SULFURYLATION OF STEROIDAL ESTROGENS BY A RAT LIVER PREPARATION.

BY

H.R. RAUD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF BIOCHEMISTRY,
McGILL UNIVERSITY.
MONTREAL, CANADA.

APRIL, 1965.

ACKNOWLEDGMENTS.

To Dr. R. HOBKIRK I would like to express my deepest appreciation of the advice, helpful criticism and support in this work as well as for the provision of laboratory facilities in the McGill University Medical Clinic, The Montreal General Hospital.

I am very grateful to Dr. O.J. LUCIS for his keen interest and especially for his many valuable suggestions.

The financial support of the Banting Research Foundation is gratefully acknowledged.

It is a pleasure to acknowledge the invaluable aid and technical assistance of Miss M. Nilsen and the preparation of several samples by Mrs. E. Purre, a fellow graduate student.

In additon, I wish to thank the typist of this manuscript, Miss D. Richards, for her skilful assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE OF STEROIDS

TRIVIAL NAME	SYSTEMATIC NAME
Dehydroisoandrosterone	3B-hydroxy-androst-5-ene-17-one
Estrone	3-hydroxy-estra-1, 3, 5 (10)-triene-17-one
Estradiol - 17β	3, 17β-dihydroxy-estra-1, 3, 5 (10)-triene
Estriol	3, 16α -17β-trihydroxy-estra-1, 3, 5 (10)-triene
16 - epiEstriol	3, 16β-17β-trihydroxy-estra-1, 3, 5 (10)-triene
16 - Ketoestradiol - 17β	3, 17β-dihydroxy-estra-1, 3, 5 (10)-triene-16-one
Estrone - 3 methyl ether	3-methoxy-estra-1, 3, 5 (10)-triene-17-one
Estradiol - 17β - 3 methyl ether	3-methoxy-17β-hydroxy-estra-1, 3, 5 (10)-triene
Estrone - 3 - sulfate	estra-1, 3, 5 (10)-triene-17-one-3-yl sulfate
Estradiol - 17β - 3 - sulfate	17β-hydroxy-estra-1, 3, 5 (10)-triene-3-yl sulfate
Estradiol - 3, 17β - disulfate	estra-1, 3, 5 (10)-triene-3, 17β-diyl sulfate
Estradiol - 17β - 17 - sulfate	3-hydroxy-estra-1, 3, 5 (10)-triene-17β-yl sulfate

INTRODUCTION

A. EARLY STUDIES

The problem of sulfate and glucuronide conjugation of naturally occurring compounds, specifically steroids, has intrigued investigators for many years. The early workers regarded conjugation as only a form of detoxification. It was not until much later that a wider significance was applied to this phenomenon, as that of an important process in the activity or metabolism of these compounds.

Due to the studies in the present work dealing almost exclusively with estrogen sulfurylation, emphasis will be placed upon the development of knowledge in this area.

As early as 1829 Henry (1) recognized that two forms of sulfate were present in human urine, one form requiring an excess of acid to be precipitated as the barium salt. Normal and pathological urines containing indigo-forming substances were found by Baumann (2,3) to be decomposed by glacial acetic or hydrochloric acids, yielding sulfuric acid, the latter being a compound of phenols, catechols and indigo - forming substances. Many of these conjugates were found to be non-toxic which led to ideas that conjugation and detoxification were related. Upon administering indole to animals, Baumann (4) found that it was combined with an organic radical in urine later found to be glucuronic acid.

In 1899 Herter and Wakeman (5) suggested the possible production of phenol sulfate by liver tissue <u>in vitro</u>. They

obtained evidence indicating combination of the phenol with the "living protoplasm" of the liver cells, "while the neutralization of the poison through combination with sulfuric acid is taking place." Further early work on phenol conjugation with sulfuric acid in both liver and intestinal tissue was carried out by Embden and Glassner (7), Embden (6) and Marenzi (8).

It was not until 1929, with the crystallization from human pregnancy urine of the first of the three classical estrogens, namely estrone (9), estriol and estradiol - 17 β (10), that the preliminary observations of the conjugation of estrogens were made by Glimm and Wadehn (11). They showed that estrogens are excreted via the kidney in a form which cannot be extracted by organic solvents. It was suggested that unextracted estrogens may have been present in combination with an acid. Subsequently Marrian (12,13) and Doisy et al. (14) found that yields of extractable estrogenic material from pregnancy urine could be increased by preliminary acidification of the urine. Veler (15) and collaborators attributed this to the formation of an ether soluble, non-hydrophilic, hydrogen compound. A tentative proposal was made that the urinary estrogens were glucuronic or sulfuric acid conjugates (13).

In 1932, Collip and associates (16) isolated ether insoluble "emmenin" from human placentas. Orally this material had the potency of estriol, but when injected into rats its activity decreased (17). It was suggested that the placental material was an estriol ester (18).

Up to 1934, scant attention had been paid to the problem of the chemical nature of the "combined" ether insoluble, acid hydrolyzable estrogenic substances present in human and equine pregnancy urines. Using the basic Kober method for "estrin" (19), Cohen and Marrian (20) modified it for the separate quantitative estimation of estrone and estriol in etheral extracts of human pregnancy urine. This allowed for a more complete study of factors influencing hydrolysis by acids of "estrin - esters" in urine (21), which in turn provided evidence regarding the stability of the compounds so that methods for their purification could be devised. Aided by this, Cohen and Marrian (22,23) isolated and identified estriol mono - glucosiduronate from human pregnancy urine. The compound was identified as a uronic acid function attached to estriol at the 16 or 17 position by a glycosidic linkage involving the aldehyde group of the former (23). The phenolic hydroxyl of estriol was unconjugated as shown by the Callow spectrophotometer technique (24). It was not until 1950, that Grant and Marrian (25) conclusively proved that the uronic acid moiety was D - glucuronic acid.

At about the same time as Marrian first isolated the estriol glucosiduronate, Schachter and Marrian (26) attempted to characterize the conjugated estrogen in pregnant mares' urine. They had only limited success, partially purifying an estrone complex from alkali insoluble material in the sodium hydroxide washed butanol extract of acidified urine. They obtained, nevertheless, some evidence on the probable chemical nature of the compound(s). The material was ether insoluble, water soluble, contained no glucuronic acid and sulfur

was present. The conjugating group blocked the phenolic hydroxyl. This was followed by the crystallization and positive identification of the potassium salt of estrone sulfate (27).

Discovery that there naturally occur two main forms of estrogen conjugates led workers to specialize, delving more deeply into the study of either sulfurylation or the problem of glucosiduronate formation. Consequently, from this point onwards, these topics will be treated separately, although there is frequent overlap due to the obvious related significance of the phenomena.

e.g. a) Trans-conjugation of estriol-3-sulfate into estriol glucosiduronates in human fetus and newborn (40).

b) Mutual competition between glucuronide and sulfate synthesis, one inhibiting the other (83).

B. CONJUGATION AS SULFATE

Following the isolation of estrone sulfate by Schachter and Marrian, a search for other endogenous steroid sulfate esters began. Venning and Brown (28,29) in 1942 crystallized a conjugated androgen, androsterone sulfate, from the urine of a man suffering from an interstitial cell tumor of the testis. Hydrolysis of the purified homogeneous conjugate indicated that the hydroxylated androgens, androsterone and androsten-17-one, were both products when the compound was heated with acid. Hydrolysis removed sulfate. Munson et al. (30) isolated a 17-ketosteroid sulfate in the form of a semicarbazone from normal mens' urine, which on hydrolysis yielded dehydroisoandrosterone.

This sulfate conjugate was later crystallized and identified as dehydroisoandrosterone sulfate (31). Drayer and co-workers (32) crystallized cholesterol sulfate from bovine adrenals, present in quantities of at least 1.5 mg/kg. of tissue. An infra-red spectrum and melting point determination confirmed the identity of the material.

These are the only endogenously occurring steroid sulfates, which to the knowledge of the author, have been isolated, crystallized and identified. However, in vivo injection and perfusion of exogenous material has resulted in the extraction and characterization of steroid ester sulfate metabolites from human and animal matter.

Butenandt and Hofstetter (33) suggested that conjugated estrone present in human pregnancy urine was identical with estrone sulfate. Cohen and Bates (34) found a large amount of conjugated estrone in the urine, which could be hydrolyzed by phenol sulfatase. However, it was found in 1952 that most of the estrone in pregnancy urine is present as the glucuronide (35). Nevertheless, McKenna et al. (36) by use of counter current partition, hydrolysis, paper chromatography and infra-red analysis showed conclusively that some estrone hydrogen sulfate could be detected in human pregnancy urine. Two hours after intravenous injection of estradiol-17β-16-^{14}C into human patients, estrone sulfate was found in the supernatant solution of Cohn fraction IV-1 of the plasma by Purdy and associates (37,38) as the primary radioactive metabolite. They also detected endogenous estrone sulfate in plasma of pregnant women. Estrone

sulfate is therefore the major human circulating estrogen. Levitz et al. (39), upon administering estrone-^{14}C to the mother, found sulfates of estrone and estradiol-17β in the umbilical circulation of the human fetus. Evidence was presented that the placenta sulfurylated the estrogens and released them into the umbilical circulation. Sodium estriol-3-sulfate has been purified and characterized in human meconium extracts, cord-blood and as a minor component in ammiotic fluid and urine of newborns (40,41). It has not been crystallized.

Diczfalusy et al. (42) studied the nature of the conjugated estrogens formed by the human fetus in vivo following intraamniotic administration or perfusion of estradiol-17β and estriol respectively. Estradiol-17β treatment yielded sodium-estradiol-3-sulfate and sodium-estrone sulfate in lungs, liver and kidneys. Fetuses after estriol treatment were found to contain sodium-estriol-3-sulfate in lungs, liver, and kidneys. Intestines of untreated fetuses contained some sodium-estriol-3-sulfate. Administered estrone-16-^{14}C in 3 human fetuses of 3-5 months gestation was found to be one-third conjugated (43). Enzymatic hydrolysis and paper chromatography, showed these to be virtually all estrogen sulfates.

If rabbits, rats and guinea pigs are dosed with 2-naphthylamine, aniline and 1-naphthylamine, isolation of 2-naphthylamine sulfamate, phenylsulfamic acid and naphthylsulfamic acid, respectively, has been effected by Boyland and associates (44). Foggitt et al. (45), after intra-muscular administration of corticotrophin to normal human subjects extracted steroid sulfate and glucuronide conjugates

from urine by adsorption on alumina. Injection of cholesterol-7α--^{3}H sulfate-^{35}S into the artery supplying the adrenal tumor of a patient is converted into urinary dehydroisoandrosterone sulfate (46).

In vitro, studies with various tissue slices, homogenates and cell-free fractions of human and animal origin have constituted by far the major type of experiment performed. Sulfurylation by the liver will be emphasized, as it pertains directly to investigations performed by the author.

Conjugation of phenols in vitro with sulfate by rat liver and intestinal tissue has been recognized for many years (5,6,7,8). On incubating rat tissues for two hours in Ringer solution at 37.5°C, Arnolt and DeMeio (47,48) found phenol conjugation in liver, intestine, spleen but none in adrenals, brain, diaphragm, heart, kidney, ovary, stomach, uterus or testicle. In the cat, liver, kidney and small intestine were involved in conjugation.

The obvious similarity of phenols and the A ring of estrogens led to investigations of sulfurylation of estrogens and other steroids by various tissues. Thus Crepy (49) on incubation of estrone, estradiol-17β and estriol with rat liver slices found estrone sulfate produced, although estradiol-17β and estriol were conjugated for the most part with glucuronic acid. Using the soluble fraction of rat liver homogenates for incubation in the presence of ATP, Mg^{++} and estrone-16-^{14}C, Segal (50) found estrone sulfate. Soluble enzymes of the spleen, kidney and heart had no conjugating activity.

Rat liver soluble enzyme fraction after ultracentrifugation and

ammonium sulfate precipitation, with added Mg^{++} and ATP was found to sulfurylate a large number of compounds including phenols, arylamines, and many different steroids (51,52,53). Schneider and Lewbart (54) incubated 37 steroids in a system consisting of the microsome-free supernatant of rabbit liver, ATP, sulfate ion, and Mg^{++}. Fourteen of these steroids including testosterone and deoxycorticosterone were conjugated with sulfate. Identifications were performed by paper chromatography in an aqueous ammonia: ethyl acetate: butanol system. Synthesis of charonin sulfuric acid in the presence of p-nitrophenyl sulfuric acid, as the sulfate donor and an acetone powder of the mucous gland of Charonia lampas (shell-fish) was observed by Suzuki et al. (55) and serves as an example of a transsulfation reaction. De Meio and associates (56) presented direct evidence for the biosynthesis of dehydroisoandrosterone, testosterone, estrone and estradiol - 17β sulfates in vitro by ox-liver preparations. Sneddon and Marrian (136) upon incubation of bovine-adrenocortical minces with estrone-16-^{14}C detected the formation of estrone sulfate.

It was not until the 1960's that much of the in vitro work with fetal tissues was done. Thus Diczfalusy et al. (57) incubated slices of human fetal liver, lungs, kidneys, adrenals and also skeletal muscle with estriol, and obtained a significantly increased concentration of conjugated estriol. The evidence suggested that this conjugated estriol might be estriol-3-sulfate. No such increase was found with adult human endometrial or myometrial tissue.

Although many of the fetal tissues were found to sulfurylate

steroids, it had been generally assumed that the liver was the major site of production of these compounds in the adult human, as well as in animals. Revision of this view became necessary with the demonstration that the steroid conjugate androstenolone sulfate can be synthesized in vitro by human adrenocortical tissue (58,59,60). Vande Wiele and associates (130) identified dehydroisoandrosterone sulfate as a secretion product of $C_{19}O_2$ androgens by adrenals or gonads. This has been followed by a further discovery that the ovary, another steroid-producing tissue, can also synthesize steroid sulfates. Androstenolone and Δ^5- androstene - 3β, 17β-diol can be converted to sulfate esters by minces and homogenates of normal human ovaries (61). This suggests that steroid sulfate synthesis may be a more general property of steroid - forming endocrine tissue than was previously recognized.

In vitro studies have elucidated the mechanism of sulfurylation, again with the aid, mainly, of rat liver preparations. From the findings it can be assumed that estrogen hydrogen sulfates are formed in the same manner as are other sulfate esters.

In 1950, De Meio and Tkacz (62) observed that a properly fortified homogenate of rat liver conjugates phenol with sulfate, while slices of liver bring about formation of both sulfate and glucuronide derivatives. The system involved in phenyl sulfate formation seemed to be localized between mitochondria and mitochondrium-free supernatant, after centrifugation at 600 g, both fractions being required for activity (62,63). The same workers (63) noticed that addition of 2,4 - dinitrophenol inhibited phenol

conjugation. A similar effect was observed with methylol gramicidin and 0.01 M azide, indicating that sulfurylation probably involves phosphorylation (63).

The requirements of the soluble enzyme system narrowed down to simple addition of ATP to the reaction mixture. The observation that mitochondria are required for activity applies only if ATP is omitted. De Meio et al. showed that "microsome-free" supernatant solutions of rat liver could be used for the in vitro synthesis of phenyl sulfate when supplemented with ATP and Mg^{++}. If AMP is added to this supernatant, mitrochondria must be present to synthesize ATP by aerobic phosphorylation (64). This was aptly confirmed by Bernstein and McGilvery (65), who, using m-aminophenol and potassium sulfate, found m-aminophenyl sulfuric acid produced by the high speed supernatant fraction of the homogenate. The entire synthetic system was found in this fraction if supplemented with ATP and Mg^{++}. Ammonium sulfate fractionation was found to partially purify the enzyme system. It was shown (66) that the enzymatic activation of inorganic sulfate by ATP is a preliminary step for conjugation with m-aminophenol. Segal (67) demonstrated that increasing amounts of ATP added to the in vitro reaction mixture inhibited the first or activating step of the reaction. Hilz and Lipmann (68) stated, in 1955, that formation of "active sulfate" is probably the common first step of biosynthesis of sulfuric acid esters.

Transfer of the sulfate from the "active sulfate" to the substrate is probably mediated by specific transferring enzymes. This was followed by De Meio and associates (69) who demonstrated

that the formation of aryl sulfates requires a sulfate-activating enzyme system and a sulfate-transferring system, which transfers sulfate from the "active sulfate" to a phenol. Hilz and Lipmann (68) described a method by which "sulfate activating" enzyme may be prepared from the whole soluble fraction by adsorption of the "sulfate transferring" enzymes (sulfokinases) on alumina. This was followed by identification of two enzymes involved in sulfate activation, as well as description of the "active sulfate" complex (70,73). The enzymes were found to be adenosine triphosphate sulfate adenyl transferase and adenosine triphosphate adenyl sulfate-3'-phospho transferase, the "active sulfate" complex being adenosine-3'-phosphate-5'-phospho sulfate. It was shown that this same system is present in yeast, that it is just as active with selenate as sulfate and that at least two separate, heat labile, protein fractions are required for sulfate activation (71,74).

Robbins and Lipmann further observed a reversible reaction between adenosine-3'-phosphate-5'-phosphosulfate (PAPS) and acceptor substrate, in this case p-nitrophenol (72). In 1956 Roy showed that the enzyme system synthesizing dehydroisoandrosterone sulfate is identical with that responsible for the biosynthesis of aryl sulfates (52). This was followed by the work of Gregory and Nose (144) who noted that transfer of sulfate from PAPS to phenolic compounds and to non-phenolic steroids was due to two different enzymes (sulfokinases). One produces sulfates of steroids containing a 3β-hydroxyl group in the A ring, and the other transfers

sulfates to many phenolic compounds. Estrogens are sulfurylated by the phenol sulfurylating fraction. Phenol sulfokinase was further separated into two separate kinases by Nose and Lipmann (75) one being estrone sulfokinase, and it was suggested that steroid sulfokinases are of a large family, with more or less sharply developed specificity. Roy (76) demonstrated the synthesis of 2-naphthyl sulfamate from PAPS, catalyzed by a specific sulfokinase, arylamine sulfokinase.

Segal (77) emphasized the necessity, at least for in vitro activity, of magnesium ion. Variations in concentration of the latter had pronounced effects on both sulfate activation and esterification.

Species differences are few and far between for the general phenomenon of sulfate activation and transfer, but to a small degree they exist. Thus Roy (78) found that enzyme preparations from guinea pig or rabbit liver synthesized aryl sulfamates at a much greater rate than rat liver preparations. 2-Naphthylsulfamate synthesis by rat liver soluble enzymes is strongly activated by the presence in the reaction mixture of 17-oxosteroids. Guinea pig liver enzymes are not affected in this way (76). The presence of 3β - methoxyandrost - 5 - en - 17 - one strongly inhibits guinea pig arylamine sulfokinase but activates rat arylamine sulfokinase (79). Pasternak and co-workers (80) found activation of sulfate in extracts of calf, rabbit, guinea pig and rat corneal epithelium and stroma. Addition of vitamin A did not decrease activation in any of the tissues except that of rabbit corneal

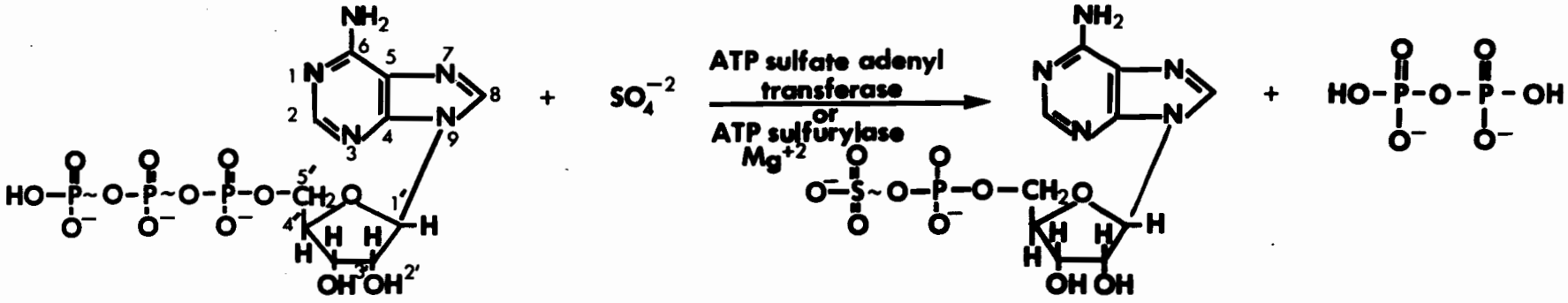
stroma.

The biosynthesis of sulfuric acid esters, both of steroids and of other compounds, is aptly summarized by Lipmann (81). He stresses that one is dealing with a process in which a central activated molecule is first elaborated. This carries the sulfate in activated form, and from it, sulfate is picked up by quite a large number of separate acceptor enzymes, which we call sulfokinases. This in fact is a repetition of a rather general scheme in biosynthesis. It has been well defined in the case of acetyl activation and transfer (82).

FIGURE I

ACTIVATION AND TRANSFER OF SULFATE

1.



Adenosine-5′-triphosphate (ATP) + SO_4^{-2} → Adenosine-5′-phosphosulfate + Pyrophosphate

1.(a) Adenosine-5′-diphosphate (ADP) + SO_4^{-2} —(ADP sulfate adenyl transferase or ADP sulfurylase/Mg^{+2})→ Adenosine-5′-phosphosulfate + Pi

2. Adenosine-5′-phosphosulfate + ATP —(ATP adenyl sulfate 3′-phosphotransferase or APS phosphokinase/Mg^{+2})→ Adenosine-3′-phosphate-5′-phosphosulfate (PAPS) + ADP

3. PAPS + ROH —(Sulfokinase; e.g. Phenol sulfokinase, Estrone sulfokinase, Steroid sulfokinase, Arylamine sulfokinase)→ R-O-SO$_3^-$ + Adenosine-3′-5′-diphosphate

C. CONJUGATION AS GLUCOSIDURONATE

The first steroid glucosiduronate was isolated and identified by Cohen and Marrian (22) and Cohen, Marrian and Odell (23), as estriol mono-glucosiduronate from human pregnancy urine. Recently Carpenter and Kellie (84,85) have shown that estriol glucosiduronate from this source contained two isomers, estriol - 16α- -glucosiduronate and estriol - 17β - glucosiduronate, although only the former could be isolated by Neeman and Hashimoto (86). Another type of estriol glucosiduronate was detected in pregnancy urine by Beling (87,88,89) using gel filtration on Sephadex. This was estriol - 3 - glucosiduronate.

The possible presence of di and tri-glucosiduronates has also been considered. Felger and Katzman (90) resolved commercial preparations of estriol glucuronide into two components by paper and column chromatography. One fraction was mainly estriol mono-glucuronide, the other mainly estriol di-glucuronide. Whether the latter is the same compound as estriol - 16 (17?) - glucosiduronate, found by Troen et al. (91) in cord blood, amniotic fluid and urine of newborn, is not known. Troen and associates also suggested the presence of estriol tri-glucosiduronate in the above body fluids.

Another kind of double conjugate has been detected by Straw and co-workers (92). The compound is estriol containing one molecule of glucuronic acid and one of sulfuric acid. Troen et al. (91) also suggested the possible presence of estriol - 3 - sulfate - 16 (17?) - -glucosiduronate or estriol - 3 - sulfo-glucuronide in cord blood,

amniotic fluid and urine of newborn.

Steroids other than estrogens have also been found to be conjugated with glucuronic acid. Thus, pregnanediol conjugate was simultaneously isolated in crystalline form, from an alkali washed butanol extract of pregnancy urine, by two groups of workers (93,94). Peterson and associates (95) proposed that androgens are also conjugated with glucuronic acid. They identified hexuronides of androsterone and dehydroandrosterone in fresh urine of man, but did not isolate them. It has been demonstrated that labelled testosterone can be conjugated by human liver slices (96,97) with glucuronic acid at C_{17} (98). Of the adrenocortical steroids, only tetrahydrocortisone glucuronide has been crystallized from human urine (99).

Conjugation of various compounds with glucosiduronic acid takes place mainly in the liver, kidney and gastrointestinal tract of both humans and animals.

In 1939 Lipschitz and Bueding (100), using rats, found the liver to be the chief organ of glucuronic acid conjugation. This occurs to a much lesser extent in kidney slices. Incubation of estrone, estradiol - 17β, and estriol with liver slices produced estradiol - 17β and estriol glucuronide as well as estrone sulfate (49). Other workers who demonstrated glucuronide synthesis by the liver were Storey (83) and De Meio and Tkacz (62,63). Using rat tissue slices, Shirai and Ohkubo (101) demonstrated active glucuronide synthesis in the liver, kidney, mesenterium and especially in the intestines. In vitro studies in the presence of O-aminophenol showed glucuronide formation by rat liver, kidney,

slices of mucosa of gastric pylorus, duodenum, jejunum, ileum and colon (102). Hartiala (103) has reported that slices of rabbit, rat, and dog intestine conjugate O-aminophenol with glucuronic acid. Schachter and associates (104) have found that the carboxyl and phenol groups of salicylate are conjugated with glucuronic acid by slices of intestine, liver, kidney, urinary bladder and spleen. Menini and Diczfalusy (105) isolated and identified estriol glucosiduronate in the human meconium using electrophoresis, enzymatic hydrolysis and partition chromatography. The area of glucosiduronate production in the gastro-intestinal tract of the human fetus has been localized (106). Most of the conjugation takes place in the jejunal loop followed by the intra-gastric area. None is found in the large intestine.

Oneson and Cohen (35) found that a large portion of estrone in human pregnancy urine is conjugated as the glucuronide. 2 - -Naphthylamine N - glucosiduronate has been isolated from urine of rabbits dosed with 2 - naphthylamine (44). Estriol - 16α - glucosiduronate and estriol - 17β-glucosiduronate have been detected in pregnancy urine (87,88). After intra-muscular administration of corticotrophin to human patients, steroid glucuronide has been extracted from urine (45).

It is known that the biochemical mechanism for formation of estrogen glucosiduronates is the same as that of acids, aliphatic alcohols, hydroxybenzenes and neutral hydroxysteroids.

Shirai and Ohkubo in 1954 (101) noticed that glucuronide conjugation is augmented sharply by the presence of glycogen and

glucose - 1 - phosphate. Dutton and Storey (107,108) isolated, in the pure state, a nucleotide containing uridylic acid, labile phosphorous and glucuronic acid, the structure of which was determined (109). Uridine diphosphate glucose has been shown to be formed in a 2 step reaction (110,111).

Uridine diphosphate (UDP) + ATP $\rightleftharpoons$ Uridine triphosphate (UTP) + ADP.

Uridine triphosphate (UTP) + Glucose-1-phosphate $\rightleftharpoons$ Uridine diphosphate glucose (UDPG) + pyrophosphate.

The uridine diphosphate glucose is oxidized to uridine diphosphate glucuronic acid (UDPGA) which is the active form of glucuronic acid.

Smith and Mills (112) found that both uridine diphosphate acetylglucosamine and uridine diphosphate glucuronic acid as well as uridine triphosphate occur in liver. There are enzymes in the liver nuclei which can act upon these compounds.

Storey and Dutton (109) also confirmed that the reaction between uridine diphosphate glucuronic acid and acceptor (ROH) consists of uridine diphosphate glucuronic acid (UDPGA) + ROH $\longrightarrow$ Uridine diphosphate + R-O-glucuronic acid.

Isselbacher (113) first described the complete enzyme system for estrogen conjugation with glucuronic acid.

$$\text{UTP + glucose-1-phosphate} \xrightleftharpoons{\text{UDPG pyrophosphorylase}} \text{UDPG + pyrophosphate}$$

$$\text{UDPG + NAD (DPN)} \xrightleftharpoons{\text{UDPG dehydrogenase}} \text{UDPGA + } NADH_2 \text{ (DPNH + } H^+\text{)}$$

$$\text{Aglycon + UDPGA} \xrightleftharpoons{\text{UDP glucuronyl transferase}} \text{glucosiduronate + UDP}$$

The enzyme glucuronyl transferase has been found in microsomes of mammalian liver, kidney-cortical and gastrointestinal mucosal cells (114). Although the steps have been elucidated by in vitro means, Dutton and Stevenson in 1959 (115) showed that this process also probably operates in intact kidney and gastrointestinal tract by the isolation of uridine diphosphate glucuronic acid from these tissues.

Smith and Breuer (116) have confirmed the work of Isselbacher by incubating estrone with rabbit liver microsomes, at pH 8.2, in the presence of uridine diphosphate glucuronic acid and isolating estrone mono-glucosiduronate. By use of this system it should be possible to determine the substrate specificity of the glucuronyl transferase for the various estrogens and to determine the chemical nature of the different conjugates.

D. MISCELLANEOUS CONJUGATION

Steroid complexes involving groups other than sulfuric or glucuronic acid are known to occur in vivo.

Rakoff and co-workers (117) presented good evidence for protein binding of the blood estrogens, since the estrogenic material did not pass through a collodion membrane, did precipitate with the protein fraction and could not be recovered from the precipitate by extraction with ether - ethanol. Estrogens were found to be mainly associated with serum albumin and with several of the fractions containing globulins, if fractionation of plasma protein was performed according to Cohn fractionation technique (118,119,120,38). It has been suggested that a rather loose

connection exists between proteins and steroids (118,122) which indicates that the complex formed is not due to conjugation but "binding" of some kind. Protein-bound circulating estrogens are present in conjugated form (estrogen sulfate) rather than the free form (38), suggesting that conjugated estrogens play an important physiological role. Rather conflicting results suggest that the problem of protein binding is far from being settled.

Possible conjugation of adrenal cortical hormones with amino acids has been reported by Eades et al. (123)

Weichselbaum and Margraf (124) isolated for the first time a C_{21} steroid acetate, 11-dehydro-corticosterone acetate, from normal human plasma. This may be significant in relation to the steroid transport mechanism. In 1961, Oertel (125) isolated conjugated 17 - oxo - steroids containing sulfate, phosphate and lipids, suggesting the possibility of conjugation of estrogens with acetate or phosphate. Finally, Layne et al. (153) isolated the N-acetylglucosamine conjugate of radio - active estradiol - 17α from rabbit urine after administration of estrone - 16 - ^{14}C.

E. SIGNIFICANCE OF CONJUGATION

Conjugation of estrogens is of great physiological importance in the metabolism of these steroids. It has been established that the liver plays the most important part in the overall metabolism of estrogen molecules. On the other hand there are certainly other tissues sharing in this activity, the intestine, the kidneys, the lungs and the blood being the most

important.

It was originally believed that conjugation was solely a form of inactivation or detoxification of steroids, but this is frequently not the case. Fishman (127) and Fishman and Fishman (126) interpreted the synthesis of estrogen glucosiduronates in the uterus as some sort of activation of the estrogens. The binding of estrogens to protein has also been expounded as an activation of the steroid. Roberts and Szego (128) showed that in partially hepatectomized rats uterine response to estrogens was enhanced during active liver regeneration. Activation of the estrogens was believed to be due to binding to a protein in the regenerating liver. Some types of conjugated estrogens have been found to be as active as the free forms.

This was followed by the suggestion that estrogen conjugation is the formation of more water soluble transport and excretion products of the steroids. It was believed that the conjugates were the end products of steroid metabolism and that the liver was the major site of their production. Now it seems that this view requires some revision due to the discovery that many conjugates undergo further transformations in vivo. This applies particularly to steroid sulfates. Twombly and Levitz (129) investigated the fate of injected estrone - ^{14}C and estrone - ^{14}C sulfate and characterized the metabolic transformation products in bile and urine. Purdy et al. in 1961 (38) noticed that although estrone sulfate is a major circulating estrogen very little was excreted as such in the urine. The fact that estrone sulfate is the

predominant estrogen metabolite in the blood suggests that this conjugated form of estrone is perhaps the main physiologically active circulating estrogen. Dehydroisoandrosterone sulfate has been identified as one of four secretion products of C_{19} O_2 androgens by the adrenals and gonads. The products are peripherally interconvertible (130). Intravenous administration of estradiol - 6, 7 - ^{3}H and estrone - 16 - ^{14}C to patients and calculation of $^{3}H/^{14}C$ ratios led Fishman et al. to imply that conjugation is involved in the oxidation of estradiol to estrone in humans (143).

Previously it had been suggested that in organs where steroids are elaborated they occur predominantly in unconjugated (free) form, whereas in other parts of the organism they appear to be present mainly in a conjugated form as glucosiduronates or sulfates. Here again some revision is required due to submission of new evidence. Androstenolone sulfate is seen to be produced by adrenocortical tissue and gonads (58,59,60,130), while Δ^5 - androstene - 3β, 17β diol and androstenolone is sulfurylated by human ovaries in vitro (61). Estrone sulfate production by bovine adrenocortical minces has been shown by Sneddon and Marrian (136).

The implications therefore are that steroid sulfates, glucuronides, and possibly other minor conjugates, while being detoxification and excretion products may also be involved, as actual intermediates, in many metabolic reactions, especially in the case of sulfate conjugates. They are not simply end products of reactions, conjugated by the liver for excretion, but appear

as valuable intermediates produced by tissues which synthesize and metabolize steroids as well as those which only metabolize them.

On the basis of knowledge outlined, the following work was undertaken.

EXPERIMENTAL

PURPOSE OF THE INVESTIGATION.

The object of this work was to obtain information on the *in vitro* sulfurylation of steroidal estrogens.

Two main aspects of the problem were considered; first, the effect of estrogen molecular structure on the degree of sulfurylation along with the mutual effect of steroids upon each other; secondly, the effect of animal age and condition on this mechanism.

MATERIALS

A. SUBSTRATES AND COFACTORS.

1. RADIOACTIVE COMPOUNDS.

Solutions of estrone - 16 - ^{14}C (specific activity - 50 uc./mg.) were purchased from the Radiochemical Centre, Amersham, England. Estriol - 16 - ^{14}C (specific activity - 22 uc./mg.) and 16 - ketoestradiol - 17β - 16 - ^{14}C (specific activity - 22 uc./mg.) was kindly donated by Dr. M. Levitz (N.Y.U.). Estrone - 6, 7 - ^{3}H (specific activity - 150 uc./ug.) and estradiol - 17β - 6, 7 - ^{3}H (specific activity - 150 uc./ug.) were obtained from the New England Nuclear Corp., Boston. Dehydroisoandrosterone - 4 - ^{14}C (specific activity - 141 uc./mg.) was acquired from Merck, Sharp and Dohme, Montreal. Standard solutions of these steroids were prepared with analytical reagent (A.R.) grade methanol (aldehyde and ketone free).

Reduction of part of the standard solution of 16 - keto-estradiol - 17β - 16 - ^{14}C, diluted with unlabelled carrier and resulting in a final specific activity - 0.44 uc./mg., yielded 16 - epiestriol - 16 - ^{14}C (specific activity - 0.23 uc./mg.). Methylation of estrone - 6, 7 - ^{3}H and estradiol - 17β - 6, 7 - ^{3}H standard solutions produced estrone - 6, 7 - ^{3}H - 3 methyl ether (specific activity - 1.22 uc./mg.) and estradiol - 17β - 6, 7 - ^{3}H- - 3 methyl ethers (specific activities - 1.31 uc./mg. - 4.18 uc./mg.), respectively. Procedures employed will be discussed in Chapt. III.

An aqueous solution of $Na_2{}^{35}SO_4$ (specific activity - 1.30 mc./mg.) was purchased from New England Nuclear Corp., Boston.

2. UNLABELLED COMPOUNDS.

Pure crystalline estrone, estradiol - 17β and estriol was supplied by Parke, Davis and Co., Ann Arbour, Mich. Dehydroisoandrosterone was purchased from Steraloids Inc., Flushing, N.Y. Dr. M. Levitz (N.Y.U.) donated the unlabelled 16 - ketoestradiol - 17β. Use was also made of the following organic and inorganic sulfate compounds: magnesium sulfate (Merck & Co. Ltd., Montreal); cadmium sulfate (Fisher Scientific Co., Montreal); ammonium sulfate (Merck & Co. Ltd., Montreal); sodium sulfate (Fisher Scientific Co., Montreal); ceric sulfate (The British Drug Houses Ltd., London); serotonin creatinine sulfate (Abbott Laboratories, Chicago); hydroxylamine sulfate (Fisher Scientific Co., Montreal); methyl isothiourea sulfate (Brickman & Co., Montreal); p - toluene sulfonic acid (Anachemia Chemicals Ltd., Montreal); 8 - amino - 1 - naphthol - 3, 6 - disulfonic acid (Brickman & Co., Montreal).

Solution of all materials in A.R. grade methanol yielded standard solutions, used to eliminate excessive weighing procedures.

Part of the crystalline estrone and estradiol - 17β was utilized in the preparation of pure estrone - 3 - sulfate, estradiol - 17β - 3 - sulfate, estradiol - 17β - 17 - sulfate and estradiol - 3, 17β - - disulfate. (See Chapt. III for details of process).

Adenosine triphosphate - disodium salt (ATP (Na_2)) was acquired from Sigma Chemical Co., St. Louis.

B. BUFFER SYSTEMS.

1. EDTA - KCl buffer, pH 7.0 used in enzyme fraction prepara-

tion. Consists of 0.15 M KCl containing 0.001 M ethylene diamine tetraacetate disodium salt ($Na_2C_{10}H_{14}O_8N_2 \cdot 2H_2O$), both obtained from Anachemia Chemicals Ltd., Montreal, as C.P. (Chemically pure).

2. Buffered ATP solution, pH 6.8. Reagents are C.P. Contains:

1 vol. 0.3 M KH_2PO_4 (pH 6.8)

1 vol. 0.03 M K_2SO_4

1 vol. 0.005 M $MgCl_2$

0.33 vol. 0.08 M ATP disodium salt (pH 6.8)

Used in the assay of steroid sulfate synthesis.

3. Buffered ATP solution, pH 6.0, 6.5, 7.0, 7.5, 8.0. All constituents are the same as 2. except for 1 vol. 0.1 M KH_2PO_4 adjusted to different pH with N - NaOH.

4. Buffered ATP solution, used in ^{35}S esterification study. All reagents are C.P. Consists of 0.25 ml. of $Na_2{}^{35}SO_4$ (specific activity = 1.30 mc./mg.) added to 37.97 mg. of Na_2SO_4 and made up to 7.6 ml. with H_2O. Equal volumes of 0.3 M KH_2PO_4 (pH 6.8); 0.005 M $MgCl_2$ and 0.33 vol. of 0.08 M ATP (pH 6.8) were added.

5. Borate buffer, pH 8.6, μ = 0.025 (ionic strength), used in electrophoretic work. Contains 333 gm. Boric acid and 68 gm. NaOH, both from Fisher Scientific Co. as C.P. grade. Dissolved in 16 litres H_2O and diluted 4 times before use.

6. 0.1 M Phosphate buffer, pH 7.2, used in the preparation of the PAPS fraction. Consists of 19.4 ml. of 0.5 M KH_2PO_4 and 26.8

ml. of 0.5 M Na_2HPO_4, both from Merck and Co., Montreal.

C. REAGENTS EMPLOYED DIRECTLY IN PRODUCT DETECTION.

1. Methylene Blue Reagent: Prepared by the method of Vlitos (135) by dissolving 250 mg. of methylene blue chloride (Merck, Sharp and Dohme, Montreal) in H_2O, adding 50 gm. of Na_2SO_4 and 10 ml. of H_2SO_4 and making up to 1 litre with H_2O. Diluted with an equal volume of H_2O before use.

2. Toluene Scintillation Fluid: Compounded by mixing 3 gm. of 2, 5 - diphenyloxazole and 100 mg. of p - Bis (2 - (5 - phenyloxazolyl) 1 - benzene), both purchased from Pilot Chemicals Inc., Watertown, Mass., as C.P. reagents. Dissolved in 1 litre of reagent grade, sulfur free Toluene from British Drug Houses, Montreal.

3. 0.1% Brilliant Blue F.C.F., used in detection of protein material, purchased from Allied Chemical and Dye Corp. and dissolved in 5% 'Baker Analyzed' C.P. acetic acid.

4. Ninhydrin Spray, used in the detection of free amino groups, was acquired from British Drug Houses, Ltd. as reagent grade material. (Contains 0.5% indane - trione hydrate in n - butanol).

5. Ittrich Reagents for accurate determination of weight of estrogen present (133).

a. Hydroquinone, obtained from British Drug Houses, Ltd. as C.P. reagent. Further powdered before use.

b. p - Nitrophenol, from Fisher Scientific Co., Montreal as C.P. grade. The material was further crystallized twice

from benzene and stored in a brown colored bottle away from light.

c. Tetrachloroethane, being A.R. (analytical reagent) grade, from Anachemia Chemicals Ltd., did not require further purification.

D. CHROMATOGRAPHIC SOLVENTS.

1. Lisboa - Diczfalusy System A (132), for thin-layer chromatography, consisting of: ethyl acetate: 45; cyclohexane: 45; ethanol: 10. All solvents were obtained from Anachemia Chemicals, Ltd. as C.P. grade. Cyclohexane was used as received. Ethyl acetate and ethanol were distilled in all-glass apparatus before usage, the latter in the presence of 2, 4 - dinitrophenylhydrazine.

2. Lisboa - Diczfalusy System C (132), containing: ethyl acetate: 50; cyclohexane: 50 and treated as in 1.

3. Paper chromatographic system described by Emerman et al. (137), consisting of: Toluene; Butanol; 2.8% ($^{v}/v$) NH_4OH (3: 1: 2). All solvents reagent grade and used as received.

4. Column partition chromatographic system, utilizing a 1 cm. x 15 cm. column of HCl washed Johns - Manville No. 545 Celite (10 g.) packed as previously described by Bauld and Greenway (156). The partition System B of Kelly et al. (157) consisting of: Isooctane; tert - butyl alcohol; 1 M NH_4OH (200: 500: 500) was used.

Some of the reagents referred to have been used also for purposes other than systems under which they are described. However, their source, grade and purification remain the same.

E. OTHER REAGENTS AND SOLVENTS.

1. Deionized water was used for preparation of buffers and other aqueous solutions.

2. The following reagents were obtained from Fisher Scientific Co. (a) Ammonium sulfate (b) Sodium sulfate, C.P. grade (c) Glacial Acetic acid, A.R. grade (d) Sulfuric acid, C.P. grade, (e) Hydrogen peroxide, A.C.S. reagent grade, (f) Pyridine, C.P. grade and (g) t - Butanol, C.P. grade.

3. Anachemia Chemicals Ltd. provided the following reagents which were C.P. grade, requiring no further purification. (a) Sodium Carbonate and (b) Propylene glycol. The (c) Chloroform and (d) Hexane were A.C.S. reagent grade and C.P. grade respectively, but were distilled twice before usage. The diethyl ether, reagent grade, was freed from peroxides by shaking with approximately 0.3 M $FeSO_4$ in 0.4 N H_2SO_4 (100 ml./l) and distilled within six hours of use as described by Bauld (139).

4. (a) Sodium borohydride ($NaBH_4$) was purchased from L. Light and Co. as reagent grade and (b) practical grade dimethyl sulfate and isooctane were acquired from Eastman Organic Chemicals. (c) Both $MgSO_4$ and K_2SO_4 were A.C.S. reagent grade materials from Merck and Co. (d) Sodium chloride was 'Baker Analyzed', C.P. grade, from J.T. Baker Chemical Co., N.J., as was n - Butanol. (e) N - glycyl-glycine was obtained from Matheson, Coleman and Bell as reagent grade compound. Iron - free chlorosulfonic acid was obtained from the same source.

5. Whatman no. 1 and 3 paper was purchased from Fisher Scientific Co., Ltd.

6. Silica Gel H, for thin layer chromatography according to Stahl, was acquired from Research Specialties Co., Richmond, Calif.

F. ANIMALS

All were white Wistar rats purchased from Robidoux Breeding Farms, Montreal.

1. Adult females (approx. 75 days old).

2. Thirty day old females.

3. Maternal females at term.

METHODS

A. PURITY AND STANDARDIZATION OF STEROIDS

Most steroids used in these studies were purchased in already purified form. However, in cases where derivatives were made re--purifications and standardizations had to be performed.

1. CHEMICAL REDUCTION OF RING D - α- KETOLS

Ring D - α- Ketols were reduced by the method of Diczfalusy and Münstermann (131). The Ketols were treated overnight in 50% aqueous ethanol with 10 mg./ml. of sodium borohydride. The reaction was stopped by addition of glacial acetic acid, and suitably extracted.

By the above method 16 - epiestriol - 16 - ^{14}C, specific activity, 305 counts per minute per ug. was produced by reducing a stock solution of 16 - ketoestradiol - 17β - 16 - ^{14}C, specific activity of 580 counts per minute per µg. prepared for this purpose by mixing labelled and unlabelled 16 - ketoestradiol. After arrest of the reduction the solution was diluted and extracted four times with equal volumes of ether. The ether was washed, dried and evaporated and the reduced 16 - ketoestradiol - 16 - ^{14}C was dissolved in methanol. Purification was accomplished by thin-layer chromatography on Silica Gel H using the Lisboa - Diczfalusy system A (132), consisting of cyclohexane: 45; ethyl acetate: 45; ethanol: 10. By this method a good separation was obtained between 16 - -epiestriol - 16 - ^{14}C and any 16 - ketoestradiol - 16 - ^{14}C or estriol - 16 - ^{14}C which may have been present. The 16 - epiestriol - 16 - ^{14}C was eluted from the glass plate with methanol

and the activity and weight were determined respectively by scintillation spectrometry and the Ittrich modification of the Kober reaction done fluorometrically (133) using the Turner Fluorometer, model 110. In this way pure 16 - epiestriol - 16 - ^{14}C of known specific activity was obtained.

Similarly 100 mg. of estrone - 3 - sulfate was reduced to estradiol - 17β - 3 - sulfate but extraction was done with n - butanol after arrest of the reduction with acetic acid. Purification of this material will be discussed in section A, 3 of this chapter (Chemical preparation of estrogen sulfates).

2. METHYLATION

In experiments where one requires that the 3 - hydroxyl group of the A ring of estrogens be non-functional, blocking was accomplished by formation of the respective 3 - methyl ether of the steroid.

Methylation was performed in borate buffer solutions PH 10.0--11.5 with dimethyl sulfate at 37°C for 30 min. by the method of Brown (134). The estrogen methyl ether was extracted with hexane. Purification was accomplished by thin-layer chromatography on Silica Gel H using the Lisboa - Diczfalusy system C, consisting of ethyl acetate: 50; cyclohexane: 50. The fraction containing the methyl ether was eluted from the glass plate with methanol and the activity and weight determined. In this way pure estrone - 6, 7 - ^{3}H - 3 methyl ether of specific activity, 428 counts per minute per ug. and several samples of estradiol - 6, 7 - ^{3}H - 3 methyl ether of specific activities, ranging from 458 counts per minute per µg. to 1470 counts

per minute per µg., were prepared.

3. CHEMICAL PREPARATION OF ESTROGEN SULFATES

a. Estrone - 3 - sulfate: To 100 mg. of estrone was added a homogenized pyridine - sulfur trioxide suspension prepared by the reaction described by Fieser (158) using 1 ml. of pyridine and 0.1 ml. of chlorosulfonic acid. After standing overnight at room temperature, the reaction mixture was taken up in distilled water, made alkaline with concentrated ammonium hydroxide and extracted with n - butyl alcohol. The butanol extracts were dried, redissolved in a small quantity of methanol and recrystallized twice with the addition of a few drops of dry diethyl ether (159). The crystals were dried in a dessicator over $CaCl_2$.

b. Estradiol - 17β - 3 - sulfate: 100 mg. of estrone were sulfurylated and crystallized as described above. The crystals were dissolved in 20 ml. of methanol and reduced by the method of Diczfalusy and Münstermann (131). The aqueous fraction containing the reduced estrone - 3 - sulfate was made alkaline with ammonium hydroxide and extracted with n - butyl alcohol. The butanol extracts were dried, redissolved in a small quantity of methanol and recrystallized as already described. The crystals were dried in a dessicator over $CaCl_2$.

Both the estrone - 3 - sulfate and estradiol - 17β - 3 - sulfate were identified by Infrared analysis, performed through the kind assistance of Dr. H. WOTIZ of Boston University.

c. Estradiol - 17β - 17 - sulfate: 100 mg. of estradiol - 17β

was sulfurylated and crystallized from methanol as described in "Section 3 (a) methods". The crystals were constituted mainly of estradiol - 17β - 17 - sulfate with some estradiol - 3, 17β - disulfate (personal communication, Dr. R. GREEN). 8 Mg. of this material was placed upon a 1 cm. x 15 cm. celite column (10 g.) and eluted with 200 ml. of Isooctane: tert - butyl alcohol: 1 M NH_4OH (200: 500: 500) prepared as outlined in "Section D materials". The first 2 ml. of eluate collected from the column was discarded. The following 20 ml. was collected and evaporated to dryness at 40°C. The material was redissolved in methanol and again recrystallized as previously described. The crystals were stored in a dessicator over $CaCl_2$.

d. Mixture of Estradiol - 3 - 17β - disulfate and Estradiol - 17β- - 17 - sulfate: To obtain an optimum yield of the labile disulfate a modified method of sulfurylation, as partially described by Sobel et al. (160), was utilized. Fundamentally this consisted of synthesizing the pyridine - sulfur trioxide in the absence of the steroid so as to prevent acid conditions during the actual steroid sulfation. 10 Ml. of pyridine were dissolved in 20 ml. of chloroform, the mixture was cooled and 4 ml. of chlorosulfonic acid were added drop by drop. Anhydrous conditions were maintained at all times. The precipitate was centrifuged and washed three times with chloroform. To 125 mg. of this pyridine sulfate was added 1.6 ml. of pyridine and 0.05 ml. of acetic anhydride. After stirring for one-half hour, a solution of 60 mg. of estradiol in 0.6 ml. of pyridine was added and stirring was continued overnight. The material was evaporated

to dryness at 40°C under vacumm. The solution was adjusted to PH 8 with ammonium hydroxide and extracted with ether. The PH was elevated to 11 - 12 and extracted 5 times with butanol (161). The rest of the procedure was the same as previously described. The crystals were approximately 30% estradiol - 3, 17β - disulfate and 70% estradiol - 17β - 17 - sulfate (personal communication, Dr. R. GREEN).

B. PREPARATION OF ENZYME FRACTION.

The high speed soluble enzyme fraction was prepared by the method of Bernstein and McGilvery (65). Pooled livers from white Wistar rats were homogenized in a Virtis "45" homogenizer with 3 - 4 volumes of EDTA - KCl. buffer (see materials) pH 7.0 for 20 sec. De Meio and Tkacz (63) showed that long homogenization of liver abolished its sulfate conjugating activity. Mitochondria are damaged by the shearing effect. The temperature of this and all subsequent stages was kept at 0°C. The homogenate was centrifuged for 1½ hrs. at 20,000 g. in a Beckman Model L ultracentrifuge, and the supernatant containing the enzyme, separated. Fractional salt precipitation was employed to partially purify the enzyme, and the fraction precipitated between 1.7 M and 2.3 M ammonium sulfate contained the bulk of the enzyme. The active fraction was dissolved in 0.7 ml. of water per gram of liver originally used. 1.0 Ml. of the solution was pipetted into each vial and the individual portions stored at -20°C. This was done to prevent rethawing and refreezing, each fraction having only been frozen and thawed once for use. At the time of incubation each 1.0 ml. aliquot was

diluted with 3 volumes of water before use. 0.4 Ml. of this suspension - solution was used per tube (equivalent to ≅ 100 mg. of liver).

In this manner enzyme fractions were prepared from various classes of animals:

(a) Adult females.
(b) Fetuses at term (not sex differentiated).
(c) Ten day old animals (not sex differentiated).
(d) Thirty day old females.
(e) Maternal females at term.
(f) Maternal females ten days after delivery.

C. ASSAY OF STEROID SULFATE SYNTHESIS.

1. INCUBATION.

Unlabelled steroid(s) in methanol, in amounts ranging from 0.50 µg. to 12 µg., depending upon the determination to be done, were pipetted into a series of glass - stoppered tubes (Quickfit). To this was added ^{3}H or ^{14}C labelled steroid(s) of high specific activity (negligible weight), normally of the magnitude of 20,000 c.p.m./tube in cases where radioactive technique was to be used. The methanol was evaporated to dryness. The steroid(s) were redissolved in 0.1 ml. propylene glycol per tube. 0.5 Ml. of buffered ATP (pH 6.8) was added to each tube, followed by 0.4 ml. of enzyme solution (equivalent to approximately 100 mg. of liver). The mixture was incubated in open tubes for 1½ hrs. at 37°C in a Blue M Magni Whirl water bath with occasional shaking. Blank tubes were incubated in exactly the same fashion except for omission of ATP from the medium which was replaced by distilled water. All incubations were done in duplicate and as described

above.

2. THE PH OF THE INCUBATION MEDIUM.

It was accepted that a pH of 6.8 was optimum for the sulfation activity of the high speed soluble enzyme fraction from adult female rat liver in vitro (52). Nevertheless, it was of interest to determine how critical this pH optimum actually was.

The entire procedure, components for incubation and arrest of reaction (sections C, 1 and C, 3 chapt. III) was unchanged. Only the KH_2PO_4 constituent of buffered ATP varied from pH 6 to 8 (see chapt. II, Materials). The weight and activity of estradiol-17β-6, 7-^3H incubated was 0.04 μ moles and 34,700 counts per minute per tube, respectively, while 0.04 μ moles of estradiol-17β-6, 7-^3H-3 methyl ether contained 16,400 counts per minute per tube. Duplicate incubations were performed at intervals of 0.5 in a pH range of 6 to 8.

3. DETECTION OF SULFATE ESTER SYNTHESIZED.

a. Use of methylene-blue as described by Roy (51, 52).

Complexes are formed between methylene-blue and certain sulfuric acid esters, rendering a blue chloroform soluble material. No such reaction takes place between unconjugated steroids and methylene-blue. Methylene-blue itself is insoluble in chloroform.

A series of glass-stoppered tubes containing 0.1 ml. of 0.4 mM estrone (10.8 μg.), 0.1 ml. 0.4 mM estradiol-17β (10.9 μg.), and 0.1 ml. 0.4 mM estriol (11.5 μg.), respectively, were incubated. All determinations were done in duplicate and a

blank was run for each individual steroid as described in section C, 1 of this chapter. The reaction was stopped by the addition of 5 ml. of ethanol, the mixture was allowed to stand for 15 minutes, and precipitated protein removed by centrifuging. 5 Ml. of the supernatant was taken for assay of the ester sulfates formed in each case. The supernatant was concentrated in a boiling - water bath until about 0.1 ml. of a viscous liquid (propylene glycol) remained. To this was added 2 ml. of methylene - blue reagent, and 5 ml. of chloroform after cooling. The mixture was shaken vigorously for 20 sec., centrifuged, the aqueous layer removed and the chloroform layer dried over anhydrous Na_2SO_4. The optical density of the chloroform layer was read at 650 m μ in a Unicam Sp .600 Spectrophotometer using standard Unicam cells of 1 cm. light path and red photocell (625 - 1000 m μ) against the corresponding blank.

The methylene - blue technique was found to be inadequate for present purposes (see results).

b. Use of radioactive tracer technique as partially described by Sneddon and Marrian (136).

Radioactivity was determined using a Packard Tri-Carb Liquid Scintillation Spectrometer model 314 AX. Liquid Scintillation counting is preferable to solid state, as it gives a higher efficiency and sensitivity.

The machine was set by discriminator controls form 10 - 100 and 10 - 100 at a high voltage of 9.5 for ^{3}H and 5.5 for ^{14}C in the case of a single isotope. In the case of a mixture of ^{14}C

and ^{3}H or ^{35}S, double label counting was done by setting discriminator controls from 10 - 50 and 100 - ∞ using split window at a high voltage of 9.0. The operational efficiency of the machine is 16% for ^{3}H and 60% for ^{14}C when counted separately.

Counts due to each isotope during double label counting requires consideration of the effect of ^{3}H on ^{14}C and vice versa. To simplify this, the following formuli were utilized for calculations, as described by Okita et al. (151).

$$^{3}H = N_1 - \frac{N_2}{b}$$

$$^{14}C = N_2 - aN_1$$

Where: N_1 = total count due to ^{3}H

N_2 = total count due to ^{14}C

$$a = \frac{\text{c.p.m. } ^{3}H \text{ in channel 2}}{\text{c.p.m. } ^{3}H \text{ in channel 1}}$$

$$b = \frac{\text{c.p.m. } ^{14}C \text{ in channel 2}}{\text{c.p.m. } ^{14}C \text{ in channel 1}}$$

The radioactivity for all samples was determined to within 5 percent probable error and corrected for background radiation. Where necessary, standards of known radioactivity were added to and counted with the experimental samples to correct for any quenching occurring.

To assay the radioactivity, an aliquot of the material to be determined was dissolved in an appropriate volume of methanol, pipetted into a 20 ml. liquid scintillation spectrometer vial and evaporated under a stream of air with care to exclude any water. It was then redissolved in 0.1 ml. of methanol to which was added

10 ml. of Toluene Scintillation fluid.

After incubation of the steroid(s) with the soluble enzyme fraction the reaction was stopped by the addition of 1 ml. of water followed by 4.7 ml. of methanol (to yield a final methanol concentration of 70%). The glass - stoppered tubes were allowed to stand for 15 min., capped, shaken vigorously, and centrifuged to remove the small amount of precipitated protein. The supernatant was decanted and a further 6.7 ml. of 70% methanol: water was added to the precipitate and the above procedure repeated. The two supernatant fractions were pooled.

The methanol in the pool was evaporated at 70°C under a constant air stream, taking care that the solution was alkaline at all times to prevent hydrolysis. The volume was made up to 3 ml. with distilled water.

After cooling, each tube was extracted twice with 15 ml. of diethyl ether. The extracts were pooled, washed with water and dried over sodium sulfate, following which they were evaporated to dryness and redissolved in 2 ml. of methanol. An aliquot of 1 ml. was pipetted into a scintillation vial and counted as outlined above. (In most cases a double label setting was utilized due to the presence of two isotopes).

The aqueous fraction was gently heated to remove any ether, while maintaining an alkaline pH, cooled, saturated with NaCl and extracted twice with 15 ml. of ethyl acetate. The extracts were dried over sodium sulfate, evaporated to dryness and redissolved in 2 ml. of methanol. An aliquot of 1 ml. was removed for counting.

Recoveries were reported for all experiments in the form of the sum of the activity in the ether and ethyl acetate fraction expressed as a percentage of total radioactivity added.

(i) Steroid sulfate synthesis by the soluble enzyme preparation from adult female rat liver.

The extent of estrogen sulfurylation by adult female liver preparations as compared with the activity towards dehydroisoandrosterone was studied. Table I indicates the amount and activity of steroid in the incubation systems. All blanks were executed in the same way except for omission of ATP.

TABLE I

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY SULFURYLATION BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

STEROID 0.04 μ moles/tube	ACTIVITY c.p.m./tube
Estrone - 16 - ^{14}C	25,900
Estrone - 6, 7 - ^{3}H	14,600
Estradiol - 17B - 6, 7 - ^{3}H	16,400
Estriol - 16 - ^{14}C	109,000
16 - epiEstriol - 16 - ^{14}C	3,500
16 - Ketoestradiol - 17β - 16 - ^{14}C	15,900
Estradiol - 17B - 6, 7 - ^{3}H - 3 - methyl ether	10,900
Dehydroisoandrosterone - 4 - ^{14}C	17,300

* Average of 3 experiments in duplicate performed with each steroid.

(ii) Steroid sulfate synthesis by the soluble enzyme preparation from adult female rat liver in the presence of (^{35}S) and (^{3}H) - steroid.

Findings of experiments (i) and (iii), described immediately after this section, were supplemented by a series of studies involving ^{35}S for a more direct detection of actual sulfate formation. To this end, incubations were performed in the presence of $Na_2\ ^{35}SO_4$ and $^{35}S/^{3}H$ ratios of final products were calculated.

The procedure and components for incubation and arrest of reaction were similar to those in experiments (i) and (iii) except for the buffered ATP, in which K_2SO_4 was replaced by $Na_2\ ^{35}SO_4$. For the purposes of efficient detection of incubation product a ratio of 100:1 $^{35}SO_4^{-2}$ to (^{3}H) - steroid per tube was required (5.4 μ moles of $Na_2\ ^{35}SO_4$/0.040 μ moles of (^{3}H) - steroid). The buffer was prepared as outlined in Chapt. II. The blanks were the same as above except that ATP was omitted.

The procedure for arrest of the reaction and detection of the final product was unaltered except for a few minor deviations from the procedure previously described. Only the ethyl acetate fraction (conjugated material) was counted. The ether fraction containing unreacted (^{3}H) - steroid and a trace of $^{35}SO_4^{-2}$ was discarded. Most of the $^{35}SO_4^{-2}$ which did not esterify remained in the aqueous fraction, being insoluble in most organic solvents. This did not allow for calculation of recoveries, which were assumed to be the same as in experiments (i) and (iii), only minor changes having been made in the technique. The series of experiments reported

were performed in the period of a week due to the short half-life of ^{35}S (87 days) with scintillation spectrometry done on standards each time a sample was counted to account for radioactive decay. The actual loss of activity of standards was also checked against loss calculated by the following formula:

CALCULATED LOSS OF ACTIVITY OF ^{35}S.

$$2.3 \log \frac{A}{X} = - \frac{0.693\ z}{y}$$

Where: A = no. of c.p.m. after z days.
X = no. of c.p.m. started with.
y = half-life (87 days).
z = no. of days disintegrated.

TABLE II illustrates the weight and activity of materials involved.

TABLE II

THE WEIGHT AND ACTIVITY OF ESTROGENS USED TO STUDY SULFURYLATION IN THE PRESENCE OF ^{35}S BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

ACTIVITY OF $Na_2\ ^{35}SO_4$ = 13×10^6 c.p.m./tube.

STEROID 0.04 μ moles/tube	ACTIVITY c.p.m./tube
Estrone - 6, 7 - ^3H	34,300
Estradiol - 6, 7 - ^3H	23,100
Estrone - 6, 7 - ^3H - 3 methyl ether	4,900
Estradiol - 17β - 6, 7 - ^3H - 3 methyl ether	5,300

* 1 experiment in duplicate performed.

(iii) Effect of varying estrone and dehydroisoandrosterone concentration upon sulfurylation of estrogens and estrone upon dehydroisoandrosterone by adult female rat liver soluble enzyme fraction.

Differences in the degree of sulfurylation of the various estrogens in experiment (i) led to speculation as to whether the differences noted are due to either, the presence of multiple sulfokinases for different estrogens, or the effect of steroid structure on a common enzyme. The former has been suggested by Nose and Lipmann (75), who claimed separation of estrone sulfokinase from phenol sulfokinase.

With this in mind, the following incubations (TABLE III - IX) were performed. Blanks in all cases consisted of one tube with 0.04 μ moles of substrate steroid and another tube with 0.04 μ moles of substrate steroid and 0.04 μ moles of dehydroisoandrosterone or estrone. Isotopically the same counts per minute per tube were present as in actual samples.

TABLE III

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF VARYING ESTRONE CONCENTRATION UPON SULFURYLATION OF DEHYDROISOANDROSTERONE BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	ESTRONE - 6, 7 - ^{3}H 29,800 c.p.m./tube	DEHYDROISOANDROSTERONE - 4 - ^{14}C 10,900 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.040
2.	0.008	0.040
3.	0.016	0.040
4.	0.024	0.040
5.	0.040	0.040

* 2 experiments in duplicate performed.

TABLE IV

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF VARYING ESTRONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	ESTRONE - 16 - ^{14}C 23,700 c.p.m./tube	ESTRADIOL - 17β - 6, 7 - ^{3}H 13,700 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.040
2.	0.040	0.040
3.	0.002	0.040
4.	0.004	0.040
5.	0.020	0.040
6.	0.040	0.040

* 2 experiments in duplicate performed.

TABLE V

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF VARYING ESTRONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β - 3 - METHYL ETHER BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	ESTRONE	ESTRADIOL - 17β - 6, 7 - ^{3}H - 3 METHYL ETHER 39,500 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.090
2.	0.002	0.090
3.	0.004	0.090
4.	0.020	0.090
5.	0.040	0.090

* 1 experiment in duplicate performed.

TABLE VI

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF VARYING DEHYDROISOANDROSTERONE CONCENTRATION UPON SULFURYLATION OF ESTRONE BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	DEHYDROISOANDROSTERONE - 4 - ^{14}C 11,500 c.p.m./tube	ESTRONE - 6, 7 - ^{3}H 32,300 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.040
2.	0.002	0.040
3.	0.004	0.040
4.	0.020	0.040
5.	0.040	0.040

* 2 experiments in duplicate performed.

TABLE VII

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF VARYING DEHYDROISOANDROSTERONE CONCENTRATION

UPON SULFURYLATION OF ESTRADIOL - 17β BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	DEHYDROISOANDROSTERONE - 4 - ^{14}C 18,300 c.p.m./tube	ESTRADIOL - 17β - 6, 7 - ^{3}H 13,900 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.040
2.	0.002	0.040
3.	0.004	0.040
4.	0.020	0.040
5.	0.040	0.040

* 2 experiments in duplicate performed.

TABLE VIII

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF VARYING DEHYDROISOANDROSTERONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β - 3 METHYL ETHER BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	DEHYDROISOANDROSTERONE - 4 - ^{14}C 17,200 c.p.m./tube	ESTRADIOL - 17β - 6, 7 - ^{3}H - 3 METHYL ETHER 10,900 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.040
2.	0.002	0.040
3.	0.004	0.040
4.	0.020	0.040
5.	0.040	0.040

* 2 experiments in duplicate performed.

TABLE IX

THE WEIGHT AND ACTIVITY OF STEROIDS USED TO STUDY THE EFFECT OF DEHYDROISOANDROSTERONE UPON SULFURYLATION OF 16 - KETOESTRADIOL - 17β AND 16 - epiESTRIOL BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION *

TUBE NO.	DEHYDROISOANDROSTERONE	16 - KETOESTRADIOL - 17β - 16 - ^{14}C 15,900 c.p.m./tube	16 - epiESTRIOL - 16 - ^{14}C 3,500 c.p.m./tube
	WEIGHT μ moles/tube	WEIGHT μ moles/tube	WEIGHT μ moles/tube
1.	-	0.040	-
2.	0.040	0.040	-
3.	-	-	0.040
4.	0.040	-	0.040

* 2 experiments in duplicate performed.

(iv) Effect of animal age on steroid sulfate synthesis by the rat soluble enzyme preparation.

It was of interest to determine how age of animal, as well as pregnancy may affect sulfurylation of steroids.

The procedures for enzyme preparation, incubation, arrest of reaction and product detection were the same as for experiments (i) and (ii) already described. The only difference relates to livers of animals at various stages of development and maternal condition being used to prepare the soluble fraction (see sect. A, chapt. III). The activity of the different enzyme fractions was compared to that of adult female animals (75 days old) of experiment (i). The blanks were prepared in the same way as in experiments (i), (ii) and (iii).

Table X lists the amount and activity of three different steroids incubated with the various soluble fractions.

TABLE X

THE WEIGHT AND ACTIVITY OF ESTRONE, ESTRADIOL - 17β AND DEHYDROISOANDROSTERONE USED TO STUDY THE EFFECT OF ANIMAL AGE AND MATERNAL CONDITION UPON STEROID SULFURYLATION BY THE RAT LIVER SOLUBLE ENZYME PREPARATION *

ANIMAL AGE	ESTRONE - 16 - ^{14}C 0.04 μ moles/tube	ESTRADIOL - 17β - 6, 7 - ^{3}H 0.04 μ moles/tube	DEHYDROISOANDROSTERONE - 4 - ^{14}C 0.04 μ moles/tube
	ACTIVITY c.p.m./tube	ACTIVITY c.p.m./tube	ACTIVITY c.p.m./tube
FETUS (TERM) +	43,700	20,900	26,800
10 DAYS +	56,700	20,500	26,700
30 DAYS	105,000	68,700	27,600
MATERNAL (TERM)	44,700	20,700	27,100
MATERNAL (10 DAYS POST DELIVERY)	56,700	20,500	26,700
MATURE (75 DAYS)	25,900	16,400	17,300

* 2 experiments in duplicate performed.

\+ Not sex differentiated; other age groups were female (see Table XXI "Results" for explanation).

D. PURIFICATION AND IDENTIFICATION OF THE RADIOACTIVE INCUBATION PRODUCTS.

A series of techniques was utilized to purify and identify the reaction products as those postulated by the individual experiments. Although no single technique suffices as positive identification, collectively the procedures used may be considered to furnish sufficient proof of product formation.

Only one-half of the water-soluble, probable sulfate ester produced by each incubation was used for scintillation spectrometry, the other half being pooled separately for identification purposes. A drop of NH_4OH was added to each pool to preserve an alkaline medium.

1. PAPER CHROMATOGRAPHY.

Parts of the various methanol pools were evaporated to minimal volumes ($\cong$ 2 ml.) and chromatographed on a series of descending paper systems (Whatman no. 1) in toluene: butanol: 2.8% ($^v/_v$) NH_4OH (3: 1: 2) (137). Areas corresponding to appropriate pure reference standards (50 µg. estrone - 3 - sulfate and dehydroisoandrosterone - 3 - sulfate mixed together) were eluted with methanol and counted by liquid scintillation spectrometry. Standards were detected by immersing the paper strips containing them in methylene - blue reagent (135) followed by chloroform washing. White areas corresponded to sulfate esters.

In similar fashion the pooled incubation product of estradiol - 17β - 6, 7 - ^3H was chromatographed on the same system with reference

standards consisting of 50 µg. each of, estradiol, estradiol - 17β - - 3 - sulfate and 100 µg. of the mixture consisting of estradiol - 17β- - 17 - sulfate and estradiol - 3, 17β - disulfate. Areas corresponding to the standards were eluted as above and counted by liquid scintillation spectrometry.

2. SOLVOLYTIC CLEAVAGE OF REACTION PRODUCTS.

Solvolysis was performed by the method of Segal et al. (138).

Another part of the methanol pool of conjugated material was evaporated to dryness and redissolved in water, made 3 M with NaCl., pH adjusted to 2 - 3, and extracted with ethyl acetate. The ethyl acetate was rendered anhydrous by drying over Na_2SO_4. Scintillation spectrometry was done on a portion of this extract. The rest was solvolyzed at 37°C for 24 hr. in the presence of 2×10^{-4} M H_2SO_4. After washing, drying, evaporation, and diethyl ether extraction of this fraction, scintillation counting was carried out. Material in this fraction required to be conjugated to be detected, being ether insoluble previous to solvolysis.

3. RECRYSTALLIZATION OF INCUBATION PRODUCTS.

For the purpose of determining radiochemical homogeneity of the reaction products of estrone - 6, 7 - ^3H and estradiol - 17β - - 6, 7 - ^3H, 15 mg. of pure estrone and estradiol - 17β - 3 - sulfate were added to 8200 c.p.m. of the presumptive conjugated reaction product of estrone - 6, 7 - ^3H and to 18,100 c.p.m. of the estradiol- - 6, 7 - ^3H incubation product, respectively. Similarly, 15 mg. of pure estradiol - 17B - 17 - sulfate were mixed with 19,000 c.p.m. of

the estradiol - 6, 7 - ^{3}H reaction product. Three successive crystallizations from methanol with the addition of a few drops of dry diethyl ether were carried out. Aliquots of nearly identical weights were removed from the crystalline and mother liquor moieties and their radioactivity assessed. This procedure led to the elucidation of the specific activities (c.p.m./mg.) of these two fractions after successive crystallizations.

4. As a final attempt at identification the incubation product of estradiol - 17β - 6, 7 - ^{3}H, the material was chromatographed on a partition column employing the system: Isooctane; tert - butyl alcohol; 1 M NH_4OH (200: 500: 500) described in "Section D Materials". The method used consisted of applying approximately 5000 c.p.m. of the reaction product along with 2 mg. of estradiol - 17β - 3 - sulfate and 5 mg. of the mixture of estradiol - 17β - 17 - sulfate and estradiol - 3, 17β - disulfate to the column. The eluate from the column was collected in 6 ml. fractions. Two-thirds of each fraction was analyzed for radioactivity. The remaining one-third was retained for the methylene - blue reaction as a weight measurement of the standard sulfates.

E. ATTEMPTS AT LOCALIZATION OF MATERIAL INTERFERING WITH THE METHYLENE - BLUE REACTION.

The major part of this work was performed using the radioactive tracer technique, already discussed. It is occasionally difficult, however, especially in the case of rare estrogen derivatives, to obtain radioactive forms of these substrates. At these times the

spectrophotometric methylene - blue technique would have been more advantageous.

Attempts to use methylene - blue as previously described were frustrated by the presence of high blank values after incubation. This was assumed to be due to non-specificity of the complexing reaction, rather than the presence of steroid sulfates in the enzyme preparation. Efforts were therefore made to localize the source of the contaminating material, study the enzyme fraction more closely and briefly examine the specificity of the methylene - blue reaction.

1. PAPER ELECTROPHORESIS.

The high speed soluble enzyme fraction from mature female Wistar rats was prepared as outlined in Chapt. III, sect. B. The diluted enzyme solution (1.0 ml., 3 times diluted) was centrifuged and the supernatant was fractionated electrophoretically as described by Sehon et al. (140).

The paper electrophoresis was performed on Whatman no. 3 paper using borate buffer, pH 8.6, and ionic strength ($\Gamma/2$) of 0.025. The paper (5.5 inches wide x 22.5 inches long) was dipped in buffer and blotted. The enzyme solution was applied centrally in a line 0.75 inches long. The paper was placed on a glass tray and two sealing strips of Whatman no. 3 paper were placed along the outside edges of the electrophoresis tray to minimize evaporation during the electrophoresis. The tray was covered with a rubber faced frame which was covered in turn with a further sheet of Whatman no. 3 paper similarly dipped in buffer and blotted. Two layers of Whatman no. 1 paper (9

inches wide) were placed in contact with the ends of the paper and dipped in the electrode vessel to complete the circuit. The input voltage of 110 - 140 volts was adjusted to pass a current of 5 - 6 ma. through the apparatus. The duration of the experiment was 16 to 18 hr. The paper was then removed, dried and cut into 3 parts, one of which was immersed for 5 min. in a solution of 0.1% Brilliant blue in 5% acetic acid. Background color was removed from the strip by rinsing in 5% acetic acid. Another strip was stained with Ninhydrin spray. The third strip was left unstained.

2. CHLOROFORM - SOLUBLE METHYLENE-BLUE COMPLEX FORMATION.

One mg. quantities of the following organic and inorganic sulfates were used:

Magnesium Sulfate

Cadmium Sulfate

Ammonium Sulfate

Sodium Sulfate

Ceric Sulfate

Serotonin Creatinine Sulfate

Hydroxylamine Sulfate

Methyl iso - Thiourea Sulfate

p - Toluene Sulfonic Acid

8 - Amino - 1 - Naphthol - 3, 6 - Disulfonic Acid.

Each of the above compounds was placed in a glass - stoppered tube containing 2 ml. of methylene-blue reagent. To this, 5 ml.

of chloroform were added, the mixture shaken vigorously, centrifuged, the aqueous layer removed and dried over Na_2SO_4. The intensity of the chloroform layer was read at 650 m μ in a Unicam Sp .600 Spectrophotometer against a chloroform blank (chloroform after shaking with methylene - blue).

3. INCUBATION OF SOLUBLE ENZYME FRACTION FOLLOWED BY DESCENDING PAPER CHROMATOGRAPHY.

The incubation and arrest of the reaction were executed as described in Chapt. III, sect. C, 1, but on a larger scale.

6.0 Ml. of buffered ATP (pH 6.8) was placed in a 100 ml. erlenmeyer flask followed by 4.8 ml. of enzyme solution. The volume was made up to 12 ml. with distilled water. The flask was incubated for 1½ hr. at 37°C in a shaking water bath. The reaction was stopped by the addition of 60 ml. of ethanol, followed by centrifugation and removal of the supernatant. A final volume of 2 to 3 ml. was obtained by evaporation of the supernatant under a constant air stream. The material was chromatographed using a descending paper system (Whatman no. 1) in toluene: butanol: 2.8% (v/v) NH_4OH (3: 1: 2) (137) previously described. (This system was designed especially for identification of steroid sulfates). The possible presence of methylene - blue complexing chloroform soluble material could be detected by immersing the paper strip in methylene-blue reagent followed by chloroform washing. No reference standards were used, this being simply a qualitative test for a chloroform soluble sulfate complex, which if present, could later be identified by more elaborate means.

4. FRACTIONATION OF THE HIGH SPEED SOLUBLE ENZYME PREPARATION.

Because of the possibility that high blanks resulted from the presence of nucleotide sulfates not commercially available, it became necessary to extract such compounds from natural sources. Only one nucleotide sulfate was known to be present in the enzyme preparation in large enough quantity to interfere by the formation of chloroform soluble methylene - blue complexes, namely, adenosine 3′ phosphate 5′ sulfatophosphate (PAPS). It was obtained from the soluble enzyme fraction as described by Roy (53).

100 Ml. of a mixture containing 50 ml. of unfractionated enzyme preparation, 10 ml. of 0.1 M phosphate buffer (pH 7.2), 10 ml. of 0.04 M Mg SO_4 - 0.06 M K_2SO_4 and 400 μ moles of ATP (pH adjusted to 7.2 with N - NaOH) were incubated for 1 hr. at 37°C. Proteins were precipitated by the addition of 3 vol. of ethanol and removal by centrifugation. The ethanol was removed from the supernatant by concentrating it in vacuo to about 25 ml. at a bath temperature of 40°C. The resulting solution was brought to pH 8 with N - NaOH, insoluble material removed by centrifuging and the volume made up to 50 ml. with water. The resulting solution was stored frozen at -10°C. Although this preparation presumably contained other nucleotides, PAPS was the main constituent.

To ascertain that none of the endogenous blue color in the controls resulted from the sulfokinase fraction of the enzyme preparation, the former was prepared from the whole enzyme fraction, as again outlined by Roy (53).

The complete enzyme system was dialysed for 48 hr. against

repeated changes of 100 vol. of water at 0°C, destroying the sulfate - activating system which is unstable in the absence of SO_4^{-2} ions. The resulting suspension was made 0.03 M in glycylglycine and adjusted to pH 8 with N - Na_2CO_3. The suspension was allowed to stand for a few hours followed by centrifugation to remove any remaining insoluble material.

The PAPS and Sulfokinase fractions were incubated separately in two glass - stoppered tubes as previously described. Each of the above fractions, corresponding to 100 mg. of rat liver, was incubated in the presence of 0.5 ml. of buffered ATP (pH 6.8) for 1½ hr. at 37°C. Reactions were stopped, and methylene - blue color assays performed by colorimetric determination using the Unicam spectrophotometer.

RESULTS

N.B.

1. It must be strongly emphasized that throughout this chapter results are expressed as percentage recovered product conjugated, rather than weight of conjugated material synthesized. This was done to simplify the readers' task in comparing the activity of the enzyme fraction with different steroids. It is obvious that the percentage basis, as applied here, is only significant for the weight of substrate(s) specified due to the reaction being zero order. (If concentration is doubled, percentage is halved due to the weight of conjugated material synthesized remaining constant or actually decreasing as a consequence of substrate inhibition of enzyme). For this reason all weights were identical (on a molar basis) from experiment to experiment, except for one notable exception, so as to have a basis for comparison. This method is acceptable as an expression of relative activity but is obviously not an absolute value, except for the concentration specified.

2. Wherever possible standard deviations (S.D.) of the mean have been reported (e.g. $14 \pm X$ %). This was done only if the mean was the result of three or more individual observations (i.e. if only one experiment in duplicate had been performed, no S.D. was reported).

A. THE EFFECT OF PH ON SULFATE SYNTHESIS.

As a preliminary attempt at incubation of the prepared adult female rat liver soluble enzyme fraction with steroid, the pH requirement was examined. It was of interest to determine whether a pH of 6.8, as employed by Roy (52), was a strict criterion or a rather flexible condition.

The pH of the incubation medium was varied from 6 to 8 and two substrates were incubated as described in 'Section C, 2 methods'.

Table XI illustrates the distribution of radioactivity in the 'free' (unconjugated) and conjugated fractions of each of the steroids as well as the percentage sulfate esterification at different pH values of the incubation medium.

From the data presented it can be clearly seen that pH 6.0 and pH 8.0 are extremes for efficient sulfurylation, the percentage ester formation dropping considerably in both cases. Although a pH of 6.8, described by Roy, was used for all experiments it is apparent that there is very little effect of pH variation upon sulfate formation, with this system, between pH 6.5 to 7.5. The 'dip' in the curve for estradiol - 17β - 6, 7 - ^{3}H at pH 7.0 is likely due to experimental error as is the rise for estradiol - 17β - 6, 7 - - ^{3}H - 3 methyl ether at pH 7.5. In both cases the effect is small, occurring due to the averaging of duplicates, only one of these showing the probable artifact.

As a result of Roy's work and the above determinations, the pH of 6.8 was retained as most favourable for the *in vitro* sulfation of estrogens with the previously discussed soluble enzyme preparation.

TABLE XI

THE EFFECT OF PH OF INCUBATION MEDIUM ON ESTER SULFATE SYNTHESIS BY THE SOLUBLE ENZYME PREPARATION FROM ADULT FEMALE RAT LIVER. Δ , *

PH	ESTRADIOL - 17B - 6, 7 - ^{3}H			ESTRADIOL - 17B - 6, 7 - ^{3}H - 3 METHYL ETHER		
	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.+	UNCONJUGATED	CONJUGATED	% RECOVERED PRODUCT CONJUGATED.+
6.0	24,800	3,700	12.6	12,300	2,700	17.6
6.5	25,100	4,700	15.2	12,000	2,900	19.3
7.0	24,600	3,900	13.3	11,200	2,700	19.3
7.5	25,000	4,100	14.6	11,200	2,900	20.1
8.0	25,500	3,600	11.9	10,900	2,200	16.6

Δ c.p.m. are averages of duplicate experiments.

\+ Figures corrected for blank value of 0.43% recovered product conjugated.

* Average recovery of incubated radioactivity is 84 ± 1.6% and 87 ± 4.2% for estradiol - 17B and estradiol - 17B - 3 methyl ether, respectively.

Standard deviations of the mean for per cent recovered product conjugated are not reported, as only one experiment in duplicate was performed.

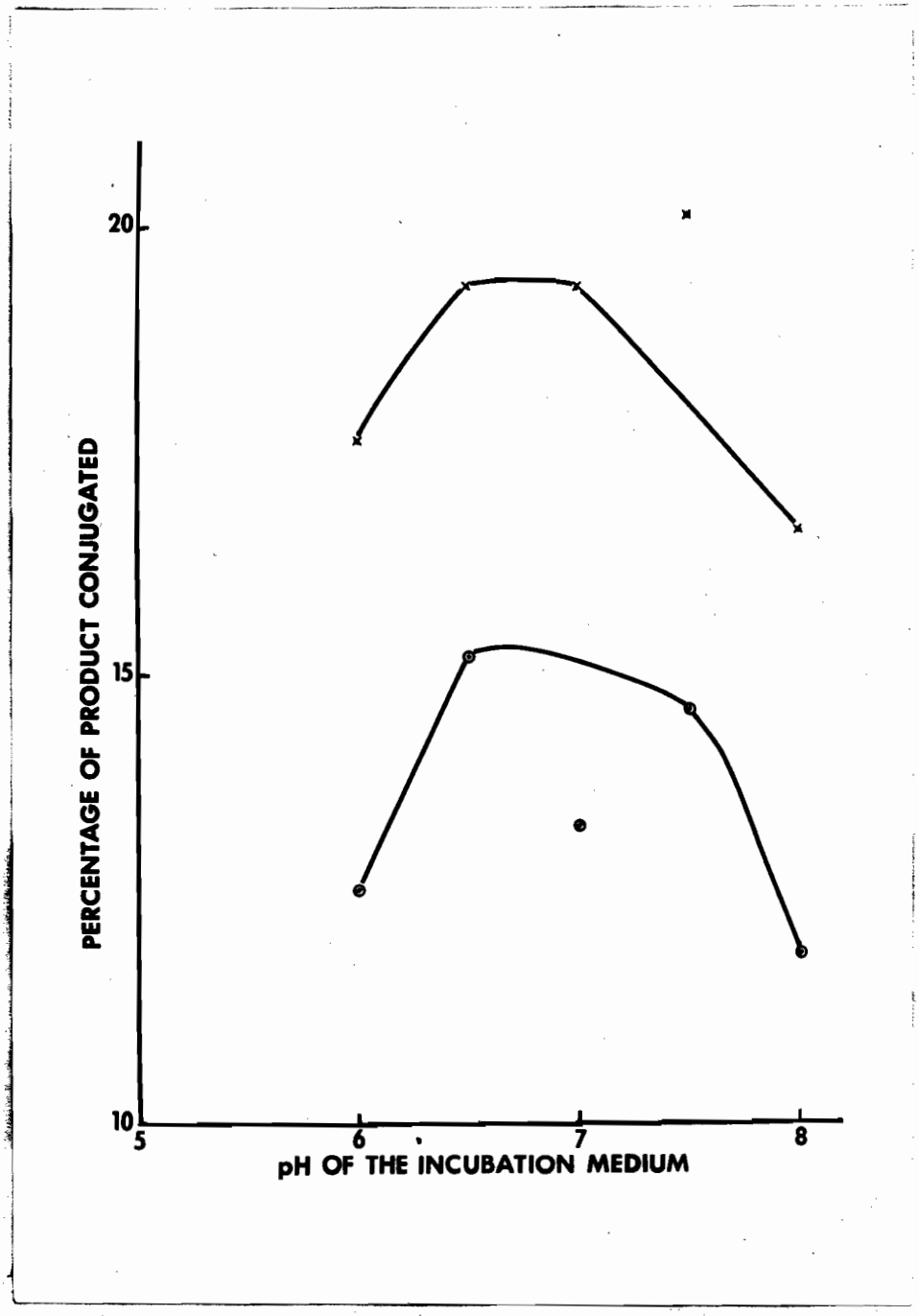


FIGURE II. The Effect Of Incubation Medium PH Upon The In Vitro Sulfurylation Of Estradiol - 17β - 6, 7 - ^{3}H And Estradiol - 17β - - 6, 7 - ^{3}H - 3 Methyl Ether.

B. MEASUREMENT OF STEROID SULFATE ESTER SYNTHESIZED EMPLOYING METHYLENE BLUE AS INDICATOR.

Methylene blue assay of estrone, estradiol - 17β and estriol ester sulfates was attempted as outlined in 'Section C, 3(a) methods'.

Reaction blanks yielded optical densities of 0.16 to 0.20 for the soluble enzyme fraction corresponding to approximately 100 mg. of rat liver as compared to a chloroform blank (chloroform after shaking with methylene blue). For steroids other than the estrogens (e.g. dehydroisoandrosterone) this may have been acceptable due to the high degree of sulfurylation of these compounds. For estrogens, however, the results for the actual incubations were only slightly higher than the reaction blanks, the difference not being large enough to be significant. As will be later shown, more than 80% of estrone, estradiol - 17β or estriol exists in the 'free' (unconjugated) form after incubation, while less than 20% of dehydroisoandrosterone is recovered in the unconjugated state.

Due to the problem of high blank values, the methylene blue technique was sacrificed for the more accurate radioactive tracer technique. However, as the former has certain advantages, already discussed in 'Section E methods', attempts were made to localize the source of the contaminating material yielding blanks of high absorbence. In 'Section E results', further data related to this will be presented.

C. MEASUREMENT OF STEROID SULFATE ESTER SYNTHESIZED EMPLOYING THE

RADIOACTIVE TRACER TECHNIQUE.

Sulfation of (^{3}H) and (^{14}C) - steroids was observed by the procedure outlined in 'Section C, 3(b) methods'. This part of the work was separated into several subdivisions.

1. Table XII contains data on the extent of radioactive estrogen sulfurylation by the enzyme preparation from adult female rat livers as compared with the activity towards dehydroisoandrosterone. This was done to obtain some preliminary information about the effect of estrogen molecular structure on the degree of sulfurylation of these compounds.

From the table it is observed that esterification of estradiol - 17β is two times greater than that of estrone. Blocking of the 3 - hydroxyl grouping of the estradiol molecule by means of methylation did not change the degree of sulfurylation as compared with that of estradiol - 17β.

Incubations performed by the addition of $Na_2{}^{35}SO_4$ to the medium containing (^{3}H) - labelled estrogen, further shed light upon the effect of molecular structure of estradiol - 17β upon the degree of sulfurylation of this compound. This involved a more direct approach to the study of the sulfurylation problem by actually following the fate of the sulfate. The ratio of $^{35}SO_4$ to (^{3}H) - steroid after incubation was determined as outlined in 'Section C, 3(b) ii methods'. Table XIII illustrates the distribution of radioactivity due to ^{35}S and ^{3}H in the conjugated fraction after double label counting, as well as the subsequently calculated $^{35}S/^{3}H$ ratio.

The table indicates that (^{3}H) - labelled estradiol - 17β and

TABLE XII

STEROID SULFATE SYNTHESIS BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

STEROID	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED †	% RECOVERY OF TOTAL INCUBATED ACTIVITY
ESTRONE-16-^{14}C	20,700	1,800	0.38	7.5 ± 0.9	85 ± 4.9
ESTRONE-6, 7-^{3}H	11,000	1,000	0.30	8.2 *	84
ESTRADIOL-17B-6, 7-^{3}H	10,400	2,100	0.69	16.1 ± 0.5	79 ± 4.1
ESTRIOL-16-^{14}C	87,900	3,300	0.49	4.3 ± 0.3	81 ± 3.5
16-epiESTRIOL-16-^{14}C	2,400	330	0.19	12.0 ± 0.9	82 ± 6.5
16 KETOESTRADIOL-17B-16-^{14}C	12,300	1,000	0.35	7.4 ± 0.4	81 ± 3.8
ESTRADIOL-17B-6, 7-^{3}H-3 METHYL ETHER	6,800	1,600	0.43	18.5 ± 0.9	80 ± 4.1
DEHYDROISOANDROSTERONE	1,300	6,200	0.26	82.9 ± 1.7	83 ± 5.0

** The weight and activity of steroids used as in TABLE I 'methods'.

Δ c.p.m. are averages of 3 experiments in duplicate.

† Figures are corrected for blank values.

* One experiment in duplicate performed, therefore no S.D. reported.

TABLE XIII

SULFURYLATION OF ESTROGENS BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION IN THE PRESENCE OF ^{35}S. **, Δ

SUBSTRATE †	CONJUGATED c.p.m.	WEIGHT EQUIVALENT TO c.p.m. mμ moles.	$^{35}S/^{3}H$ RATIO
ESTRONE-6, 7-^{3}H	2,000	2.4	1.23
SULFATE (^{35}S)	500	2.9	
ESTRONE-6, 7-^{3}H-3 METHYL ETHER	6 *	-	0
SULFATE (^{35}S)	140 *	-	
ESTRADIOL-17β-6, 7-^{3}H	2,000	3.4	2.05
SULFATE (^{35}S)	1,200	7.0	
ESTRADIOL-17B-6, 7-^{3}H-3 METHYL ETHER	160 *	0.61	1.03
SULFATE (^{35}S)	1,900 *	0.63	

** The weight and activity of steroids used as in TABLE II 'methods'.
Δ c.p.m. are averages of one experiment in duplicate (no S.D. reported).
† Figures are corrected for blank values.
* ^{35}S used in these two sections had a specific activity 10 times that of the remaining sections. 140 c.p.m. of ^{35}S was therefore considered to be of negligible weight. Also due to the low specific activity of estradiol methyl ether, 160 c.p.m. was considered to accurately represent a weight of 0.61 u moles.

its methyl ether incubated with $Na_2\ ^{35}SO_4$ resulted in $^{35}S/^{3}H$ isotope ratios of 2.05 and 1.03 respectively. This indicates that in the case of the former, two molecules of sulfate are esterified with one molecule of estradiol - 17β while in the latter only one molecule of sulfate attaches to the steroid. It is also seen that one molecule of sulfate conjugates with one molecule of estrone ($^{35}S/^{3}H$ = 1.23). Blocking of the C_3 position of the latter steroid with a methyl group yielded no sulfate or estrone methyl ether in the aqueous fraction, strongly indicating that sulfate ester formation, as measured by the present procedure by this worker, is not an artifact but an actuality.

Returning to Table XII it is observed that the degree of sulfate ester formation was found to be affected by C_{16} substitution. Thus the percentage of recovered estriol in conjugated form was only approximately one-half that seen with estrone as substrate, while the sulfurylation of 16 - ketoestradiol - 17β was equal to that of estrone. The recovered 16 - epiestriol as its conjugate was 1½ times that of estrone but only 0.7 times as great as when estradiol - 17β was the substrate.

The conjugation of dehydroisoandrosterone, containing a saturated A ring and angular methyl group, was discovered to be approximately 11 times that of estrone, containing a planar, unsaturated A ring with no methyl group at C_{10}. The significance of all the above findings will be discussed in Chapt. V.

2. To obtain further information concerning the possible action of the enzyme or enzymes in the soluble fraction, studies were performed

involving inhibition or possible activation of sulfation by incubating two different steroids simultaneously in the presence of the liver preparation from mature female rats as described in 'Section C, 3(b) iii methods'.

Roy (53), upon study of the effects of various steroids on 2 - naphthyl sulfamate synthesis, noted that steroids with a keto group in the C_{17} or possibly C_{16} position exhibited activation of this synthesis by rat-liver preparations. He also noted that there appeared to be antagonism between 17 - oxo and 17 - hydroxy steroids, the latter depressing the activity of the former.

Nose and Lipmann in 1958 suggested that phenol sulfokinases can further be fractionated into more specific enzymes; e.g. estrone sulfokinase (75). However, in 1960, Gregory and Robbins suggested that all phenolic compounds are sulfurylated by one sulfokinase (141).

No work of any significance, however, had been done with ring A unsaturated steroids regarding inhibition or activation of sulfation. This was most probably due to the inapplicability of the methylene blue technique for measuring small differences in esterification of steroids which in themselves are not sulfated to a large degree (see Chapt. IV sect. B).

The aim was, therefore, to study these effects upon estrogen sulfate synthesis to obtain some information regarding the behaviour of the enzyme fraction with steroids. The effect of estrone on dehydroisoandrosterone conjugation was examined for purposes of comparison.

Using estrone or dehydroisoandrosterone (both with 17 - oxo functions) along with the substrate steroid (e.g. estradiol - 17B)

in the reaction mixture, results were obtained from which some conclusions regarding the singularity or multiplicity of enzymes in the fraction as well as specificity of the enzyme(s) could be drawn. Tables XIV to XX contain data on the extent of radioactive estrogen and dehydroisoandrosterone sulfate synthesis by the enzyme preparation from adult female rat livers under the influence of the 17 - oxo steroids, estrone and dehydroisoandrosterone. The oxo steroids are also isotopically labelled, allowing for the calculation of degree of esterification of both the steroids present in the incubation mixture.

It should be noted that the percentage of the recovered product conjugated in the case of the "competing steroid" (17 - oxo steroid) has been calculated at each concentration of this compound. It is obvious, however, that the values shown in any one of the Tables XIV to XX for the conjugation of the competing steroid cannot be directly compared because of the part played by the increasing steroid concentration in the computation of the percentage values. Thus, increasing amounts of competing steroid are conjugated as the steroid concentration is increased but this is not obvious from simple inspection of Tables XIV to XX. However, it is of course possible, from one table to another, to compare the conjugation of the competing steroid (e.g. estrone or dehydroisoandrosterone) at any one concentration (0.002 - 0.04 μ moles) when incubated with a constant amount of any of the substrate steroids.

It is also pertinent to note that the percentage recovered product column of Table XVI contains values which are considerably below normal. This arose mainly due to the necessity of having had

to employ 0.09 μ moles of substrate per tube rather than the usual 0.04 μ moles. As zero order substrate kinetics are found to apply in all of these experiments, the percentage recovered product decreases according to increase of substrate concentration. Further decrease may have resulted from substrate inhibition of enzyme which in the case of some steroids is considerable. However, as the aim of this experiment was to detect inhibition of sulfurylation of estradiol - 17β - 3 methyl ether by estrone, this was of no consequence.

From Table XIV it is noted that 0.008 μ moles of estrone increases the sulfurylation of 0.04 μ moles of dehydroisoandrosterone by a factor of approximately 1.2 times the normal. However, larger quantities of 0.016 μ moles, 0.024 μ moles and 0.040 μ moles of estrone inhibit the sulfation. Esterification reaches a low of 0.85 of the normal 84.0% sulfurylation with 0.04 μ moles of estrone.

No activation of esterification of 0.04 μ moles of estradiol - 17β takes place in the presence of estrone in any concentration, as illustrated by the data of Table XV. Increasing amounts of estrone (0.002 - 0.04 μ moles) inhibit sulfation up to a maximum of 33% from a normal of 15.6% recovered product conjugated.

Table XVI demonstrates a definite inhibition of estradiol - 17β - 3 methyl ether esterification by increasing concentration of estrone. 0.04 μ moles of estrone decreases sulfation of the methyl ether by 53% from the normal which in this case is only 3.8% (already explained).

Figure III summarizes the above described effects.

If another 17 - oxo steroid, dehydroisoandrosterone, is used

TABLE XIV

THE EFFECT OF VARYING ESTRONE CONCENTRATION UPON SULFURYLATION OF DEHYDROISOANDROSTERONE BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	ESTRONE - 6, 7 - ^{3}H *				DEHYDROISOANDROSTERONE - 4 - ^{14}C *			
	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED †	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED †
1.	-	-	-	-	1,300	7,200	0.54	84.0 ± 2.1
2.	16,600	7,600	0.54	30.9 ± 1.1	340	8,500	0.52	95.7 ± 1.8
3.	17,000	6,700	0.79	27.5 ± 1.4	1,900	6,000	0.67	75.0 ± 3.1
4.	19,700	5,300	0.63	20.5 ± 0.6	2,200	6,100	0.52	72.8 ± 2.6
5.	21,100	2,500	0.70	9.7 ± 0.6	1,800	4,700	0.58	71.7 ± 0.7

** The weight and activity of steroids used as in TABLE III 'methods'.

Δ c.p.m. are averages of 2 experiments in duplicate.

† Figures are corrected for blank values.

* Average recovery of incubated radioactivity is 81 ± 2.2% and 73 ± 9.1% for estrone and dehydroisoandrosterone, respectively. It should be emphasized that the S.D. ± 9.1 resulted due to the recovery of a single determination being very low. Generally agreement of recoveries was satisfactory.

TABLE XV

THE EFFECT OF VARYING ESTRONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	ESTRONE - 16 - ^{14}C *				ESTRADIOL - 17β - 6, 7 - ^{3}H *			
	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED+	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED+
1.	-	-	-	-	7,900	1,600	0.82	15.6 ± 0.5
2.	18,100	1,500	0.40	7.2 ± 0.3	-	-	-	-
3.	17,700	1,500	0.61	7.4 ± 0.3	8,400	1,800	0.89	14.2 ± 0.3
4.	18,400	1,600	0.61	7.6 ± 0.2	8,600	1,700	0.89	14.9 ± 0.3
5.	19,100	1,300	0.61	5.8 ± 0.4	9,200	1,800	0.89	11.0 ± 0.5
6.	17,000	1,000	0.61	5.1 ± 0.3	8,200	960	0.89	10.4 ± 0.2

** The weight and activity of steroids used as in Table IV 'methods'.
Δ c.p.m. are averages of 2 experiments in duplicate.
+ Figures are corrected for blank values.
* Average recovery of incubated radioactivity is 82 ± 3.7% and 73 ± 5.3% for estrone and estradiol, respectively.

TABLE XVI

THE EFFECT OF VARYING ESTRONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β - 3 METHYL ETHER BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	ESTRADIOL - 17β - 6, 7 - ^{3}H - 3 METHYL ETHER. *		
	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED. +, 0
1.	31,000	1,300	3.8
2.	33,900	1,400	3.6
3.	35,400	1,100	2.8
4.	33,900	1,100	2.9
5.	33,800	690	1.8

** The weight and activity of steroids used as in Table V 'methods'.

Δ c.p.m. are averages of 1 experiment in duplicate.

+ Figures are corrected for a blank value of 0.24%

* Average recovery of incubated radioactivity is 88 ± 3.7%.

0 See 'Section C, 2 results' for explanation concerning low values.

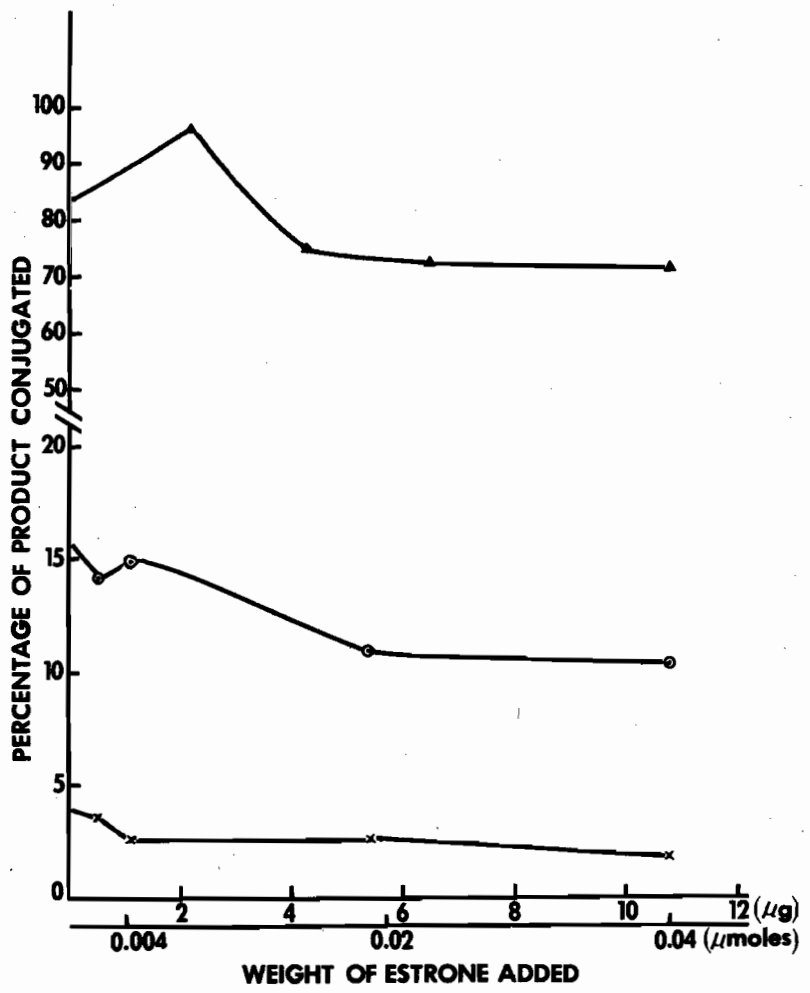


FIGURE III. The Effect Of Varying Estrone Concentration Upon Sulfurylation Of 0.04 μ Moles Of Dehydroisoandrosterone - 4 - ^{14}C, Estradiol - 17β - 6, 7 - ^{3}H And 0.09 μ Moles Of Estradiol - 17β - - 6, 7 - ^{3}H - 3 Methyl Ether In Vitro.

similar results are perceived.

Dehydroisoandrosterone in amounts below one-tenth substrate steroid concentration (estrone) by weight stimulates sulfation of the latter. Thus, 0.002 and 0.004 μ moles of dehydroisoandrosterone increase esterification while 0.02 and 0.04 μ mole amounts depress sulfation of 0.04 μ moles of estrone, reading a low of 1.3%, a drop of 80% from the normal value of 6.4%.

No activation of sulfation is found with either estradiol - 17β or estradiol - 17β - 3 methyl ether, both being considerably inhibited by increasing concentrations of dehydroisoandrosterone, attaining lows of 6.4% and 4.6% respectively. This represents a decrease of esterification from the normal by 62% and 75% respectively.

It is noted that the presence of 0.04 μ moles of dehydroisoandrosterone significantly depresses the degree of sulfurylation of both 16 - ketoestradiol - 17β and 16 - epiestriol, especially in the case of the latter. As shown in Table XX, esterification of 16 - ketoestradiol - 17β is lowered by 18% from the normal while 16 - epiestriol is decreased by 52%.

All these effects are illustrated in Figures IV and V.

In all cases where initial activation of sulfurylation takes place, as with dehydroisoandrosterone influencing estrone sulfurylation and vice versa, it only occurs in the presence of less than one-fifth of activating steroid to substrate steroid by weight. Amounts in excess of one-fifth inhibit sulfation of substrate steroid.

This series of experiments, the data from which was illustrated in Tables XIV to XX, inclusively, was designed to

TABLE XVII

THE EFFECT OF VARYING DEHYDROISOANDROSTERONE CONCENTRATION UPON SULFURYLATION OF ESTRONE BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	DEHYDROISOANDROSTERONE - 4 - ^{14}C *			ESTRONE - 6, 7 - ^{3}H *		
	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†
1.	-	-	-	24,600	1,800	6.4 ± 0.2
2.	1,200	3,400	73.9 ± 1.8	24,800	2,100	7.5 ± 0.3
3.	1,300	5,800	81.6 ± 2.8	24,800	2,000	6.8 ± 0.2
4.	920	6,100	86.5 ± 2.1	25,700	1,100	3.5 ± 0.4
5.	680	4,400	86.2 ± 2.4	28,200	500	1.3 ± 0.4

** The weight and activity of steroids used as in TABLE VI 'methods'.

Δ c.p.m. are averages of 2 experiments in duplicate.

† Figures are corrected for blank values of 0.40% and 0.46% for dehydroisoandrosterone and estrone, respectively.

* Average recovery of incubated radioactivity is 83 ± 3.3% and 83 ± 4.5% for dehydroisoandrosterone and estrone, respectively.

TABLE XVIII

THE EFFECT OF VARYING DEHYDROISOANDROSTERONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	DEHYDROISOANDROSTERONE - 4 - ^{14}C *			ESTRADIOL - 17β - 6, 7 - ^{3}H *		
	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†
1.	-	-	-	9,200	2,000	16.9 ± 0.5
2.	2,900	10,300	77.5 ± 2.2	10,500	1,300	10.5 ± 0.7
3.	1,500	10,600	87.2 ± 1.4	9,700	1,300	11.2 ± 0.4
4.	3,500	11,400	75.9 ± 2.5	11,200	890	6.8 ± 0.4
5.	4,600	10,700	69.4 ± 2.5	11,300	840	6.4 ± 0.4

** The weight and activity of steroids used as in Table VII 'methods'.

Δ c.p.m. are averages of 2 experiments in duplicate.

† Figures are corrected for blank values of 0.51% and 0.55% for dehydroisoandrosterone and estradiol - 17β, respectively.

* Average recovery of incubated radioactivity is 74 ± 4.2% and 84 ± 5.6% for dehydroisoandrosterone and estradiol - 17β, respectively.

TABLE XIX

THE EFFECT OF VARYING DEHYDROISOANDROSTERONE CONCENTRATION UPON SULFURYLATION OF ESTRADIOL - 17β - - 3 METHYL ETHER BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	DEHYDROISOANDROSTERONE - 4 - ^{14}C *			ESTRADIOL - 17β - 6, 7 - ^{3}H - 3 METHYL ETHER *		
	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†
1.	-	-	-	6,800	1,600	18.5 ± 0.4
2.	460	9,700	94.8 ± 2.7	7,300	980	11.5 ± 0.7
3.	500	12,600	95.5 ± 2.0	7,400	1,100	12.9 ± 0.4
4.	700	9,300	92.7 ± 2.6	7,800	630	7.0 ± 0.6
5.	2,400	11,400	82.1 ± 1.4	8,100	430	4.6 ± 0.4

** The weight and activity of steroids used as in Table VIII 'methods'.

Δ c.p.m. are averages of 2 experiments in duplicate.

† Figures are corrected for blank values of 0.65% and 0.46% for dehydroisoandrosterone and estradiol - 17β - - 3 methyl ether, respectively.

* Average recovery of incubated radioactivity is 74 ± 4.7% and 80 ± 4.4% for dehydroisoandrosterone and estradiol - 17β - 3 methyl ether, respectively.

TABLE XX

THE EFFECT OF DEHYDROISOANDROSTERONE UPON SULFURYLATION OF 16 - KETOESTRADIOL - 17β AND 16 - epiESTRIOL BY ADULT FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. **, Δ

TUBE NO.	16 - KETOESTRADIOL - 17β - 16 - ^{14}C *			16 epiESTRIOL - 16 - ^{14}C *		
	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†	UNCONJUGATED c.p.m.	CONJUGATED c.p.m.	% RECOVERED PRODUCT CONJUGATED.†
1.	12,300	1,000	7.4 ± 0.4	-	-	-
2.	11,700	810	6.1 ± 0.2	-	-	-
3.	-	-	-	2,400	330	12.0 ± 0.9
4.	-	-	-	2,800	180	5.8 ± 0.4

** The weight and activity of steroids used as in TABLE IX 'methods'.

Δ c.p.m. are averages of 2 experiments in duplicate.

† Figures are corrected for blank values of 0.40% and 0.20% for 16 - ketoestradiol - 17β and 16 - epiestriol, respectively.

* Average recovery of incubated radioactivity is 81 ± 2.7% and 82 ± 4.0% for 16 - ketoestradiol - 17β and 16 - epiestriol, respectively.

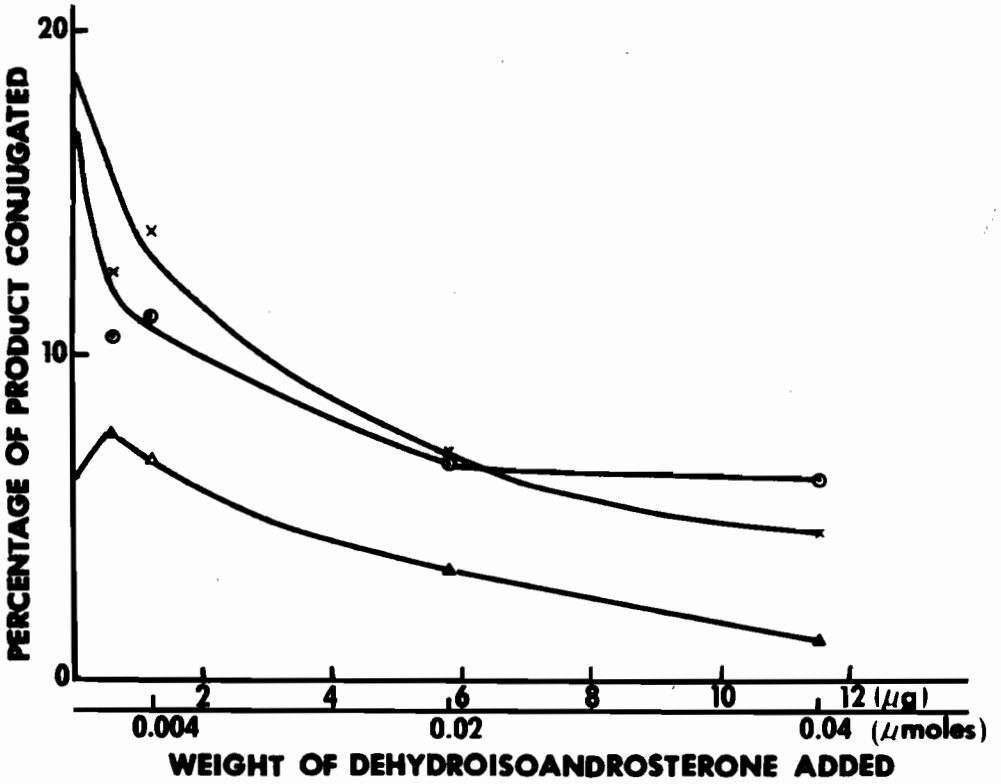


FIGURE IV. The Effect Of Varying Dehydroisoandrosterone Concentration Upon Sulfurylation Of 0.04 µ Moles Of Estrone - 6, 7 - ^{3}H, Estradiol - 17β - 6, 7 - - ^{3}H And Estradiol - 17β - 6, 7 - ^{3}H - 3 Methyl Ether In Vitro.

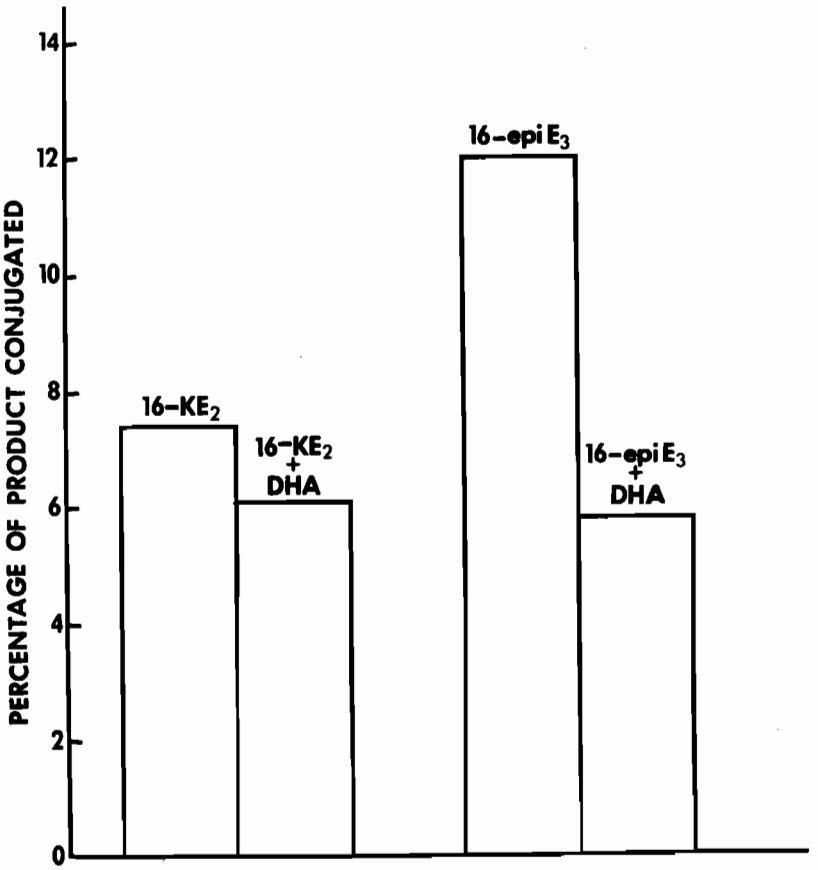


FIGURE V. The Effect Of 0.04 μ Moles Of Dehydroisoandrosterone Upon Sulfurylation Of 0.04 μ Moles Of 16 - Ketoestradiol - 17β - 16 - ^{14}C And 16 - epiEstriol - 16 - ^{14}C In Vitro.

complement the results of Table XII. The differences in esterification noted between the various estrogens in Table XII could have been due either to the presence of multiple sulfokinases in the crude enzyme fraction (a more or less specific enzyme for each estrogen) or to the effect of steroid structure on a common enzyme. The results obtained in this section, at present, indicate the latter to be more likely. A more detailed discussion of this matter will be found in Chapt. V.

3. Because it is well known that sulfation by many human fetal tissues, including the liver, is high in vitro (57), it was of importance to study conjugation by rat fetal liver preparations in order to determine the suitability of the rat as an experimental animal reflecting human metabolic processes by noting any differences between the two species. Obvious dissimilarity of rat fetal liver conjugation with respect to that of human fetuses (see Table XXI) led to studies using animals of different ages and maternal livers at different periods during and after pregnancy. The degree of sulfation was compared to that of normal adult female animals in each case. Also by contrasting esterification of estrone, estradiol - 17β- and dehydroisoandrosterone by livers of rats of increasing age, it was hoped that some further conception as to the singularity or multiplicity of the sulfokinases in the crude enzyme fraction could be obtained.

Table XXI contains data concerning esterification of estrone, estradiol - 17β and dehydroisoandrosterone by rat liver preparations of various ages as well as maternal preparations at different stages before and after pregnancy. Studies were carried out as described in

TABLE XXI

THE EFFECT OF ANIMAL AGE AND MATERNAL CONDITION UPON STEROID SULFATE SYNTHESIS BY THE RAT LIVER SOLUBLE ENZYME PREPARATION. **, Δ

ANIMAL AGE	ESTRONE - 16 - ^{14}C *				ESTRADIOL - 17β - 6, 7 - ^{3}H *				DEHYDROISOANDROSTERONE - 4 - ^{14}C *			
	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED†	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED†	UNCONJ. c.p.m.	CONJ. c.p.m.	% RECOVERED PRODUCT CONJUGATED BLANK	% RECOVERED PRODUCT CONJUGATED†
FETUS (TERM)	37,600	240	0.41	0	17,000	96	0.31	0	21,600	860	0.51	3.3 ± 1.1
10 DAYS	49,800	370	0.41	0	17,700	380	0.31	1.8 ± 0.5	17,900	3,600	0.47	16.2 ± 0.9
30 DAYS	101,000	1,100	0.32	0.8 ± 0.3	59,200	4,000	0.55	5.7 ± 0.4	10,900	12,700	0.17	53.7 ± 1.8
MATERNAL (TERM)	40,400	850	0.41	1.6 ± 0.3	15,900	2,300	0.31	12.3 ± 1.0	980	20,400	0.47	94.9 ± 2.1
MATERNAL (10 DAYS POST DELIVERY)	45,400	1,500	0.41	2.9 ± 0.1	15,300	1,200	0.31	7.1 ± 1.4	4,500	18,800	0.47	80.2 ± 2.1
MATURE (75 DAYS)	20,700	1,800	0.38	7.5 ± 0.9	10,400	2,100	0.69	16.1 ± 0.5	1,300	6,200	0.26	82.9 ± 1.7

** The weight and activity of steroids used as in TABLE X 'methods'.
Δ c.p.m. are averages of 2 experiments in duplicate.
† Figures are corrected for blank values.
* Average recovery of incubated radioactivity is 82 ± 3.2%.
It should be noted that due to a statement by Wengle (142) indicating that dehydroisoandrosterone activity of the liver increases from 0 to 25 days without any demonstrable sex differences, the fetal and 10 day old animals in our studies were not sex differentiated.

'Section C, 3(b) iv methods'.

It is obvious that the conjugating activity was virtually absent from fetal liver but that it increased with age. Table XXII displays the rate of development of sulfurylating activity towards each of the three steroids with increasing age.

TABLE XXII

DEVELOPMENT OF SULFATING SYSTEM WITH AGE AS A PERCENTAGE OF THE LEVEL IN THE MATURE FEMALE RAT LIVER PREPARATION.

STEROID	FETUS (TERM)	10 DAYS	30 DAYS	MATURE
ESTRONE	0	0	10.7	100
ESTRADIOL - 17β	0	11.2	35.4	100
DEHYDROISOANDROSTERONE	3.8	19.5	64.8	100

The above data clearly display an apparent difference in the rate of development of sulfurylating activity towards the three substrates in the order dehydroisoandrosterone > estradiol - 17β > - estrone. At an age of 30 days the liver enzyme preparation appeared not to have reached mature esterification activity with any of the three steroids studied. Complete activity appears sometime between this period and maturity.

The maternal liver preparation also undergoes a loss of enzyme activity at term, as assayed by esterification of estrone and estradiol - 17β, which is still not completely regained after 10 days. The sulfurylation of dehydroisoandrosterone appears normal with both maternal preparations, the sulfation being greater than 80% in both

cases.

Further discussion regarding the significance of the sub-normal activity of the enzyme fraction under different conditions will be found in Chapter V.

D. PURIFICATION AND IDENTIFICATION OF THE RADIOACTIVE INCUBATION PRODUCTS.

One-half of the water soluble material from each series of incubations performed using radioactive substrate was separately pooled for identification purposes. Parts of these solutions were taken and submitted to various forms of examination.

Not all of the pools were subjected to every form of identification due to the abundant number of incubations performed. Instead, random samples were considered typical of the remaining unidentified ones. This was justifiable due to the large cross-section of different pools of incubation products tested.

1. PAPER CHROMATOGRAPHY.

Data are presented regarding the chromatography of two separate pools of water soluble derivatives of dehydroisoandrosterone - 4 - ^{14}C and estrone - 6, 7 - ^{3}H obtained from studies involving the effect of dehydroisoandrosterone concentration upon estrone sulfurylation and vice versa (Tables III and VI 'methods') performed as described in 'Section D, 1 methods'. No attempt was made to actually separate the two steroid conjugates, present in each pool, from each other, the aim being simply to ascertain that the area, on the paper, containing the two incubation products corresponds to the region of the standard sulfates. This was warranted, preliminary studies having shown that unconjugated

estrone and dehydroisoandrosterone run with the solvent front in this system, there thus being a large difference between retention factors of the free and conjugated materials. The purpose of this part of the chromatography was to merely determine whether the incubation products were sulfates, not to identify the metabolites. Table XXIII indicates the activity of incubation product corresponding to the two standard sulfates (standards do not separate from each other in this system).

It is apparent that virtually all the material identified as being conjugated by counting of ethyl acetate extracts at the finish of each individual experiment had the same mobility in this system as sulfate ester standard. The compounds are therefore not in the free state and are most probably sulfates.

TABLE XXIII

PERCENTAGE OF INCUBATION PRODUCT CORRESPONDING TO SULFATE ESTER STANDARD AS DETERMINED BY PAPER CHROMATOGRAPHY Δ.

POOL NO.	ACTIVITY OF MATERIAL CHROMATOGRAPHED c.p.m.		ACTIVITY OF MATERIAL ELUTED CORRESPONDING TO STANDARDS c.p.m.		PERCENTAGE OF ACTIVITY CORRESPONDING TO STANDARDS	
	ESTRONE -6,7-^{3}H	DEHYDROISOANDROSTERONE-4-^{14}C	ESTRONE -6, 7-^{3}H	DEHYDROISOANDROSTERONE-4-^{14}C	ESTRONE -6,7-^{3}H	DEHYDROISOANDROSTERONE-4-^{14}C
1.	149,000	52,000	131,000	45,000	88.1	87.0
2.	69,400	22,600	60,000	19,000	86.3	85.6

Δ c.p.m. are averages of one experiment in duplicate.

TableXXIV displays the distribution of radioactivity upon chromatography of the incubation product(s) of estradiol - 17β - - 6, 7 - ^{3}H on a descending paper system described in 'Section D, 1 methods'.

TABLE XXIV

PAPER CHROMATOGRAPHY OF THE CONJUGATED FRACTION OF ESTRADIOL - 17β - 6, 7 - ^{3}H INCUBATION PRODUCT.

RETENTION FACTOR (RF)	ACTIVITY OF MATERIAL ELUTED c.p.m. *
0 - 0.05	2220 (444)
0.05 - 0.19	500 (100)
0.19 - 0.35	940 (188)
0.35 - 0.51	915 (183)
0.51 - 0.73	430 (86)
0.73 - 1.0	2120 (424)

* Corrected for background of 82 c.p.m. for an aliquot of 1/5 of reported value. Value in brackets is the activity of aliquot corrected for background.

The majority of the counts are found at the opposite ends of the paper while a fraction lies in between. In the area of Rf 0 to 0.05, 2220 counts per minute correspond to estradiol - 3, 17β - - disulfate standard. From Rf 0.05 to Rf 0.73 the counts appear to indicate the presence of some monosulfate (estradiol - 17β - 3 - - sulfate and estradiol - 17β - 17 - sulfate). In the Rf 0.73 to 1.0

area free estradiol - 17β was localized. Ether extraction of counts in this region was almost complete indicating that the radioactive material in this fraction was the unconjugated (free) estradiol - - 17β - 6, 7 - ^{3}H. Since this work was done, further careful examination of the behaviour of estradiol - 3, 17β - disulfate has indicated that it is quite unstable even in a nearly dry state, hydrolyzing quite readily. As pools of material applied to paper had been stored for several weeks, it is indicated that the compound detected at the solvent front may have been hydrolyzed radioactive estradiol - 3, 17β - disulfate. This is further confirmed by previous data reported in this thesis which indicates that the radioactivity of the ethyl acetate fraction is a quantitative reflection of the presence of steroid sulfates.

Paper chromatographic studies therefore further confirm that while some monosulfate of estradiol - 17β may be produced by in vitro incubation under the defined conditions, most of the material appears to be disulfate.

2. SOLVOLYTIC CLEAVAGE OF REACTION PRODUCTS.

Solvolysis is a technique involving a reaction between the substrate and a nucleophilic solvent molecule causing a deesterification of steroid sulfates. In this work it was used as a method of partial identification of products.

It was demonstrated that the aqueous incubation products of the series of experiments performed during this work could be rendered ether soluble after solvolysis. It is a well known fact that steroid conjugates exhibit a partition in favor of water in an ether/water system whereas free estrogens show the opposite behaviour.

TABLE XXV

AMOUNT OF RADIOACTIVE INCUBATION PRODUCT ETHER SOLUBILIZED BY SOLVOLYSIS. Δ

POOL NO.	PRE - SOLVOLYSIS (ETHER INSOLUBLE) c.p.m.		POST - SOLVOLYSIS (ETHER SOLUBLE) c.p.m.	
	^{3}H	^{14}C	^{3}H	^{14}C
1.	16,100	2,400	13,600	2,000
2.	12,900	12,400	10,500	9,900
3.	37,800	5,600	32,900	5,000
4.	17,300	46,800	14,900	39,500
5.	2,100	6,800	1,800	- +

Δ Only one determination per pool performed.

† Sample lost.

Table XXV displays the radioactivity of the ether insoluble material in ethyl acetate before solvolysis and the counts per minute of compound rendered ether soluble after solvolysis of a number of randomly chosen pools of incubation products. Only parts of each pool were utilized, other portions being conserved for further examination.

NOTE: ^{3}H and ^{14}C in the table represent mixtures of products of a multitude of incubations and do not designate any two specific steroids. The aim of this section was to prove that all the material in each pool was conjugated, thus indicating that the single steroids from each individual experiment must have been esterified.

Solvolytic cleavage indicated that 85.1 $\pm$ 2.2% of the (^{3}H) - material and 83.5 $\pm$ 4.1% of the (^{14}C) - labelled compounds accounted for as esterified during the individual incubation experiments was actually conjugated. The remaining 14.9% and 16.5%, respectively, can be accounted for as experimental loss as well as possible break-down of the moderately labile sulfate ester during storage.

NOTE: Only the pooled incubation products of estrone - 6, 7 - ^{3}H and dehydroisoandrosterone - 4 - ^{14}C were solvolyzed.

3. SUCCESSIVE CRYSTALLIZATION OF INCUBATION PRODUCTS.

Recrystallization as a means of determining radiochemical homogeneity of incubation products was performed as described in 'Section D, 3 methods'. Successive crystallizations of the reaction product of estrone - 6, 7 - ^{3}H after ethyl acetate extraction indicated upon the first crystallization that the incubation product and pure estrone - 3 - sulfate standard behaved similarly (Table XXVI). Specific activities of crystals and mother liquor were almost exactly

the same. Furthermore, successive crystallizations did not appreciably decrease the specific activity, indicating lack of radioactive impurity.

Labelled incubation product of estradiol - 17β - 6, 7 - ^{3}H, obtained by ethyl acetate extraction, was mixed with unlabelled estradiol - 17β - 3 - sulfate and estradiol - 17β - 17 - sulfate, respectively. Table XXVI indicates that 3 times and 5 times as much activity was found in the mother liquors as in the crystals, respectively, upon the first crystallization. Further crystallization caused a decrease in the specific activity approaching zero. These results seem to indicate that most of the incubation product was neither estradiol - 17β - 3 - sulfate nor estradiol - 17β - 17 - sulfate. If either of these compounds was present, they would exist only in very small quantities. The product of incubation could have been estradiol - 17β - 6, 7 - ^{3}H (unconjugated) as the presence of diethyl ether in methanol would have prevented its crystallization. However a single crystallization with 15 mg. of estradiol - 17β indicated appreciably no activity in the crystals. The only alternative appears therefore to be estradiol - 3, 17β - disulfate. Due to the tedious procedure of purification involved in the synthesis of the latter, direct identification by crystallization of the incubation product following dilution with carrier was not undertaken for the present.

Finally, comparison of the radioactivity of the ethyl acetate fraction, in reference to estrone - 6, 7 - ^{3}H, and the specific activity of the crystals clearly indicates the acceptability of utilizing radioactive data obtained directly from ethyl acetate without further purification as reflective of the degree of sulfurylation.

TABLE XXVI

PURIFICATION OF REACTION PRODUCTS FOLLOWING INCUBATION OF ESTRONE - 6, 7 - ^{3}H AND ESTRADIOL - 17β - 6, 7 - ^{3}H WITH MATURE FEMALE RAT LIVER SOLUBLE ENZYME FRACTION. *

STAGE OF PURIFICATION	SPECIFIC ACTIVITY c.p.m./mg. ◊		
	ESTRONE-3-SULFATE **	ESTRADIOL-17B-3-SULFATE Δ	ESTRADIOL-17B-17-SULFATE Δ
ETHYL ACETATE EXTRACT OF INCUBATION PRODUCT.	547	1207	1267
CRYSTALS #1	536	447	206
MOTHER LIQUOR #1	525	1231	1001
CRYSTALS #2	526	125	175
MOTHER LIQUOR #2	538	310	-
CRYSTALS #3	511	103	-
MOTHER LIQUOR #3	527	-	-

* For method see 'Section D, 3 methods'.
** Recrystallization with incubation product of estrone - 6, 7 - ^{3}H.
Δ Recrystallization with incubation product of estradiol - 17β - 6, 7 - ^{3}H.
◊ Corrected for background of 82 c.p.m.

4. COLUMN PARTITION CHROMATOGRAPHY OF CONJUGATED INCUBATION PRODUCTS.

Approximately 5000 c.p.m. of the conjugated incubation product (ethyl acetate extract) of estradiol - 17β - 6, 7 - ^{3}H was applied to a Celite column and eluted with Isooctane: tert - butanol: NH_4OH as described in 'Section D, 4 methods'. Approximately one-half of the activity appeared in the region corresponding to pure estradiol - 3, 17β - disulfate standard. The other half of the radioactive material was eluted with the first 5 ml. of eluant indicating the presence of unconjugated material. The lability of the estradiol - 17β disulfate ester is again implied, confirming the results of paper chromatography previously reported. It is of course possible that this hydrolyzed material arose from estradiol - 17β - 3 - sulfate or estradiol - 17β - 17 - sulfate but results from successive crystallizations appear to contraindicate this.

It should be noted that while both paper and column chromatography indicated the presence of unconjugated as well as conjugated material in the ethyl acetate fraction, none was found on crystallization. This may have been due to two reasons. Incubation products for recrystallization were immediately dissolved in methanol and used, while material for paper and column work was pooled and allowed to stand for longer periods of time. Also the procedure of crystallization was a very rapid one, while both paper and column partition chromatography took several days to complete.

In summary it could be stated that ethyl acetate extracts appear to accurately represent the degree of sulfurylation of estrogens without further purification. Indeed, purification may underestimate the degree of sulfation in the case of labile end products such as

estradiol - 3, 17β - disulfate. Only the incubation products of estrone - 6, 7 - ^{3}H and estradiol - 17β - 6, 7 - ^{3}H were identified by the four techniques outlined. Dehydroisoandrosterone - 4 - ^{14}C incubation product was shown to be a water soluble material which yielded free material upon solvolysis and which on descending paper chromatography behaved as a sulfate. Sneddon and Marrian (136) proved by less distinct means and more by implication that estrone sulfate was the incubation product of estrone with bovine-adrenocortical minces. It is, therefore, tentatively suggested that proof of the reaction products of estrone - 6, 7 - ^{3}H, estradiol - 6, 7 - - ^{3}H and dehydroisoandrosterone - 4 - ^{14}C applies to all other estrogens studied by the author.

E. LOCALIZATION OF MATERIAL INTERFERING WITH THE METHYLENE BLUE REACTION.

It was noted that the soluble enzyme preparation of rat liver contained a material which produced a chloroform soluble methylene blue complex. This unidentified compound(s) rendered it impossible to obtain accurate control values for estrogen esterification.

As a first step, the enzyme solution was submitted to electrophoresis to obtain information about the behaviour of the crude fraction in an electric field.

1. PAPER ELECTROPHORESIS.

This was performed as described in 'Section E, 1 methods'.

The crude high speed soluble enzyme fraction was found to separate into two main bands. These patterns fluoresced in the presence of ultraviolet light and were yellow in colour in the

visible region of the spectrum. This suggests that the protein fractions were of a conjugated form, rather than being simple in structure.

Studies of the electrophoresis patterns by staining with Ninhydrin and Brilliant Blue indicated two distinct fractions with a weakly stained area between them. Both bands were basic in nature, their mobility being directed toward the cathode.

2. CHLOROFORM-SOLUBLE METHYLENE BLUE COMPLEX FORMATION.

In an attempt to investigate the specificity of the methylene blue reaction for sulfate esters, a series of sulfates including inorganic and organic compounds were subjected to the methylene blue reaction, followed by chloroform extraction as outlined in 'Section E, 2 methods'.

Table XXVII indicates the absorbence of the methylene blue colour in the chloroform layer for various sulfates.

As far as could be determined from the results as well as from previous work, the methylene blue complexing and chloroform solubilization was limited to phenolic ester sulfates and steroid sulfates. However, suspicions concerning nucleotide sulfates rendering colour in chloroform made possible more direct work in localization of the endogenous colour.

3. INCUBATION OF THE SOLUBLE ENZYME FRACTION FOLLOWED BY DESCENDING PAPER CHROMATOGRAPHY.

The commercial non-availability of nucleotide sulfates led to paper chromatographic work in an attempt to qualitatively detect the compound X which yields methylene blue complexes. This was done with

TABLE XXVII

THE DEGREE OF CHLOROFORM SOLUBLE METHYLENE BLUE COMPLEXING WITH VARIOUS SULFATE COMPOUNDS AS MEASURED SPECTROPHOTOMETRICALLY.

COMPOUND 1 mg./tube	OPTICAL DENSITY 650 m μ.
MAGNESIUM SULFATE	0.00
CADMIUM SULFATE	0.00
AMMONIUM SULFATE	0.00
SODIUM SULFATE	0.01
CERIC SULFATE	0.00
SEROTONIN CREATININE SULFATE	0.01
HYDROXYLAMINE SULFATE	0.01
METHYL iso - THIOUREA SULFATE	0.01
p - TOLUENE SULFONIC ACID	1.50
8 - AMINO - 1 - NAPHTHOL - 3, 6 - DISULFONIC ACID	0.01

the hope of being able to elute the material in large enough quantities for more elaborate identification. Unfortunately not enough of the interfering substance was present for detection by this method, unless incubations on a larger scale were undertaken. This did not seem worthwhile for the present.

4. FRACTIONATION OF THE HIGH SPEED SOLUBLE ENZYME FRACTION.

As an alternative to 'Section 3' above, the enzyme preparation was further fractionated as described in 'Section E, 4 methods' and each fraction was incubated, followed by methylene blue reaction and chloroform extraction.

Table XXVIII presents data on the intensity of the chloroform layer of the 'active sulfate' (adenosine 3′ phosphate 5′ sulfatophosphate or PAPS) fraction as well as that of the sulfokinase fraction. Each of the fractions incubated corresponded to 100 mg. of rat liver. Two trials were performed.

TABLE XXVIII

CHLOROFORM SOLUBLE METHYLENE BLUE COMPLEXING BY THE FRACTIONS OF THE HIGH SPEED SOLUBLE ENZYME PREPARATION OF FEMALE RAT LIVER.

TRIAL NO.	PAPS FRACTION OPTICAL DENSITY (650 m μ)	SULFOKINASE FRACTION OPTICAL DENSITY (650 m μ)
1.	0.24	0.02
2.	0.31	0.06

Although neither of the two fractions incubated was entirely pure, each of the two compounds indicated (PAPS and Sulfokinase) was

probably the most abundant in its respective fraction. Due to the relatively high optical density of the PAPS fraction, there remains little doubt that the adenosine 3′ phosphate 5′ sulfatophosphate is the colour rendering material. It is this compound which invalidates the methylene blue technique, as used above, for the measurement of sulfation of the steroids which are esterified to a slight degree (e.g. estrogen).

DISCUSSION OF RESULTS

The purpose of the inquiry described in this thesis was to gain some insight into the *in vitro* sulfurylation of steroidal estrogens. It was of consequence to determine; (a) how the changes in molecular structure of estrogens affected the degree of their esterification; (b) whether other steroids, either phenolic or non-phenolic, competed for or activated the enzyme system; (c) how changes due to age or maternal condition of animals may have influenced this latter activity. Finally, a practical problem of technique was briefly envisaged by directly examining the enzyme fraction itself.

Since this work was begun, other publications relating to this topic have appeared. These will be included in the subsequent discussion, whether corroborative or contradictory to the author's results.

The most favourable pH conditions for this *in vitro* system was found to be 6.5 to 7.5. This agreed with the published value of pH 6.8 by Roy (52). Decrease of conjugated product below pH 6.0 was considered as a consequence of two separate phenomena. Primarily, it was probably due to direct changes of enzyme molecular structure (e.g. effect of pH upon the ionization states of component amino acids). Secondarily, acid media coupled with heat are known to hydrolyze ester sulfates, thus again decreasing conjugate formation by acting upon the actual product. In the alkaline range ($pH \geq 8.0$), decrease in sulfation is likely due to the former. A pH of 6.8 was therefore maintained in all incubation studies.

For several years controversy has developed concerning whether the soluble enzyme fraction contains one sulfokinase of low

specificity for steroid substrates or whether it is composed of multiple, specific "steroid sulfokinases". Roy (52) indicated that the enzyme system showed a relatively low degree of specificity for steroid substrates as all sterols tested were conjugated with sulfate. However, it has not been decisively demonstrated that only one enzyme is involved in the esterification of all steroids. Up to the time this work was undertaken, there had been only one indication as to the presence of more than one sulfokinase involved in the sulfation of distinct estrogens. Nose and Lipmann (75) claimed to have succeeded in separating estrone sulfokinase from phenol sulfokinase, the latter having previously been considered to be a single enzyme sulfurylating all phenolic compounds including estrogens. However, in 1960, Gregory and Robbins (141) disputed this finding, suggesting that phenolic compounds are all sulfurylated by a single phenol sulfokinase.

The present author performed a series of experiments, examining the effect of estrone and dehydroisoandrosterone upon the esterification of various estrogens which may partially contribute to the solution of this problem. It was of interest to observe whether inhibition of "substrate" steroid sulfation occurred in the presence of these above two 17 - oxo steroids.

A mathematical treatment by Dixon (145) indicated the acceptability of studying inhibition of enzyme activity with respect to substrate by maintaining a constant concentration of the latter while varying inhibitor concentration and vice versa. Roy (53, 79) studied the effect of steroids upon 2 - naphthyl sulfamate syn-

thesis. He noted activation of esterification in the presence of low concentrations of 17 - oxo steroid which subsequently decreased at higher concentrations, using rat liver arylamine sulfokinase. Guinea pig preparations were inhibited by the steroid. In either case, an activation or an inhibition both indicate the presence of a single enzyme which can sulfurylate both the steroid and 2 - naphthylamine. Roy explained both phenomena by basing them upon a mechanism formally similar to partial competitive inhibition. He suggested that a ternary enzyme - steroid - substrate complex (dissociation constant K_s') can be formed. The rate at which this complex decomposed to yield reaction products was identical with that of the breakdown of the normal enzyme - substrate complex (dissociation constant K_s). He proposed that if K_s' is greater than K_s in such a system (i.e. addition of steroid decreases the apparent affinity of the enzyme for the substrate) then the steroid is an inhibitor of the enzyme. If K_s' is less than K_s then the steroid is an activator. Activation appeared to involve the D ring of steroids, as 17 - oxo steroid singularly produced this effect.

By observing the effect of dehydroisoandrosterone concentration upon estrone sulfation and vice versa, the present author noted a very small initial activation followed by inhibition. The activation occurred only if less than one-fifth of oxo steroid relative to substrate steroid by weight was present. If Roy's explanation is adhered to, then low estrone and dehydroisoandrosterone concentrations increased the affinity of the enzyme for the substrate steroid, while higher concentrations decreased it. Activation may possibly involve the presence of more than one site of

attachment of 17 - oxo steroid being present on the enzyme molecule. Both estrone and dehydroisoandrosterone were found to considerably inhibit the function of the enzyme toward sulfurylation of estradiol-17β and estradiol - 17β - 3 methyl ether.

The above information suggested that a common enzyme esterifies estrone, estradiol - 17β, estradiol - 17β - 3 methyl ether and dehydroisoandrosterone, tending to support the view of Gregory and Robbins mentioned earlier in this chapter. It is especially interesting to observe that, if in fact, a common enzyme is present, a phenolic A ring is not a factor determining its specificity as dehydroisoandrosterone appears to compete for enzyme activity with the estrogens. Furthermore, the enzyme appears to sulfurylate both the phenolic and alcohol hydroxyl functions indiscriminately (C_3 and C_{17} groups). This is borne out by noting inhibition of estradiol - 17β - 3 methyl ether sulfation in the presence of estrone, indicating a competition between the C_3 and C_{17} positions. In addition, it is seen from TABLE XII 'results' that blocking of the phenolic group of estradiol - 17β by methylation resulted in no appreciable change in the extent of sulfurylation, again strongly suggesting that the C_{17} hydroxyl function was equally esterified. As a final test of this phenomenon, incubations including the addition of $Na_2{}^{35}SO_4$ to the medium containing (^3H) - labelled estrogen were performed. This resulted in $^{35}S/^3H$ isotope ratios of 2.05 and 1.03 for estradiol - 17β and estradiol - 17β - 3 methyl ether as substrates, respectively, strongly suggesting disulfate formation from estradiol - 17β. (Recrystallization, column and paper chromatography further substantiated this.) When estrone and its 3 - methyl ether were employed in

similar experiments, the isotope ratios were 1.23 and zero, respectively, confirming that isotopic measurements are a reflection of actual conjugation and that blocking the functional group of estrone yielded no conjugated product.

From these experiments, performed by the author, one can further suggest that disulfate formation of estradiol - 17β results under the conditions employed.

Wengle and Boström (146) in 1963 performed similar studies involving in vitro synthesis of disulfates of certain dihydroxy steroids in unfractionated cell-free rat liver enzyme systems containing (^{35}S) - labelled sulfate. They noted that the ratio of disulfate to monosulfate formed was greatest at low substrate levels. Disulfurylation of 3, 17 dihydroxy steroids also was increased if the configuration was 3β 17β rather than any other isomeric form. Payne and Mason (152) on incubation of estradiol - 17β with microsome-free extracts of rat liver demonstrated the formation of estradiol - 3, 17β - disulfate.

In summary, up to this point, it is proposed that one enzyme in the rat liver supernatant fraction is involved in sulfurylation of the estrogens and dehydroisoandrosterone and that this enzyme readily sulfurylates the phenolic C_3 hydroxyl as well as the non-phenolic C_{17} hydroxyl function of estradiol - 17β.

If a common sulfokinase catalyzes the conjugation of all the estrogens shown in TABLE XII 'results', further information concerning the effect of estrogen molecular structure on the degree of sulfurylation can then be obtained from this data.

It must be considered that a keto group at position 17, a 16 keto, 17β - hydroxy grouping or a 16 α, 17β - dihydroxy grouping can

cause a marked decrease in sulfurylation as compared with compounds possessing a 17β - hydroxy or a 16β, 17β - dihydroxy function. From the data in Table XII it is further evident that a C_{16} grouping in the α configuration interferes with sulfation of the molecule to a greater degree than does a planar function (e.g. 16 - keto). A group in the β configuration also interferes, but to a much lesser extent than either of the above (e.g. 16 - epiestriol).

The differing degrees of esterification resulting from the presence of α, β and planar groupings in the C_{16} position of the estrogen molecule prompts the consideration that it may be the α surface of the D ring which binds to the enzyme, although there may be other points of attachment. A similar phenomenon has been described by Munck (147), who suggested that the α sides of rings C and D of steroid molecules in general form complexes with purines. Langer et al. (154) made extensive studies on the influence of steroid structure on its reactivity and affinity for estradiol - 17β-dehydrogenase. They concluded that it is the rear or α - surface of the steroids that binds to the enzyme.

Additional information related to molecular effects upon enzyme activity was published by Wengle and Boström (146) and by Boström and Wengle (148). They discerned that an ethyl or methyl group in the 17 α position of steroids inhibited sulfurylation of the 17β - hydroxyl grouping by rat liver preparations. An ethinyl function in the corresponding position had no effect on esterification of such a group. Their observation that a double bond between the fourth and fifth carbon atoms inhibits sulfurylation of the 3β-hydroxyl group may explain why estrogens are sulfated to a lesser degree by rat liver preparations than the majority of other steroids.

The above workers also noted that 21 - hydroxy steroids were esterified to a fairly large extent by adult human liver extracts, while sulfation of the corresponding 17 α - hydroxy steroids proceeded in low or insignificant amounts.

Table XXI 'results' contains data regarding the effect of animal age and maternal condition upon the activity of the liver enzyme preparation. It was obvious that the conjugating activity was virtually absent from fetal liver but that it increased with age. This has been confirmed by Wengle (142) who, using liver supernatant fluid, found no dehydroisoandrosterone or phenol sulfating activity in the fetal liver.

The author has also shown that although sulfating activity toward each of the substrates used in the assay increased with age, full activity was not attained in 30 day old animals. A similar finding has been discussed by Wengle (142) who indicated that dehydroisoandrosterone sulfating activity of rat liver increased from 0 days to 25 days without demonstratable sex difference. From 25 days to adulthood, activity was still increasing in the female whereas in the male liver it did not increase but actually showed a slight decline. On the basis of both works it appears that full female activity must be reached in the period of 30 to 75 days (adulthood).

A further interesting fact regarding elaboration of enzyme activity has been demonstrated by the author. There was an apparent difference in the rate of development of sulfurylating activity towards the three substrates in the order dehydroisoandrosterone > - estradiol - 17β > estrone. This could conceivably relate to the presence of several sulfokinases specific for each of the steroids

assayed or to unknown physiological factors possibly influencing this activity at different stages of animal development. The latter view is more plausible as a multitude of previous experiments by this writer have indicated the sulfokinase for these substrates to be a single enzyme.

A similar effect was observed with maternal liver at term followed by assays of the activity of the liver ten days post delivery. Sulfation of dehydroisoandrosterone appeared normal at ten days while that of estrone and estradiol - 17β was sub-normal. Again unknown physiological factors seemed to have been involved. In addition, the gross appearance of the liver from maternal animals included a pale, spotted organ as compared to the rich smooth red colour of the normal adult female liver.

As an introductory step in an attempt to study the difference in activity of the enzyme fraction, used throughout this work, from different tissues of the same species, the author performed a preliminary experiment, not described in this thesis, but which may serve as an important basis for further work.

Several implications that the enzyme fractions differ in their activity from species to species as well as within tissues of the same species have been presented in publications. Thus Roy (52, 53) noticed that liver preparations from female rats were considerably more active toward esterification of dehydroisoandrosterone than male preparations. He also observed that incubation of 17 - oxo steroid in the presence of 2 - naphthylamine yielded inhibition of 2 - naphthyl sulfamate synthesis with guinea pig liver preparations but an activation with the rat system. Payne and Mason (155) found that both human and canine adrenal extracts formed the 3 and the

17 - sulfate esters of estradiol - 17β but bovine adrenals yielded only the 3 - sulfate. The author, in a pilot experiment, incubated female rat adrenal minces with estrone, estradiol - 17β and estriol. Due to the obvious difficulty in obtaining sufficient weight of tissue for the assays, this resulted in a situation of First Order Substrate Kinetics. The esterification of the above substrates increased with weight of adrenal tissue in the order estriol > - estradiol - 17β > estrone. Thus, even if actual sulfurylation values relating to each of the above steroids were not available, this work was an indication of the "tendency" to esterify the substrates. Partial corraboration of the preceding has been published by Wengle (149) who using human fetal adrenals has disclosed that estriol is indeed esterified to a larger extent than estrone in vitro. He also noted that adult human adrenal extracts, although having 33 - 70% of adult human liver activity toward steroids, produced twice as much estriol ester sulfate as estrone sulfate (150).

Vlitos (135) employed methylene blue in estimation of dichlorophenoxy ethyl sulfate in soil, indicating that this technique utilizes the solubility of methylene blue complexes of sulfuric acid esters. The method was applied by Roy (51) in steroid sulfate determination. The conjugated steroids complexed with methylene blue while free materials did not. The present author in attempts to use this technique noticed high control values rendering assays of materials of low sulfate esterification potential inadequate.

As a result, preliminary work was done in attempts to localize the interfering compound(s) in the enzyme fraction. An experi-

ment indicated that chloroform soluble methylene blue complexes seem to be formed only with sulfuric acid esters of phenolic and steroidal compounds. However, a different approach to the problem implied that nucleotide sulfates, specifically adenosine 3′phosphate 5′sulfatophosphate (PAPS), also render the soluble complexes. Furthermore, it was confirmed that it is probably the latter compound, present in the whole enzyme fraction, which interferes in the assay of the steroid incubation products. Not much could be done concerning this since PAPS is required as the active intermediate in the biosynthesis of steroid sulfates. For this reason, the author has preferred using the radioactive tracer technique in esterification assays.

SUMMARY

1. Incubation of estrogens singly or in the presence of other steroids and suitable cofactors with an ammonium sulfate precipitate of a high speed soluble enzyme fraction prepared from adult female white Wistar rats indicated that; (a) a common type of enzyme activity was found for both estrogen as well as dehydroisoandrosterone sulfurylation. This was contradictory to the possibility that multiple specific sulfokinases, each acting upon a different estrogen, were present in the preparation; (b) a keto group at position 17, a 16-keto, 17β - hydroxy grouping or a 16 α, 17β - dihydroxy function caused a marked decrease in sulfurylation as compared with steroids possessing a 17β - hydroxy or a 16β, 17β - dihydroxy group; (c) double label studies, performed in the same way as all previous incubations and involving (^{35}S) - labelled Na_2SO_4 and (^{3}H) - labelled estrogen, strongly suggested disulfate formation from estradiol - 17β. This was confirmed by blocking of the phenolic hydroxyl as well as by chromatographic and crystallization experiments.

2. Liver enzyme preparations from rats of various ages and maternal states were incubated with estrone, estradiol - 17β and dehydroisoandrosterone. Conjugating activity was absent from fetal liver but increased with age. Full adult female activity was reached during the period from 30 to 75 days after birth. The rate of development of sulfurylating activity towards the three substrates was in the order dehydroisoandrosterone > - estradiol - 17β > estrone which was probably a result of unknown physiological factors possibly influencing the activity at different stages of animal development. A similar effect was observed

with preparations of maternal livers obtained during and at different times after delivery of the fetus.

3. Preliminary evidence indicating a difference between the behaviour of rat adrenal minces and liver enzyme preparations towards sulfurylation of estrogens has been presented.

4. A material interfering with the methylene blue reaction was localized in the adult female rat liver enzyme fraction. It was tentatively suggested that it was adenosine 3′phosphate 5′sulfatophosphate (PAPS).

BIBLIOGRAPHY

1. HENRY, W.,
The Elements of Experimental Chemistry, 11th, ed. 2, 491,
London (1829)

2. BAUMANN, E.,
Pflügers Arch., 12, 63, 69 (1876)

3. BAUMANN, E.,
Ber. dtsch. chem. Ges., 9, 54 (1876)

4. BAUMANN, E.,
Hoppe-Seylers Z., 1, 68 (1877)

5. HERTER, C.A. and WAKEMAN, A.J.,
J. Exp. Med., 4, 307 (1899)

6. EMBDEN, G.,
Beitr. chem. Physiol. Path., 2, 591 (1902)

7. EMBDEN, G. and GLASSNER, K.,
Beitr. chem. Physiol. Path., 1, 310 (1902)

8. MARENZI, A.D.,
C.R. Soc. Biol., Paris, 107, 737 (1931)

9. DOISY, E.A., VELER, C.D. and THAYER, S.,
Am. J. Physiol., 90, 329 (1929)

10. BUTENANDT, A.,
Naturwissenschaften, 17, 878 (1929)

11. GLIMM, E. and WADEHN, F.,
Biochem. Z., 207, 361 (1929)

12. MARRIAN, G.F.,
Nature, 125, 90 (1930)

13. MARRIAN, G.F.,
Physiol. Rev., 13, 185 (1933)

14. DOISY, E.A., VELER, C.D. and THAYER, S.,
J. Biol. Chem., 86, 499 (1930)

15. VELER, C.D., THAYER, S. and DOISY, E.A.,
J. Biol. Chem., 87, 357 (1930)

16. COLLIP, J.B., BROWNE, J.S.L. and THOMSON, D.L.,
J. Biol. Chem., 97, XVII (1932)

17. BROWNE, J.S.L.,
Can. J. Research, 8, 180 (1933)

18. BUTENANDT, A. and BROWNE, J.S.L.,
Hoppe-Seylers Z., Physiol. Chem. 216, 49 (1933)

19. KOBER, S.,
Biochem. Z., 239, 209 (1931)

20. COHEN, S.L. and MARRIAN, G.F.,
Biochem. J., 28, 1603 (1934)

21. BORCHARDT, H., DINGEMANSE, E. and LAQUEUR, E.,
Naturwissenschaften, 22, 190 (1934)

22. COHEN, S.L. and MARRIAN, G.F.,
Biochem. J., 30, 57 (1936)

23. COHEN, S.L., MARRIAN, G.F. and ODELL, A.D.,
Biochem. J., 30, 2250 (1936)

24. CALLOW, R.K.,
Biochem. J., 30, 906 (1936)

25. GRANT, J.K. and MARRIAN, G.F.,
Biochem. J., 47, 1 (1950)

26. SCHACHTER, B. and MARRIAN, G.F.,
Proc. Soc. Exp. Biol. and Med., 35, 222 (1936)

27. SCHACHTER, B. and MARRIAN, G.F.,
J. Biol. Chem., 126, 663 (1938)

28. VENNING, E.H., HOFFMAN, M.M. and BROWNE, J.S.L.,
Fed. Proc., 1, 139 (1942)

29. VENNING, E.H., HOFFMAN, M.M., and BROWNE, J.S.L.,
J. Biol. Chem., 146, 369 (1942)

30. MUNSON, P.L., GALLAGHER, T.F. and KOCH, F.C.,
Endocrinology, 30, S 1036 (1942)

31. MUNSON, P.L., GALLAGHER, T.F. and KOCH, F.C.,
J. Biol. Chem., 152, 67 (1944)

32. DRAYER, N.M., ROBERTS, K.D., BANDI, L and LIEBERMAN, S.,
J. Biol. Chem., 239, PC 3112 (1964)

33. BUTENANDT, A. and HOFSTETTER, H.,
Hoppe-Seylers Z. Physiol. Chem., 259, 222 (1939)

34. COHEN, H. and BATES, R.W.,
Endocrinology, 45, 86 (1949)

35. ONESON, I.B. and COHEN, S.L.,
Endocrinology, 51, 173 (1952)

36. McKENNA, J., MENINI, E. and NORYMBERSKI, J.K.,
Biochem. J., 79, 11 p (1961)

37. PURDY, R.H., ENGEL, L.L. and ONCLEY, J.L.,
Fed. Proc., 18, 305 (1959)

38. PURDY, R.H., ENGEL, L.L. and ONCLEY, J.L.,
J. Biol. Chem. 236, 1043 (1961)

39. LEVITZ, M., CONDON, G.P., MONEY, W.L. and DANCIS, J.,
J. Biol. Chem., 235, 973 (1960)

40. MENINI, E. and DICZFALUSY, E.,
Endocrinology, 68, 492 (1961)

41. TROEN, P., NILSSON, B., WIQVIST, N. and DICZFALUSY, E.,
Acta Endocrin., 38, 361 (1961)

42. DICZFALUSY, E., CASSMER, O., ALONSO, C. and de MIQUEL, M.,
Acta Endocrin., 38, 31 (1961)

43. LEVITZ, M., CONDON, G.P. and DANCIS, J.,
Endocrinology, 68, 825 (1961)

44. BOYLAND, E., MANSON, D. and ORR, S.F.D.,
Biochem. J., 65, 417 (1957)

45. FOGGITT, F. and KELLIE, A.E.,
Biochem. J., 91, 209 (1964)

46. ROBERTS, K.D., BANDI, L., CALVIN, H.I., DRUCKER, W.D. and LIEBERMAN, S.,
J. Am. Chem. Soc., 86, 958 (1964)

47. ARNOLT, R.J. and DE MEIO, R.H.,
Rev. Soc. Argent. Biol., 17, 570 (1941)

48. DE MEIO, R.H. and ARNOLT, R.J.,
J. Biol. Chem., 156, 577 (1944)

49. CREPY, O.,
Compt. Rend. Acad. Sci., 223, 646 (1946)

50. SEGAL, H.L.,
J. Biol. Chem., 213, 161 (1955)

51. ROY, A.B.,
Biochem. J., 62, 41 (1956)

52. ROY, A.B.,
Biochem. J., 63, 294 (1956)

53. ROY, A.B.,
Biochem. J., 74, 49 (1960)

54. SCHNEIDER, J.J. and LEWBART, M.L.,
J. Biol. Chem., 222, 787 (1956)

55. SUZUKI, S., TAKAHASHI, N. and EGAMI, F.,
Biochim. Biophys. Acta, 24, 444 (1957)

56. DE MEIO, R.H., LEWYCKA, C., WIZERKANIUK, M. and SALCIUNAS, O.,
Biochem. J., 68, 1 (1958)

57. DICZFALUSY, E., CASSMER, O., ALONSO, C., de MIQUEL, M. and WESTIN, B.,
Acta Endocrin., 37, 516 (1961)

58. WALLACE, E. and LIEBERMAN, S.,
J. Clin. Endocrin. and Metab., 23, 90 (1963)

59. COHN, G.L., MULROW, P.J. and DUNN, V.C.,
J. Clin. Endocrin. and Metab., 23, 671 (1963)

60. GUAL, C., ROJO, B., LEMUS, A.E. and RIVERA, R.,
Sociedad Mexicana de Nutricion y Endocrinologia, 3rd Meeting, 1962, p. 126.

61. WALLACE, E. and SILBERMAN, N.,
J. Biol. Chem., 239, 2809 (1964)

62. DE MEIO, R.H. and TKACZ, L.,
Arch. Biochem., 27, 242 (1950)

63. DE MEIO, R.H. and TKACZ, L.,
J. Biol. Chem., 195, 175 (1952)

64. DE MEIO, R.H., WIZERKANIUK, M. and FABIANI, E.,
J. Biol. Chem., 203, 257 (1953)

65. BERNSTEIN, S. and McGILVERY, R.W.,
J. Biol. Chem., 198, 195 (1952)

66. BERNSTEIN, S. and McGILVERY, R.W.,
J. Biol. Chem., 199, 745 (1952)

67. SEGAL, H.L.,
J. Biol. Chem., 213, 161 (1955)

68. HILZ, H. and LIPMANN, F.,
Proc. Nat. Acad. Sci., 41, 880 (1955)

69. DE MEIO, R.H., WIZERKANIUK, M. and SCHREIBMAN, I.,
J. Biol. Chem., 213, 439 (1955)

70. ROBBINS, P.W. and LIPMANN, F.,
J. Am. Chem. Soc., 78, 2652 (1956)

71. WILSON, L.G. and BANDURSKI, R.S.,
Arch. Biochem. Biophys., 62, 503 (1956)

72. ROBBINS, P.W. and LIPMANN, F.,
J. Am. Chem. Soc., 78, 6409 (1956)

73. ROBBINS, P.W. and LIPMANN, F.,
J. Biol. Chem., 229, 837 (1957)

74. ROBBINS, P.W. and LIPMANN, F.,
J. Biol. Chem., 233, 681 (1958)

75. NOSE, Y. and LIPMANN, F.,
J. Biol. Chem., 233, 1348 (1958)

76. ROY, A.B.,
Biochem. J. 72, 19 p (1959)

77. SEGAL, H.,
Fed. Proc. 16, 245 (1957)

78. ROY, A.B.,
Biochim. Biophys. Acta, 30, 193 (1958)

79. ROY, A.B.,
Biochem. J., 79, 253 (1961)

80. PASTERNAK, C.A., HUMPHRIES, S.K. and PIRIE, A.,
Biochem. J., 86, 382 (1963)

81. LIPMANN, F.,
Science, 128, 575 (1958)

82. LIPMANN, F.,
Science, 120, 855 (1954)

83. STOREY, I.D.E.,
Biochem. J., 47, 212 (1950)

84. CARPENTER, J.G.D. and KELLIE, A.E.,
Biochem. J., 78, 1 P (1960)

85. CARPENTER, J.G.D. and KELLIE, A.E.,
Biochem. J., 84, 303 (1962)

86. NEEMAN, M. and HASHIMOTO, Y.,
Tetrahedron Letters, 5, 183 (1961)

87. BELING, C.G.,
V International Congress of Biochemistry, Moscow 1961,
Oxford and Warsaw (1961) p. 249

88. BELING, C.G.,
Nature, 192, 326 (1961)

89. BELING, C.G.,
Acta Endocrin., Suppl. 67, 79 (1962)

90. FELGER, C.B. and KATZMAN, P.A.,
Fed. Proc., 20, 199 (1961)

91. TROEN, P., NILSSON, B., WIQVIST, N. and DICZFALUSY, E.,
Acta Endocrin., 38, 361 (1961)

92. STRAW, R.F., KATZMAN, P.A. and DOISY, E.A.,
Endocrinology, 57, 87 (1955)

93. ODELL, A.D. and MARRIAN, G.F.,
Biochem. J., 30, 1533 (1936)

94. VENNING, E.H. and BROWNE, J.S.L.,
Proc. Soc. Exp. Biol. and Med., 34, 792 (1936)

95. PETERSON, D.H., HOSKINS, W.H., COFFMAN, S.R. and KOCH, F.C.,
J. Biol. Chem., 123, XCIII (1938)

96. FISHMAN, W.H. and SIE, H.G.,
J. Biol. Chem., 218, 335 (1956)

97. WOTIZ, H.R., ZISKIND, B.S., LEMON, H.M. and GUT, M.,
Biochim. Biophys. Acta, 22, 266 (1956)

98. WOTIZ, H.R., SIE, H.G. and FISHMAN, W.H.,
J. Biol. Chem., 232, 723 (1958)

99. SCHNEIDER, J.J., LEWBART, M.L., LEVITEN, P. and LIEBERMAN, S.,
J. Am. Chem. Soc., 77, 4185 (1955)

100. LIPSCHITZ, W.I. and BUEDING, E.,
J. Biol. Chem., 126, 663 (1939)

101. SHIRAI, Y. and OHKUBO, T.,
J. Biochem., 41, 341 (1954)

102. HARTIALA, K.J.V.,
Acta Physiol. Scand., Suppl. 114 (1954)

103. HARTIALA, K.J.,
Ann. Med. Exptl. Et Biol. Fenniae, Helsinki, 33, 239 (1955)

104. SCHACHTER, B., KASS, D.J. and LANNON, T.J.,
J. Biol. Chem., 234, 201 (1959)

105. MENINI, E. and DICZFALUSY, E.,
Endocrinology, 67, 500 (1960)

106. DICZFALUSY, E., FRANKSSON, C. and MARTINSEN, B.,
Acta Endocrin., 38, 59 (1961)

107. DUTTON, G.J. and STOREY, I.D.E.,
Biochem. J., 53, XXXVIII (1953)

108. DUTTON, G.J. and STOREY, I.D.E.,
Biochem. J., 57, 275 (1954)

109. STOREY, I.D.E. and DUTTON, G.J.,
Biochem. J., 59, 279 (1955)

110. BERG, P. and JOKLIK, W.K.,
Nature, 172, 1008 (1953)

111. MUNCH - PETERSEN, A., KALCKAR, H.M., CUTOLO, E. and SMITH, E.E.B.,
Nature, 172, 1036 (1953)

112. SMITH, E.E.B. and MILLS, G.T.,
Biochim. Biophys. Acta, 13, 386 (1954)

113. ISSELBACHER, K.J.,
Rec. Prog. Horm. Res., 12, 134 (1956)

114. STEVENSON, I.H. and DUNCON, G.J.,
Biochem. J., 82, 330 (1962)

115. DUTTON, G.J. and STEVENSON, I.M.,
Biochim. Biophys. Acta, 31, 568 (1959)

116. SMITH, E.R. and BREUER, H.,
Biochem. J., 88, 168 (1963)

117. RAKOKOFF, A.E., PASCHKIS, K.E. and CANTAROW, A.,
Amer. J. Obstet. and Gynec., 46, 856 (1943)

118. ROBERTS, S. and SZEGO, C.M.,
Endocrinology, 39, 183 (1946)

119. BISCHOFF, F., STAUFFER, R.D. and GRAY, C.L.,
Amer. J. Physiol., 177, 65 (1954)

120. SANDBERG, A.A. and SLAUNWHITE, W.R., JR.,
J. Clin. Endocrin., 16, 923 (1956)

121. ANTONIADES, H.N., McARTHUR, J.W., PENNELL, R.B., INGERSOLL, F.M., ULFELDER, H. and ONCLEY, J.L.,
Amer. J. Physiol., 189, 455 (1957)

122. SZEGO, C.M. and ROBERTS, S.,
Proc. Soc. Exp. Biol., (N.Y.), 61, 161 (1946)

123. EADES, C.H., POLLACK, R.L. and KING, J.S.,
Fed. Proc., 13, 201 (1954)

124. WEICHSELBAUM, T.E. and MARGRAF, H.W.,
J. Clin. Endocrin., 20, 1341 (1960)

125. OERTEL, G.W.,
Biochem. Z., 334, 431 (1961)

126. FISHMAN, W.H. and FISHMAN, L.W.,
J. Biol. Chem., 152, 487 (1944)

127. FISHMAN, W.H.,
J. Biol. Chem., 169, 7 (1947)

128. ROBERTS, S. and SZEGO, C.M.,
Endocrinology, 40, 73 (1947)

129. TWOMBLY, G.H. and LEVITZ, M.,
Am. J. Obstet. Gynec., 80, 889 (1960)

130. VANDEWIELE, R.L., MacDONALD, P.C., GURPIDE, E. and LIEBERMAN, S.,
Rec. Prog. Horm. Res., 19, 275 (1963)

131. DICZFALUSY, E. and MÜNSTERMANN, A.M.,
Acta Endocrin., 32, 195 (1959)

132. LISBOA, B.P. and DICZFALUSY, E.,
Acta Endocrin., 40, 60 (1962)

133. ITTRICH, G.,
Ztsch. Physiol. Chem., 312, 1 (1958)

134. BROWN, J.B.,
Biochem. J., 60, 185 (1955)

135. VLITOS, A.J.,
Contr. Boyce Thompson Inst., 17, 127 (1953)

136. SNEDDON, A. and MARRIAN, G.F.,
Biochem. J., 86, 385 (1963)

137. EMERMAN, S., LEVITZ, M., DANCIS, J., WIQVIST, N. and DICZFALUSY, E.,
Proc. 46th Meeting Endocrin. Soc., San Francisco, 1964, P. 36

138. SEGAL, L., SEGAL, B. and NES, W.R.,
J. Biol. Chem., 235, 3108 (1960)

139. BAULD, W.S. and GREENWAY, R.M.,
Methods of Biochem. Anal., 5, 337 (1957)

140. SEHON, A.H., RICHTER, M., HARTER, J.G. and ROSE, B.,
J. Allergy, 27, 236 (1956)

141. GREGORY, J.D. and ROBBINS, P.W.,
Ann. Rev. Biochem., 29, 347 (1960)

142. WENGLE, B.,
Acta Societatis Medicorum Upsaliensis, 68, 154 (1963)

143. FISHMAN, J., BRADLOW, H.L. and GALLAGHER, T.F.,
J. Biol. Chem., 235, 3104 (1960)

144. GREGORY, J.D. and NOSE, Y.,
Fed. Proc., 16, 189 (1957)

145. DIXON, M.,
Biochem. J., 55, 170 (1953)

146. WENGLE, B. and BOSTRÖM, H.,
Acta Chem. Scand., 17, 1203 (1963)

147. MUNCK, A., SCOTT, J.F. and ENGEL, L.L.,
Biochim. et Biophys. Acta, 26, 397 (1957)

148. BOSTRÖM, H. and WENGLE, B.,
Acta Societatis Medicorum Upsaliensis, 69, 41 (1964)

149. WENGLE, B.,
Acta Societatis Medicorum Upsaliensis, 69, 105 (1964)

150. BOSTRÖM, H., FRANKSSON, C. and WENGLE, B.,
Acta Endocrin., 47, 633 (1964)

151. OKITA, G.T., KABARA, J.J., RICHARDSON, F. and LE ROY, G.V.,
Nucleonics, 15, 111 (1957)

152. PAYNE, A.H. and MASON, M.,
Biochim. Biophys. Acta., 71, 719 (1963)

153. LAYNE, D.S., SHETH, N.A. and KIRDANI, R.Y.,
J. Biol. Chem., 239, 3221 (1964)

154. LANGER, L.J., ALEXANDER, J.A. and ENGEL, L.L.,
J. Biol. Chem., 234, 2609 (1959)

155. PAYNE, A.H. and MASON, M.,
Steroids, 5, 21 (1965)

156. BAULD, W.S.B. and GREENWAY, R.,
Methods of Biochemical Analysis. New York, N.Y.
Interscience Publishers, Inc., 157. Vol. 5, p. 345

157. KELLY, W.G., BANDI, L., SHOOLERY, J.M. and LIEBERMAN, S.,
Biochemistry, 1, 172 (1962)

158. FIESER, L.F.,
J. Am. Chem. Soc., 70, 3232 (1948)

159. JENSEN, H., LAIVIERE, M. and ELIE, J.J.,
J. Rev. Canad. Biol., 4, 535 (1945)

160. SOBEL, A.E., DREKTER, I.J. and NATELSON, S.,
J. Biol. Chem., 115, 381 (1936)

161. LEVITZ, M.,
Steroids, 1, 117 (1963)