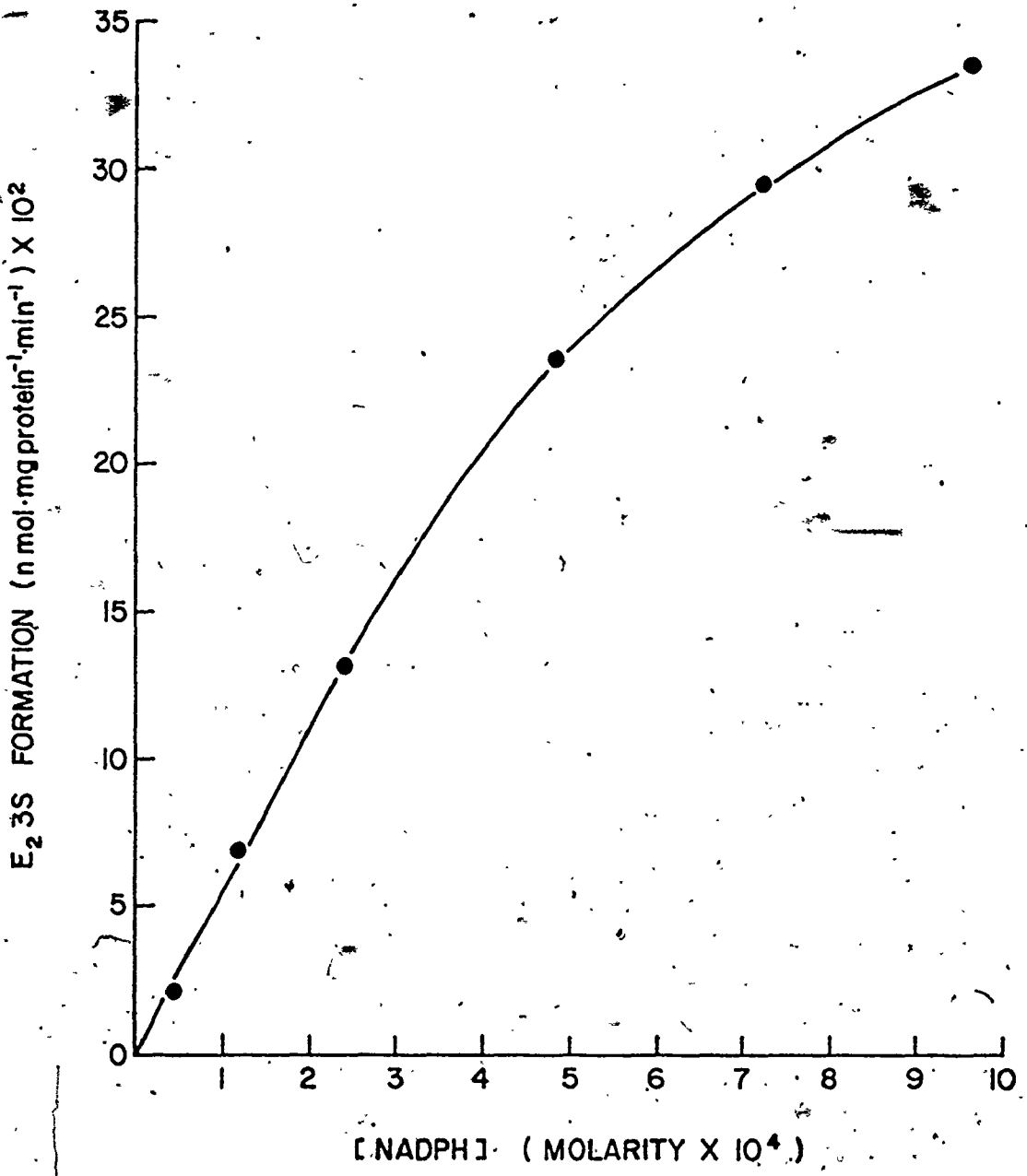

Figure 33. Effect of cofactor NADPH concentration on the rate of formation of $E_2$3S from $E_1$3S (Michaelis-Menten plot). Incubations were performed for 30 min. at 37°C, pH 7.5, in the presence of 8.0 mg of microsomal protein. $E_1$3S was 285 nmol per 5.0 ml of incubation mixture.

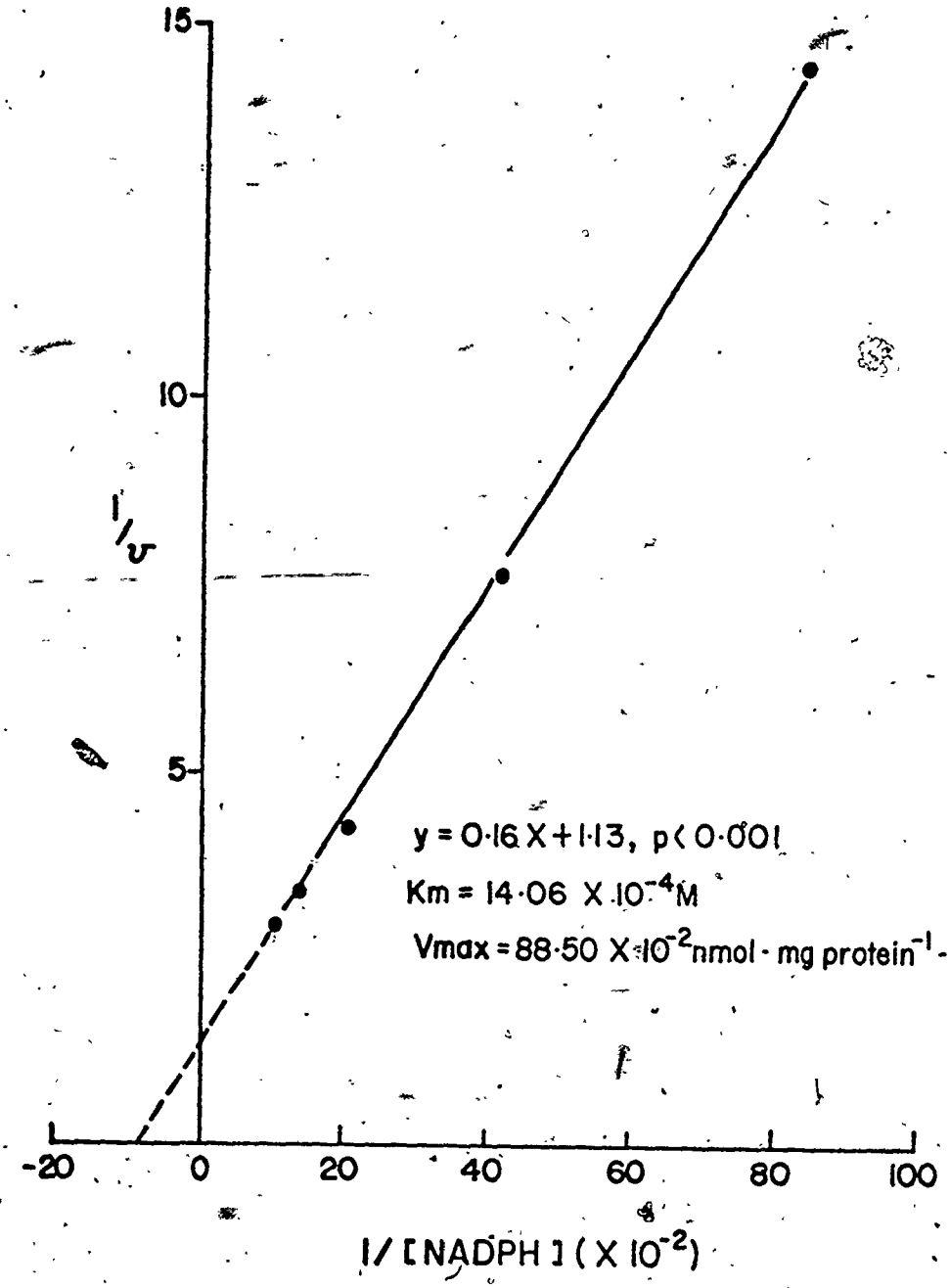

Figure 34. Effect of cofactor NADPH concentration on the rate of formation of $E_2$3S from $E_1$3S (Lineweaver-Burk plot). Experimental conditions as described in Figure 33.

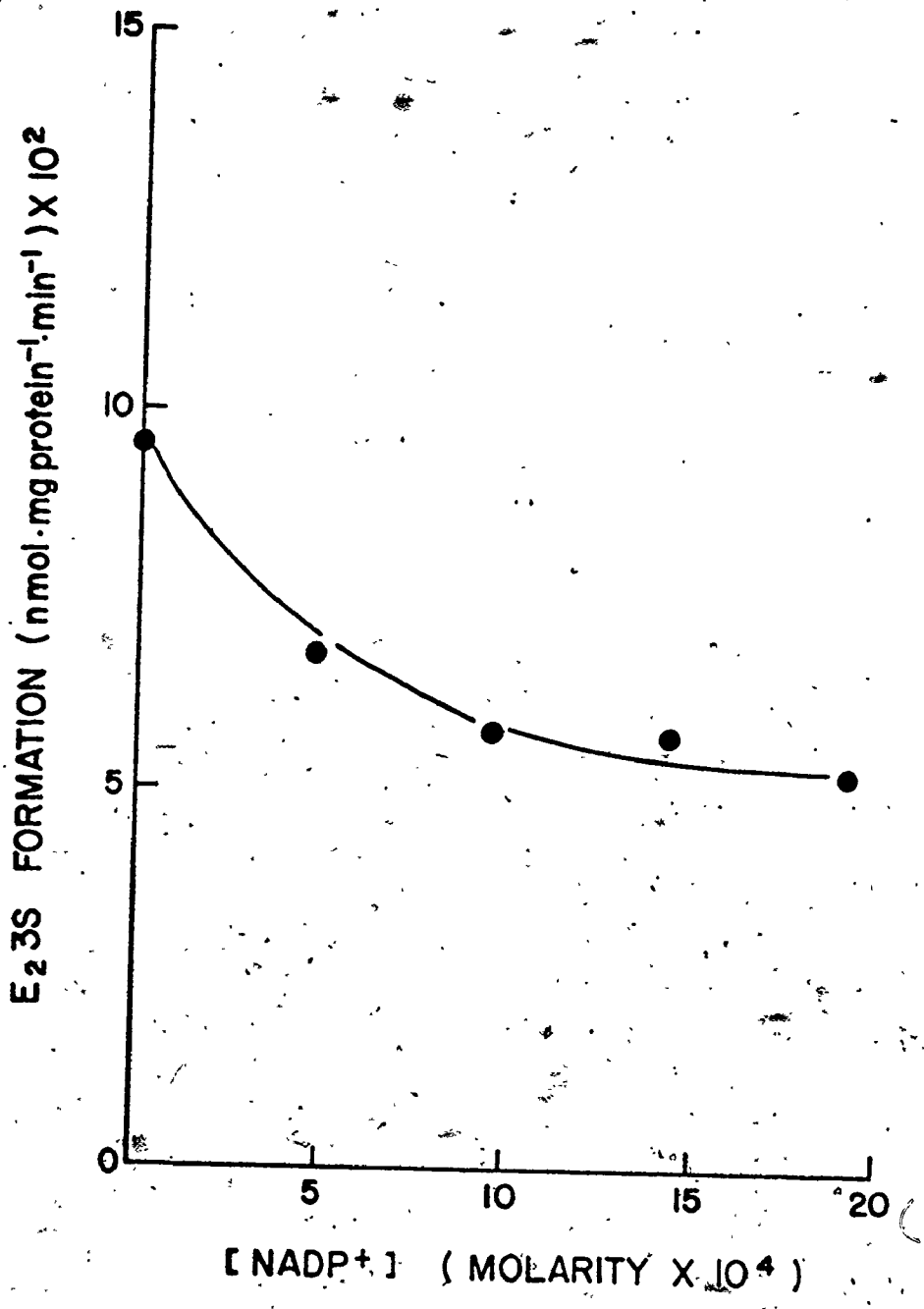

Figure 35. Effect of oxidized form of cofactor NADPH ($NADP^+$) on reduction of $E_1$3S to $E_2$3S. Incubations were performed at 37°C for 35 min., pH 7.5, in the presence of 9.0 mg of microsomal protein. $E_1$3S was 142.5 nmol and NADPH 4.2 µmol per 5.0 ml of incubation mixture.

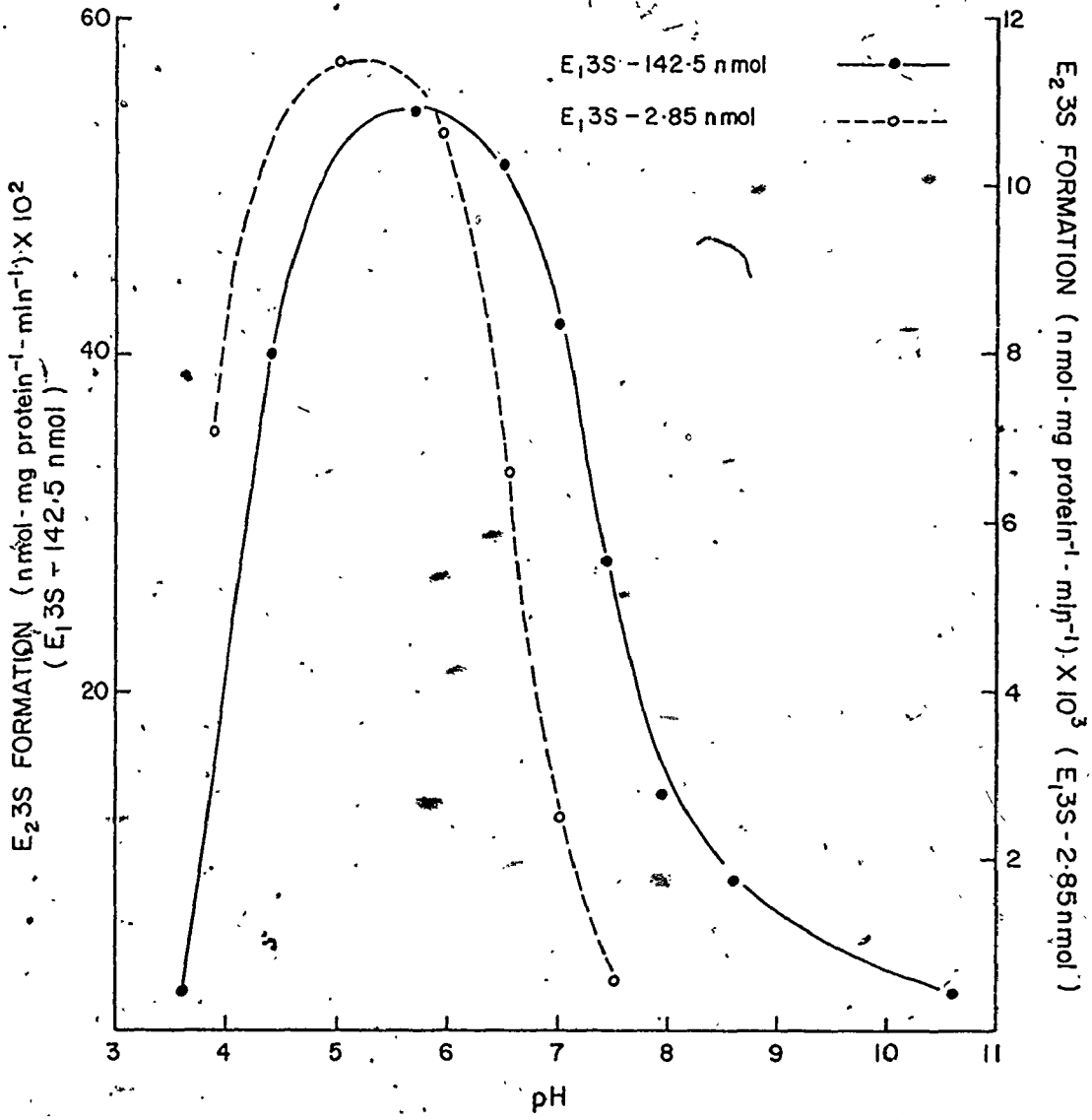

Figure 36. Effect of pH on the reduction of $E_1$3S to $E_2$3S at two different substrate concentrations (2.85 nmol and 142.5 nmol per 5.0 ml of incubation mixture). Incubations were performed at 37°C for 30 min. For the incubation with 2.85 nmol of $E_1$3S, NADPH was 2.4 µmol while that for the increased substrate concentration was 4.2 µmol per 5.0 ml of incubation mixture. Microsomal protein of 7.0 mg was employed for the $E_1$3S-2.85 nmol incubation compared to 8.5 mg of microsomal protein used for incubations with $E_1$3S-142.5 nmol.

courage $E_2$3S formation and promote the 16α-hydroxylase reaction, a pH of 7.6 was employed in the inhibition studies [see section 5.6 (f)].

Results from the kinetic studies are shown in Figure 37 (Michaelis-Menten plot) and Figure 38 (Lineweaver-Burk plot). These results are the average of three experiments with microsomes obtained from two different guinea pigs. The km value of the reductase as determined from the Lineweaver-Burk plot is 15.1 x 10^{-5}M or 4.3 times the Km for the hydroxylase reaction.

(b) INHIBITION STUDIES

Experimental data for the effect of other steroids on the reductase activity is given in Table XVI. These results were obtained from the same incubations as those already described dealing with the effects upon 16α-hydroxylation of $E_1$3S. Therefore, experimental conditions employed were those that optimized 16α-hydroxylation (incubation time 35 min; microsomal protein concentration of 6-8 mg/5 ml of incubation mixture; pH 7.6; substrate concentration $E_1$3S of 2.85 x 10^{-5}M and a cofactor NADPH concentration of 8.4 x 10^{-4}M).

Averaged results from duplicated experiments to give a general pattern of inhibition, if it existed, are

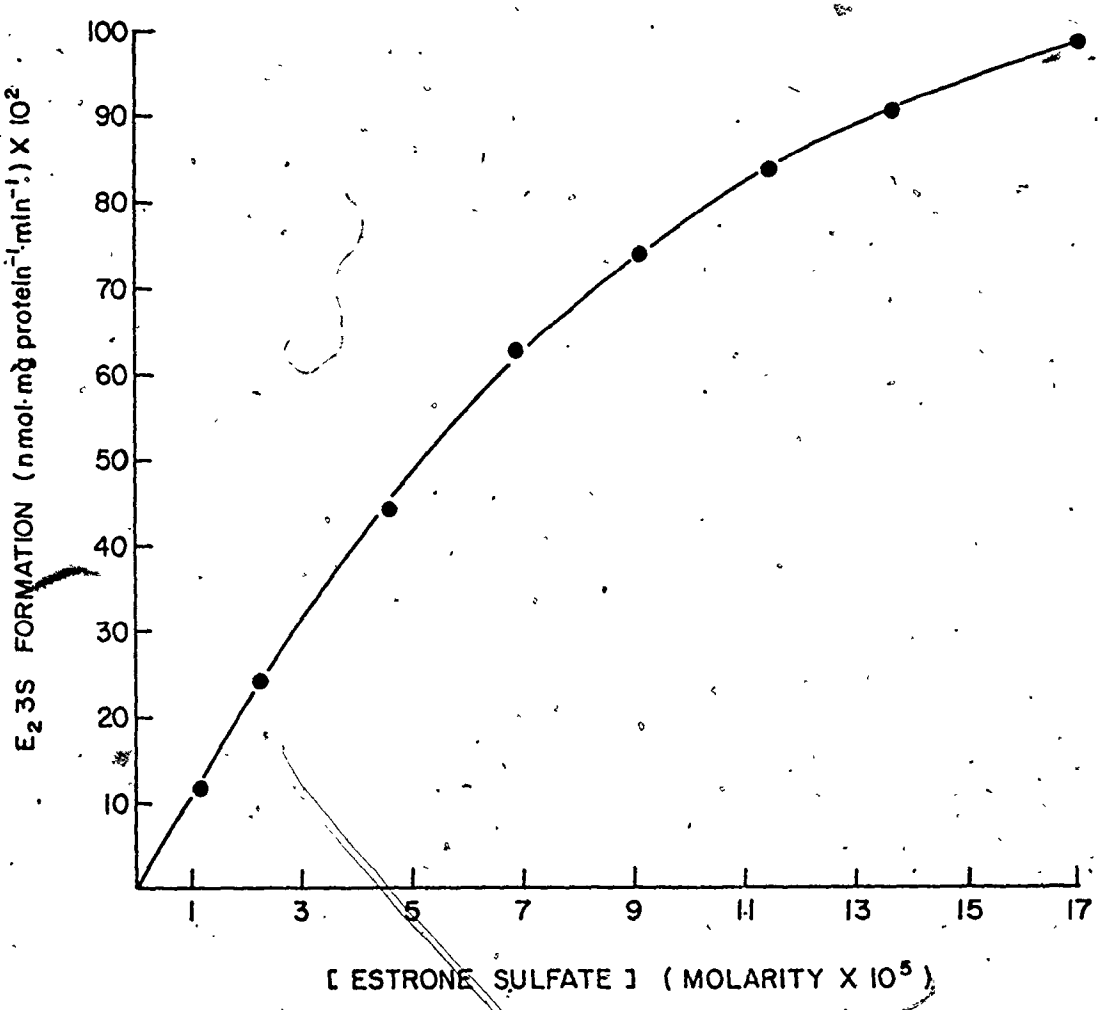

Figure 37. Effect of $E_1$3S concentration on the rate of formation of $E_2$3S (Michaelis-Menten plot). Incubations were performed at 37°C for 30 min., pH 7.5 in the presence of 7.5 mg of microsomal protein. NADPH was 4.2 µmol per 5.0 ml of incubation mixture.

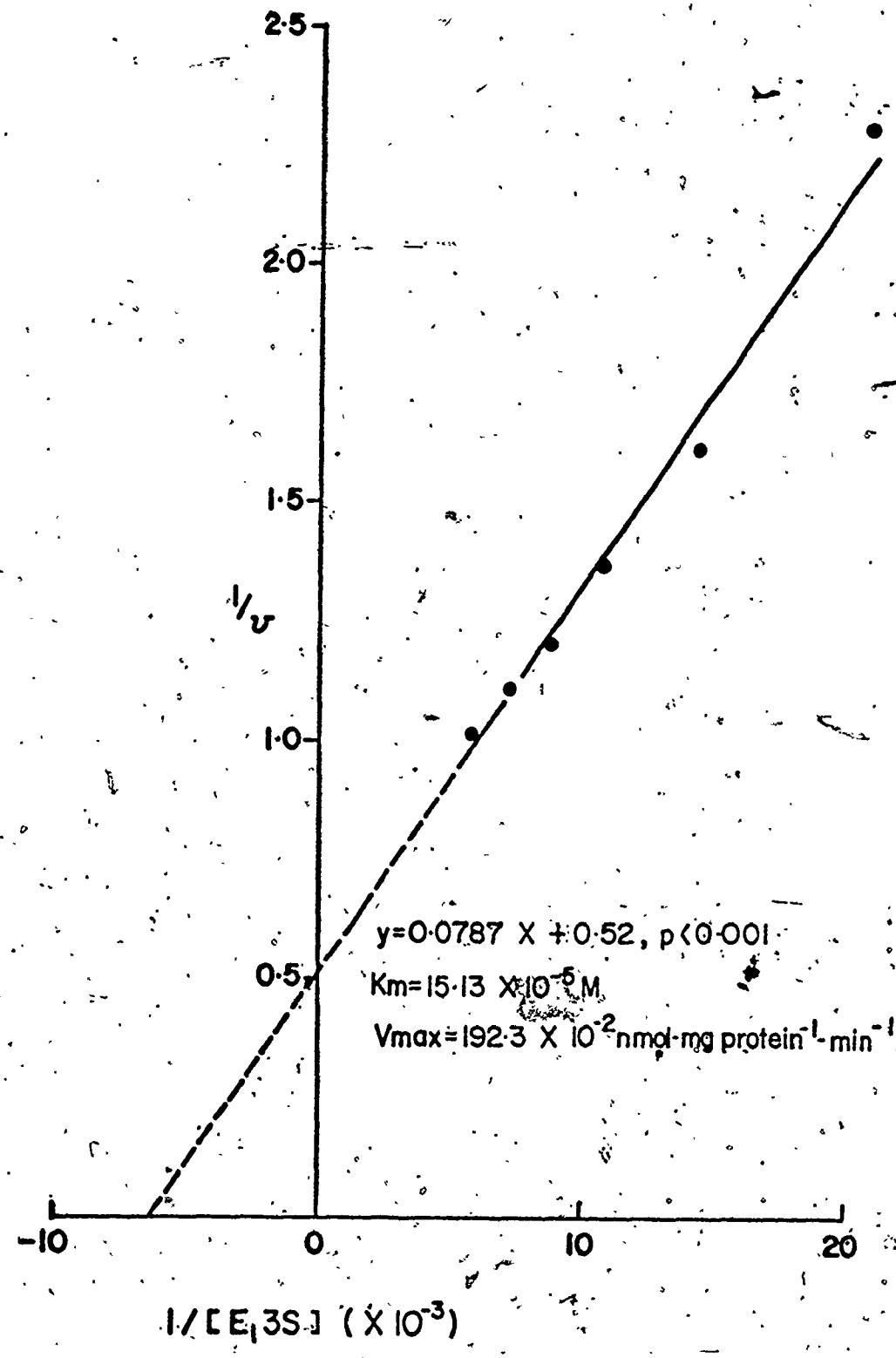

Figure 38. Effect of $E_1$3S concentration on the rate of formation of $E_2$3S. (Lineweaver-Burk plot). Experimental conditions as described in Figure 37.

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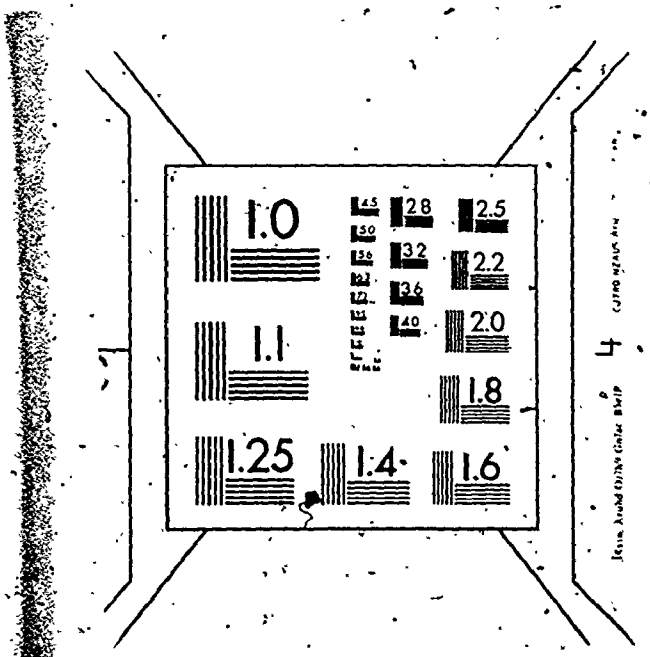

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TABLE XVI

The Effect of Other Steroids on Reduction of $E_1$3S to $E_2$3S

Steroid		Reductase Activity (nmol·mg protein^{-1}·min^{-1}) x 10^2 Amount of Steroid Added to Incubation (ug) 0	25	50	75	100	150
Progestins:							
Norethynodrel	1	13.3	13.8	13.8	14.5	15.4	12.6
	2	12.6	11.7	10.1	9.9	8.8	8.3
Average[a]		13.0	12.7	11.9	12.7	12.1	10.4
Medroxyprogesterone Acetate	1	13.1	13.9	15.3	15.2	15.8	16.3
	2	12.5	11.5	11.5	10.4	10.5	10.0
Average		12.8	12.7	13.4	12.8	13.2	13.2
Estrogens:							
Ethinyl Estradiol	3	14.3	14.8	15.2	15.7	15.6	15.6
	4	9.8	12.4	13.6	13.8	13.7	14.3
Average		12.0	13.6	14.4	14.7	14.6	14.9
Mestranol	3	13.7	14.0	14.1	14.8	14.1	14.1
	4	10.6	10.6	10.9	10.5	10.4	10.6
Average		12.2	12.3	12.5	12.6	12.3	12.4
Other:							
DHA	5	13.4	13.7	12.7	12.7	12.5	12.2
	6	12.1	8.8	7.5	6.7	7.0	6.7
	7	12.1	10.1	8.7	8.8	9.0	7.9
Average		12.5	10.9	9.7	9.4	9.5	8.9
DHAS	5	13.5	14.5	15.3	15.0	14.5	13.3
	6	12.3	9.9	8.6	7.5	6.8	5.4
Average		12.9	12.2	11.9	11.2	10.6	9.3

[a] Average results from the different guinea pigs (i.e. #1 and #2).

shown in Figures 39 to 41 respectively for progestins, estrogens and DHA and DHAS. Medroxyprogesterone acetate had no influence on the formation of $E_2$3S (Fig. 39) while norethynodrel decreased $E_2$3S formation to a small extent from a control value of 13 nmol/min-mg protein to about 11 nmol/min-mg protein when 150 µg of steroid were present (three times the weight of the substrate). This corresponds to a decrease of about 15%. The results of the effects of these synthetic progestins on the reduction of $E_1$3S to $E_2$3S is similar to their effect on 16α-hydroxylation of the substrate. Inhibition caused by norethynodrel, however, on the reductase reaction, is not as great as that on the hydroxylase reaction.

Ethinyl estradiol increased $E_2$3S formation from a control value of 12 nmol/min-mg protein to 15 nmol/min-mg protein (an increase of 25%) (Fig. 40). This result is opposite to its effect on the hydroxylase reaction. Mestranol neither increased nor decreased the reductase reaction and this was similar to its action on the hydroxylase activity.

Both DHA and DHAS decreased the rate of formation of $E_2$3S (Fig. 41). The decreased reductase activity effect was more rapid with DHA than with DHAS. The percent

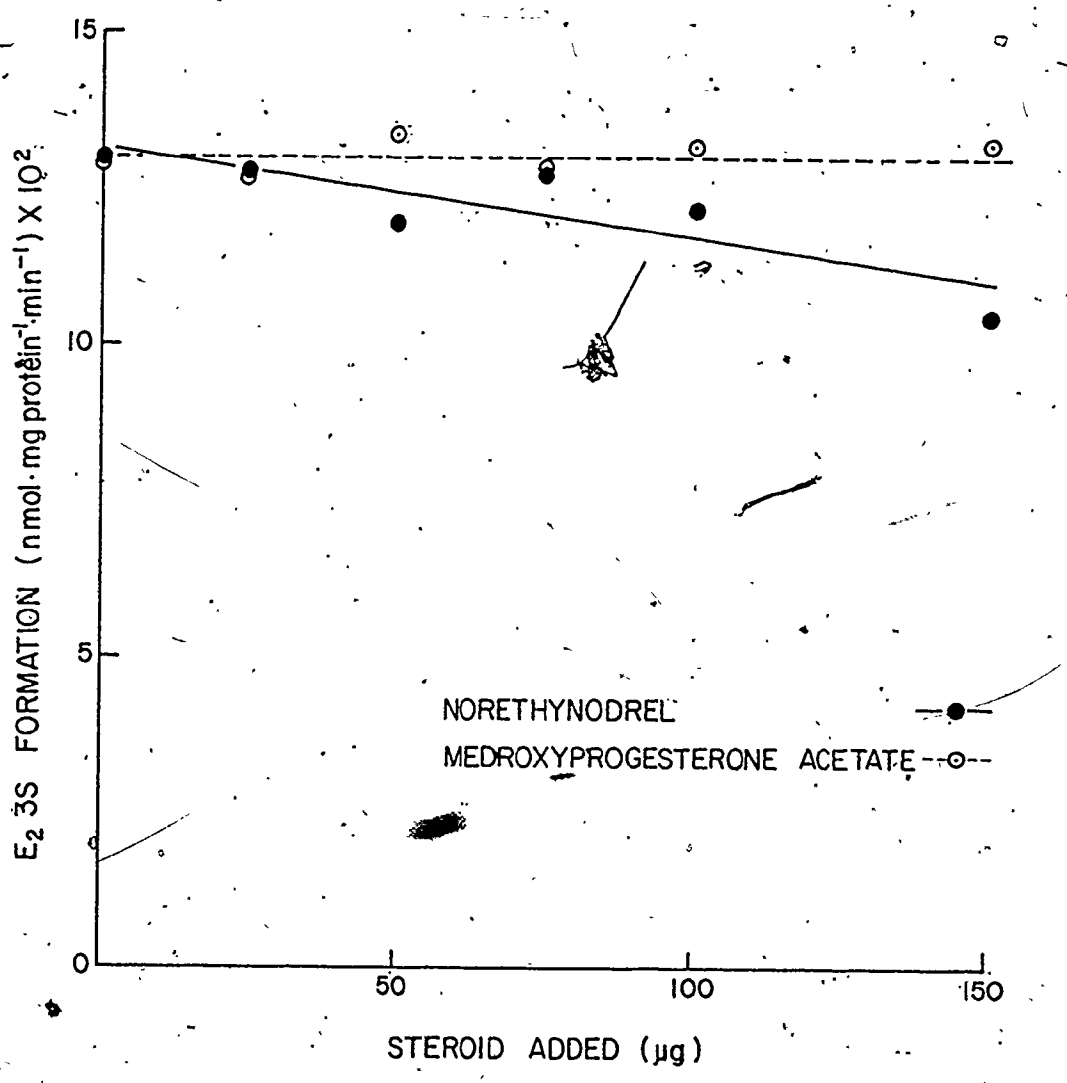


Figure 39. Effect of progestins (norethynodrel and medroxyprogesterone acetate) on the reduction of $E_1$3S to $E_2$3S. Incubation conditions described in text, section 5.7 (b).

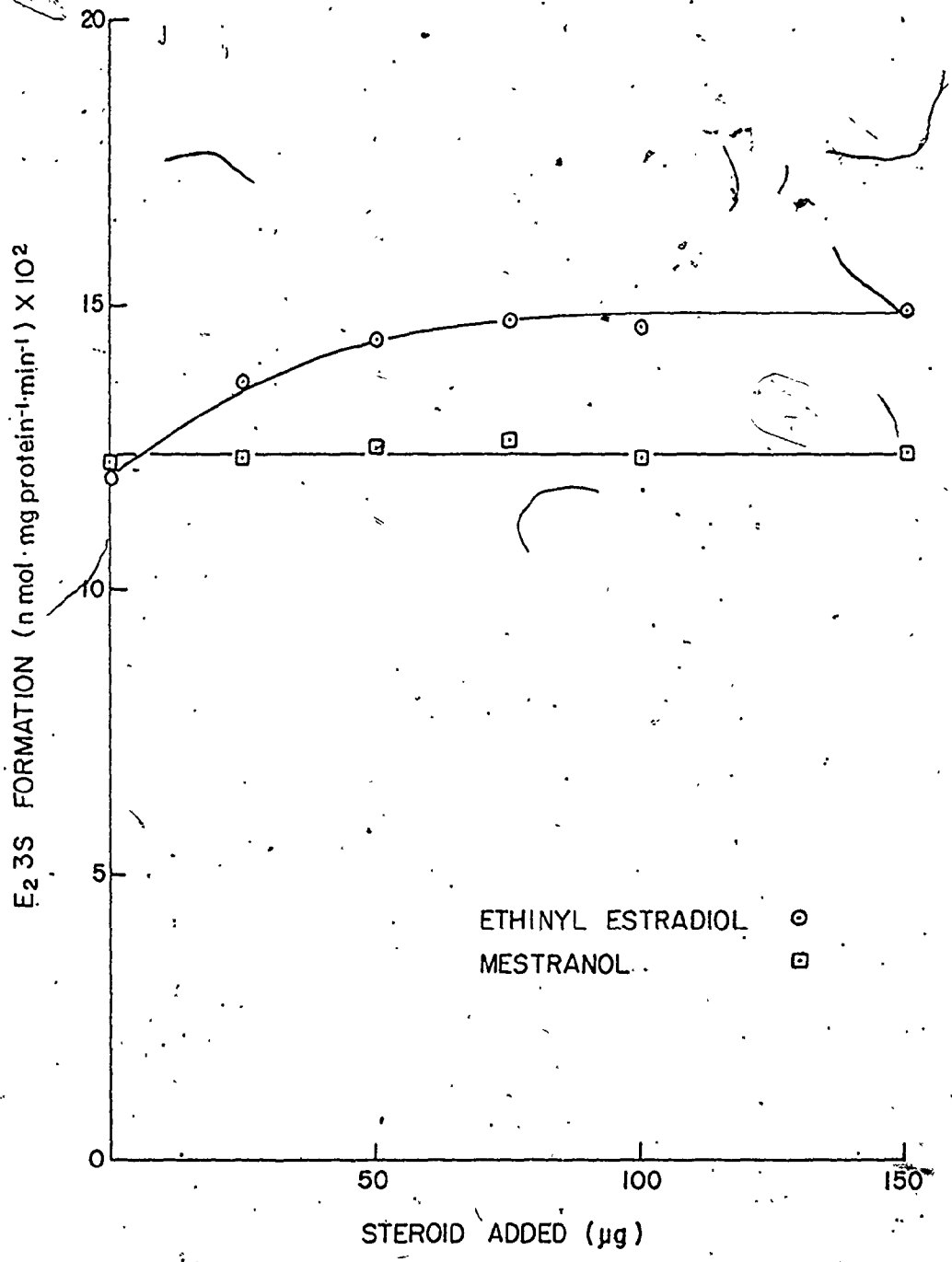


Figure 40. Effect of estrogens (ethinyl estradiol and mestranol) on the reduction of $E_1$3S to $E_2$3S. Incubation conditions described in text, section 5.7. (b).

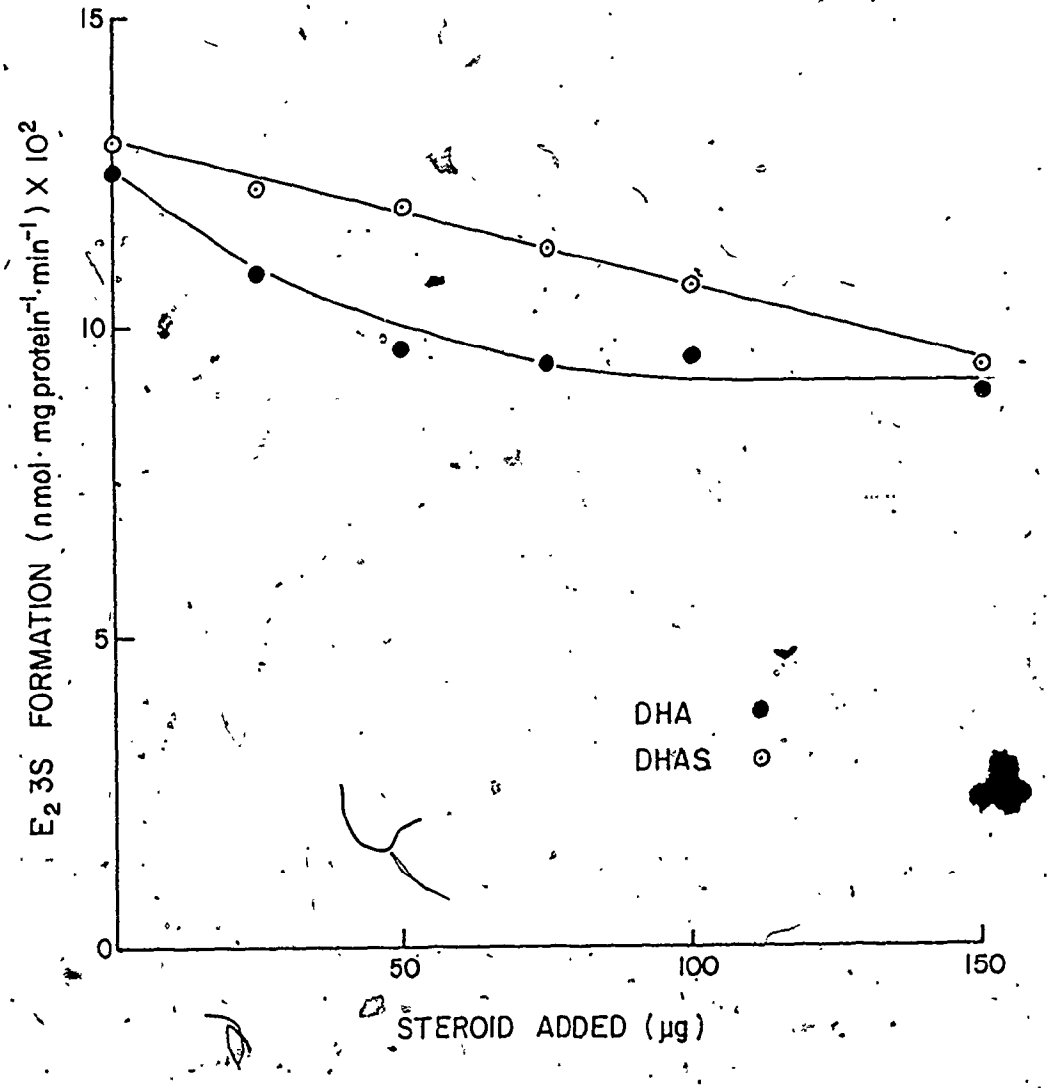


Figure 41. Effect of DHA and DHAS on the reduction of E_1 3S to E_2 3S. Incubation conditions described in text, section 5.7 (b).

change in both cases was about 30% (from control value of 12.5 nmol/min-mg protein to 9.0 nmol/min-mg protein with 150 µg of steroid present).

CHAPTER 6

DISCUSSION

6.1 LIVER TISSUE SLICE EXPERIMENTS

(a) UPTAKE STUDIES

Results from the uptake studies indicated that E_2 and $E_1$3S could possibly enter liver cells and be potentially metabolized. Association of $E_1$3S with liver tissue to comparable levels of E_2 required longer incubation times.

To obtain a more physiological picture, plasma proteins were added to the incubation medium to study their effect on the uptake and possible metabolism of the steroids. This work was stimulated by a publication of Anderson et al (1974) who reported the effects of serum albumin on the ability of E_2 and E_3 to cause the translocation of the estrogen receptor complex to the nucleus of uterine cells of the rat. It was found that, in the absence of albumin, E_2 was more potent in causing the translocation of the receptor to the nucleus. In the presence of albumin in the incubation medium, the ability of E_3 to cause translocation in vitro was equal to or greater than that of E_2. These results suggested that albumin or other serum proteins may play an

important role in determining the relative potencies of estrogens in vivo with regard to the translocation process. The possibility thus existed that serum proteins may influence the metabolism of chemically different phenolic steroids by preventing the uptake of a particular steroidal structure by a metabolizing tissue, such as the liver. Reports (Rosenthal et al, 1972, Savu et al, 1973) that estrogen sulfates were strongly bound with high capacity to serum albumin supported such a hypothesis. Data observed in the present work [section 5.2 (a)] indicated that albumin and plasma prevent, to a greater extent, the uptake of $E_1$3S in comparison with that of E_2 by guinea pig liver tissue, suggesting that, in vivo, serum proteins may result in a low supply of steroid sulfates to tissues with the capacity to metabolize these steroidal forms. This finding supports Longcope's (1972) and Fishman and Hellman's (1973) suggestions that estrogen sulfates are not metabolized as rapidly as the unconjugated estrogens.

(b) METABOLISM BY SLICES

Only two conjugating mechanisms were shown to be present in the guinea pig liver slice, namely, glucuronidation and sulfurylation. These two activities are already known to exist in the guinea pig liver (Sa'at and Slaunwhite,

1969; Goebelsman et al, 1965; Quamme et al, 1972).

The lesser glucuronide formation with ^{3}H-$E_1$3S as substrate compared with the substrate ^{14}C-E_2 is due to the sulfate group at C-3, preventing glucuronidation at this site. Glucuronidation in the guinea pig, as with other species, may represent a final step in estrogen metabolism which is followed by excretion. Rapid excretion of glucuronides was shown to occur following the intravenous injection of labeled E_2 (Stoa and Borjesson, 1971) and labeled E_1 and E_3 (Sandberg et al, 1967) in the guinea pig. The sulfate moiety on the phenolic steroid partially prevents glucuronidation and thus elimination of estrogens from the animal. Estrogen sulfates would appear to be retained in the animal to be further metabolized or to serve some unknown function. Recent work by Hobkirk et al has shown that $E_1$3S is further metabolized to 16α-hydroxylated products (particularly to a disulfate form) in guinea pig liver slices (Hobkirk, Nilsen and Jennings, 1975, Hobkirk et al, 1977).

Previous work has shown that estrogen metabolism is influenced to some degree by sex in other species, such as human (Eren et al, 1967; Zumoff et al, 1968) and rat (Gustafsson and Ingelmann-Sundberg, 1974). Evidence

has also been given (Stoa and Borjesson, 1971) indicating a sex difference in guinea pigs as comparatively greater amounts of $E_1$3G were excreted in the urine of male animals following intravenous injection of ^{14}C-E_2. The report by Gustaffson and Ingelman-Sundberg (1974) of a sex specific 15β-hydroxylase activity with a substrate preference for a sulfated steroid (5α-androstane-3α,17β diol-3,17-disulfate) present in female rat livers but absent in males, stimulated an investigation to compare the metabolism of estrogens in female and male guinea pigs. Employing disulfate formation as an indication of hydroxylation, preliminary results suggested a difference in estrogen metabolism between female and male guinea pigs (i.e. no disulfate formation was detected in male animals) [Table IV].

Gustafsson and Ingelmann-Sundberg (1974) described the influence of other steroids on the 15β-hydroxylase activity. For example, treatment of postpubertally castrated male rats with estradiol benzoate led to a partial "feminization" of the liver with a 15β-hydroxylase activity of about 30% of that present in normal female rats. Pretreatment of young male guinea pigs in the present investigation had little influence on the metabolic picture and failed to induce disulfate formation in

those guinea pigs lacking the activity. Studies have now shown (Hobkirk et al, 1977) that some young male animals are able to produce disulfates indicating the presence of the hydroxylase system. The disulfate consisted 83-94% of 16α-hydroxylated estrogens in both male and female animals. Thus, no clear-cut sex difference appears in the metabolism of estrogens with regard to hydroxylation in the guinea pig.

(c) LIVER SUPERFUSION

The experimental design of superfusion offers a number of advantages over batch incubations (Orti et al, 1965). Products of the metabolic reaction are removed preventing altered reaction rates by mass action or other inhibitory effects, such as feed back inhibitions. Removal of metabolites may also prevent their further processing by the continuous contact with the tissue which may give rise to abnormal end products. Cellular debris caused by tissue disruption as a result of agitation remains in the medium in batch incubations. The metabolic potentialities of this cellular debris may be quite different from the whole tissue. This latter phenomenon has been demonstrated by Hobkirk et al (1977) who showed that media separated from guinea pig liver tissue slices following agitation in similar conditions as an incubation

had the ability to metabolize estrogens. Superfusion eliminates this limitation by constantly infusing fresh media removing any cellular debris. A perfusion study was therefore employed to determine a temporal relationship of product formation by collecting perfusates at definite timed intervals from the same tissue slice.

Perfusion with E_2 indicates its rapid glucuronidation and sulfurylation. Decrease of $E_2$3G with a simultaneous increase of $E_1$3G points to the preferred direction of the oxido-reduction reaction of $E_2$3G → $E_1$3G in the guinea pig liver. The possibility of $E_1$3G formation via $E_2 \rightarrow E_1 \rightarrow E_1$3G, however, must not be overlooked.

The steady level of $E_1$3S via $E_2 \rightarrow E_2$3S → $E_1$3S and/or $E_2 \rightarrow E_1 \rightarrow E_1$3S throughout the perfusion indicates a central role of $E_1$3S in estrogen metabolism. Hobkirk et al (1977), employing batch incubations, have also noted the constant level of $E_1$3S from E_2 with increasing incubation time. Further metabolism of $E_1$3S to disulfate or deconjugation to unconjugated estrogen may occur. The perfusion with $E_1$3S has confirmed this latter possibility of deconjugation. Deconjugation of $E_1$3S to a more physiologically active estrogen via $E_1$3S → $E_1 \rightarrow E_2$ could represent one of the main functions of $E_1$3S.

(d) COMMENT ON TISSUE SLICE EXPERIEMTNS.

The observations noted in this investigation of guinea pig liver slices (decreased uptake of $E_1$3S by a metabolizing tissue in the presence of plasma proteins, less conversion to glucuronides for excretion and a constant level of $E_1$3S during the metabolism by the tissue) indicate the potential physiological importance of $E_1$3S.

Could it be possible that $E_1$3S acts as a buffer system for estrogen action? The rapid sulfation of estrogens by liver tissue controls the subsequent metabolic pathway preventing excessive unconjugated estrogens from being glucuronidated and lost by excretion. Cells at all times must be under delicate control. A pathological condition arises when control is absent. To protect against absence of control by estrogens just as the body has a mechanism to control pH, it may be possible that $E_1$3S could serve as a "hormonal buffer system". If, for some reason, such as an environmental effect, the pool of an active estrogen decreases, the reservoir of $E_1$3S is able to restore the temporarily decreased hormone. For example, Premarin, a conjugated sulfated estrogen preparation containing $E_1$3S as one of the major components, is employed widely to relieve post menopausal symptoms. This proposed buffer system may not only be true

for the most biologically active estrogen, E_2, but for other estrogen compounds, such as hydroxylated products which may possess biological activity other than the typical estrogenic activity.

6.2 MICROSOMAL INCUBATIONS

The observations that loss of radioactivity following a microsomal incubation with E_1 or E_2 required a supply of NADPH and adequate incubation time suggested that low recovery could be due to the formation of a metabolite (eg. a hydroxy product) binding to a microsomal protein. Investigation of the metabolites formed from a 105,000 xg supernatant plus microsomes following incubation with the same substrates (recovery was 90-100% in these incubations)† revealed no obvious hydroxy products other than 16α- or 16β-forms. Thus it would appear that the isotope "lost" or bound in microsomal incubations with free substrate is not likely to represent an additional major metabolite. Indeed, it may be that the loss is associated with the formation of 16βOHE_1, a steroid recognized to be rather labile, en route to the production of 16epiE_3.

Similar cases of irreversible binding of a metabolite of E_2 and E_1 to a microsomal protein fraction in rat liver have been reported (Jellinck et al, 1965).

† The increased recovery from a microsomal plus high speed supernatant incubation can be explained by the rapid sulfurylation of unconjugated substrate which seems to prevent "binding" to microsomal proteins.

These irreversibly bound metabolites in the rat have been shown to be 2-hydroxylated estrogens (Marks and Hecker, 1969; Kappus et al, 1973). Marks and Hecker (1969) have suggested that the 2-hydroxyestrogen metabolites are bound to proteins by thiol linkage. These studies have been extended to human liver microsomes with similar results (Bolt et al, 1974).

The physiological significance of this microsomal protein binding is unknown. Much emphasis has been placed on the intranuclear binding of estrogen as an indication of hormonal action. The possibility exists that estrogens in the form of a hydroxylated metabolite may exert an as yet unknown biological function at the level of the microsomes. Means and O'Malley (1972) in their review article on the mechanism of estrogen action on target tissues point out that, besides influencing transcriptional processes, estrogens may act at the translational level. Therefore, it may not always be necessary for the steroid to enter the nucleus to exert its activity. The liver may be unique in this respect. King et al (1965) compared the intracellular localization of estrogens in rat tissues. The major portion of the tritium labeled estrogen in the target tissue of anterior pituitary was associated with the nuclei, whereas in the liver it was

the soluble fraction which contained most of the tritium. Liver microsomes contained about 16% of the tissue tritium, compared to a nuclear content of only 5%. In further support of estrogens acting at the microsome level, Blyth et al (1971) have shown that rat hepatic microsomes contain sites that bind E_2. The binding sites were located on the smooth membrane of the endoplasmic reticulum and were more numerous in microsomes from male than from female rats. It is thought that this sex difference may be a result of the blocking of sites by endogenous estrogen in the adult female rat.

Binding to liver microsomal proteins may alter an enzymic activity, possibly modifying estrogen or some other metabolism. Hepatic carbonic anhydrase in rats has been reported to be induced by estrogen treatment (Garg, 1975). Plasma proteins have also been noted to be elevated following estrogen administration in the human (Musa et al, 1965). It may also be possible that some of the known effects of estrogens on lipid (Hagopian and Robinson, 1965; Fewster et al, 1972; Pertsemlidis et al, 1974) and carbohydrate (Sankaran and Prasad, 1974; Sladek, 1975) metabolism are mediated through hepatic changes.

Excessive modification or specificity may be achieved by sulfation of the estrogens which prevents binding to

the microsomes. Sulfated steroids are now known to possess specific hydroxylation pathways as will be discussed below.

It was originally thought that some 6-hydroxylation was occurring in the guinea pig (Harvey and Hobkirk, 1976). It appears that 16β-hydroxy steroids or artifacts of these formed by chemical manipulations could have led to that tentative conclusion. As stated earlier, 16βOHE$_1$ is a steroid recognized to be rather labile in a chemical sense.

The enzymes responsible for steroid sulfate synthesis are known to be located in the microsome-free supernatant (Roy, 1970). Thus, any considerable production of monosulfate metabolites from E$_1$3S by microsomes must arise without prior removal of the sulfate group and not by deconjugation followed by resulfurylation. The minor sulfurylation of unconjugated substrates from microsomal incubations, due probably to contamination of high speed supernatant supports this assumption. Direct hydroxylation of E$_1$3S has also been clearly shown in guinea pig liver slices (Hobkirk, Nilsen and Jennings, 1975; Hobkirk et al, 1977). Thus, in the guinea pig liver system employed in this study, the "steroid sulfate pathway" of estrogen hydroxylations is distinct from the "free steroid pathway" as described on the next page.

Free steroid pathway:-

Hydroxylation at carbon 16 in the β-configuration

$$E_2 \rightleftharpoons E_1 \rightarrow 16\beta OHE_1 \rightarrow 16epiE$$

Sulfated steroid pathway:-

Hydroxylation at carbon 16 in the α-configuration

$$E_2 3S \rightleftharpoons E_1 3S \rightarrow 16\alpha OHE_1 3S \rightarrow E_3 3S \rightarrow E_3 3,16diS$$

$$16\alpha OHE_1 3S \rightarrow 16\alpha OHE_1 3,16diS \rightarrow E_3 3,16diS$$

Conjugation with the sulfate molecule resulted in a major stereoisomeric change from the β- to the α- hydroxylated product but did not alter the specific carbon atom, namely C-16, hydroxylated. Data appear to indicate a small degree of 16α-hydroxylation of free steroid, although whether this could be due to activity towards $E_1 3S$, formed in minor amounts by contaminating high speed supernatant followed by sulfatase hydrolysis, is debatable. Certainly, no obviously direct 16β-hydroxylation of $E_1 3S$ has been detected. The only other studies indicating a difference in the hydroxylation between unconjugated and sulfurylated steroids is that of Gustafsson et al who have shown that the sulfate moiety has a directing influence on steroid hydroxylations in rat (Gustafsson and Ingelman-Sundberg, Rane and Gustafsson, 1975; Einarsson, Gustafsson, Ihre and Ingelman-Sundberg, 1976). These workers have shown that

sulfurylation of the steroid molecule tends to result in an alteration in the specific carbon atom(s) hydroxylated. The present investigation, which compared the metabolism of E_1 and E_2 and their 3-sulfates in female guinea pig liver microsomes, has extended these observations at least for the phenolic steroids, showing that sulfation results in a stereoisomeric change of hydroxylation and not merely in the particular carbon atom(s) hydroxylated.

The biological function of a distinct hydroxylating enzyme system for estrogen sulfates is at present unknown, although suggestions have been made. It has been suggested in the case of the human fetal liver (Ingelman-Sundberg et al, 1975) that hydroxylation of sulfated estrogens is a protective mechanism preventing the subsequent release of highly active estrogens by the placental sulfatase.

Excessive unconjugated estrogens reaching the endoplasmic reticulum may be hydroxylated in a specific position causing irreversible binding in extreme. It seems possible that estrogen sulfates, in particular $E_1$3S, may control this process of irreversible binding. In mammalian tissues, the endoplasmic reticulum is the dominant site of estrone sulfate hydrolysis, via sulfatase C, an arylsulfatase (arylsulfate sulfohydrolases, EC 3.1.6.1) (Roy, 1970).

$E_1$3S, reaching the endoplasmic reticulum undisturbed by attack from other enzymes (eg. glucuronidating mechanism), may be acted upon by sulfatase C releasing a controlled amount of unconjugated estrogen to be specifically hydroxylated for irreversible binding. A hydroxylated estrogen product required at a distal location and not at the endoplasmic reticulum may prefer the steroid sulfate pathway of hydroxylation. Thus, the sulfate molecule may act as a transport vehicle or a protective agent until the hydroxylated product reaches its destination. This postulated in vivo mechanism may explain the existence of a "steroid sulfate pathway" of hydroxylation distinct from a "free steroid pathway" of hydroxylation.

6.3 THE 16α-HYDROXYLATION PATHWAY OF ESTRONE SULFATE

The 16α-hydroxylation of E_1S by guinea pig liver microsomes offered a useful, relatively simple model for further study of this enzymatic activity. Although 16α-hydroxy metabolites of the estrogens have been recognized for more than forty years and are of major quantitative significance in the human, little specific information has been available regarding the properties of the hydroxylase responsible for their formation. One reason for this has been the lack of an adequate model for investigation purposes.

Initial studies of monitoring hydroxylase activity by disulfate formation were abandoned due to the indirect approach of this method. Too many variables were involved, such as the sulfate conjugation mechanism. Although it was believed that the rate-limiting step to the disulfate was hydroxylation followed by rapid sulfurylation, the possibility existed that in some guinea pig preparations the rate-limiting step might be one of sulfurylation. Incomplete analysis of hydroxylated product formation would, therefore, occur. Initially it was thought that the disulfate represented only one type of hydroxylated estrogen metabolite. In incubations with microsomes plus high speed supernatant, this is almost the case (see Table X)

as the major disulfate was $E_3$3,16αdiS. It should be noted that in guinea pig liver tissue slice experiments (Hobkirk et al, 1977), the disulfate was composed of greater amounts of 16α$OHE_1$3,16diS as compared to that found in microsomes plus high speed supernatant incubations. This, no doubt, is due to a variance in co-factor availability in the two methods of incubation. The presence of excessive NADPH in microsomes plus high speed supernatant incubations would cause the reduction of 16α$OHE_1$3S or 16α$OHE_1$3,16diS to $E_3$3S or $E_3$3,16αdiS, respectively.

The discovery of minor amounts of 16β-hydroxy-estrogen disulfates caused greater concern. Variation from one guinea pig to another with regard to its extent of 16β- vs 16α-hydroxylation would make it difficult to follow one particular hydroxylating system. Therefore, methodology had to be developed to separate the 16α-hydroxy products from the 16β-hydroxy products. The methodology of investigating monosulfates for 16α-hydroxy products following a microsomal incubation with $E_1$3S was employed as previously described. This approach enabled the 16α-hydroxylation pathway to be followed without interference or overshadowing by any other hydroxylase system. Difficulties encountered with solvolysis (low recoveries)

also favored using monosulfates for investigation.

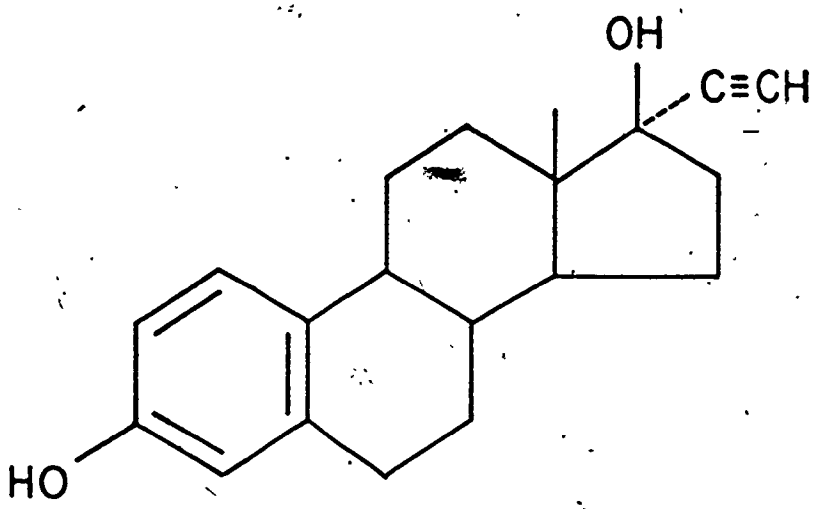
Properties of other steroid hydroxylating systems have been reported (Bolt et al, 1974; Gustafsson and Ingelman-Sundberg, 1974, 1975; Ingelman-Sundberg et al, 1975). The present investigation, however, appears to be the first detailed study of the 16α-hydroxylation of estrogens. The apparent Km value for $E_1$3S (3.55×10^{-5}M; Fig. 23) is the same order of magnitude as those reported by Bolt et al (1974) for the hydroxylations of 2,4,6,7-^{3}H estradiol and 2,4,6,7-^{3}H ethinyl estradiol as determined by the displacement of tritium in human liver microsomes. These workers reported that both compounds had identical Km values of 5.0×10^{-5}M for the microsomal ring A hydroxylases. It was assumed in their studies, because of the limited hydroxylation at C-6 and C-7 (only 4-5% of total tritium being displaced) that the total amount of hydrogen mobilized was a measure of the aromatic hydroxylation at C-2 and C-4. The Km for ethinyl estradiol was 3.6×10^{-5}M in rat liver microsomes from rats which had previously been treated with phenobarbital to induce the synthesis of the microsomal mixed function oxidases (Bolt et al, 1974).

Vmax determination for $E_1$3S in this study was 0.25 (Fig. 23) compared with that of 0.37 (E_2) and 0.74 (ethinyl estradiol) nmol/mg microsomal protein per min as

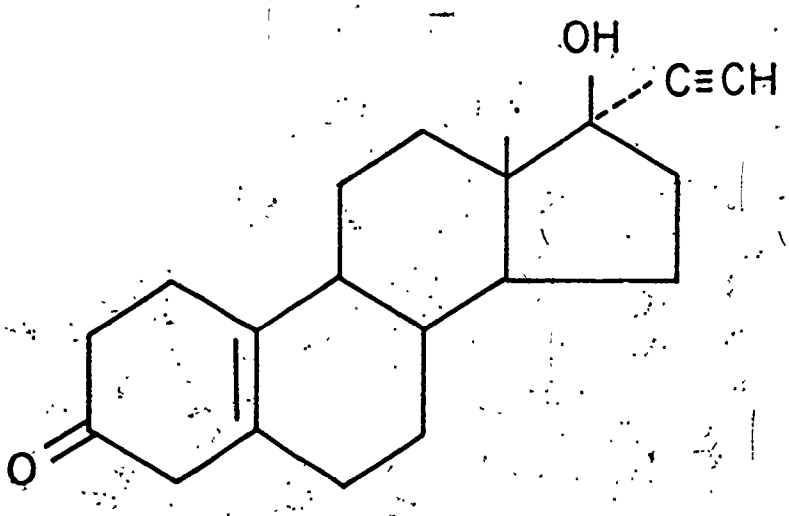
reported by Bolt et al (1974).

(a) INHIBITION STUDIES

Of the steroids tested, only norethynodrel and ethinyl estradiol had marked influence on 16α-hydroxylase activity. The similarity in structure of these two steroids is shown in Figure 42. Oral contraceptive steroids, such as ethinyl estradiol and norethynodrel, are themselves metabolized by microsomal enzymes (Bolt et al, 1974; Freudenthal et al, 1971). Although care was taken to ensure that sufficient cofactor (NADPH) was present in the incubation medium, the possibility could not be overlooked that inhibition may have been due to competition for available NADPH. The reported observation that the oxidized form of NADPH ($NADP^+$) inhibits drug hydroxylation in a competitive manner (Orrenius, 1965a) increased this possibility. The study of adding $NADP^+$ in excess of NADPH (Figure 19), however, indicated that the inhibition of 16α-hydroxylation caused by ethinyl estradiol and norethynodrel was far greater than that caused by the added $NADP^+$. In further support of the suggestion that the cofactor was not limiting was the observation that ethinyl estradiol, by blocking the hydroxylation reaction, increased the NADPH-dependent formation of $E_2$3S.
This latter observation is no doubt due to a mass action



ETHINYL ESTRADIOL



NORETHYNODREL

Figure 42. Structure of the synthetic steroids contained in some oral contraceptive preparations that were found to inhibit 16α-hydroxylase activity in this investigation.

effect.

It is striking that mestranol, differing from ethinyl estradiol only by the presence of a three methoxy group, had little effect on the 16α-hydroxylation. Femino et al (1974) reported similar observations in women, in vivo, who had been taking oral contraceptives. Urinary metabolites of radioactive E_1 and E_2 were measured in young women on oral contraceptive therapy. Subjects taking ethinyl estradiol-containing preparations were found to have a decrease in the 16α-hydroxylated urinary metabolites in the glucuronide fraction (i.e. 16αOHE_1 plus E_3) as compared to those taking preparations containing mestranol and those subjects who were taking no oral contraceptives. Femino et al suggested that their observations may be caused by decreased 16α-hydroxylation or to impairment of the reabsorption of 16α-hydroxylated metabolites from the gut. The present observations from this investigation suggest that a decrease in 16α-hydroxylated metabolites by inhibition of the 16α-hydroxylase might be a cause.

The kinetic data indicating competitive inhibition by ethinyl estradiol suggest both $E_1$3S and inhibitor have a common microsomal hydroxylase system, or at least a common part of the system. This latter pos-

sibility seems more logical in view of the recent findings of distinct hydroxylase systems (Lu et al, 1973; Guengerich, 1977) and the wide variety of substrates that may be hydroxylated. It is quite possible that distinct enzyme systems could share a common rate-limiting enzyme component.

The present investigation gives further support to previous reports that oral contraceptive steroids may interfere with the hepatic metabolism of endogenous substrates (Watanabe, 1969; Einarson et al, 1975). Although these observations in the present investigation have been made in vitro, an experimental in vivo model could be quite easily adopted. Recently, it has been shown (Hobkirk and Nilsen, personal communication) that following the injection of ^{3}H-E$_1$3S into guinea pigs, significant amounts of estrogen disulfates (primary 16α-hydroxylated metabolites) are excreted in the urine. Intact guinea pigs could therefore be used to monitor hydroxylation activity and the possible influence of administered steroids or other substances on this activity. This experimental model would offer the advantage of each guinea pig acting as its own control. The disadvantage of variability of hydroxylase activity would also be eliminated as selected strains of guinea pigs could be employed.

The widespread use of natural and synthetic steroids for therapy, especially in the form of oral contraceptives and for postmenopausal women, requires an understanding of the biochemical alterations which these hormones may produce in the organism. Alterations in natural pathways of steroid metabolism, such as E_2, might be related to etiological factors of a particular pathogenic state. Strong association between synthetic female sex hormones and various pathological manifestations have been reported (Boston Collaborative Drug Surveillance Program, 1974; Drill, 1974; Adlercreutz and Tenhunen, 1970). Further insight on how synthetic steroids, as well as other factors, may interfere with the metabolism of endogenous estrogens may be important in the elucidation of the mechanisms involved in these physiological changes of the organism.

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