RUNNING TITLE

"RENAL METABOLISM OF ESTROGENS IN VITRO"

P. I. MUSEY

ABSTRACT

M.Sc. BIOCHEMISTRY

PAUL ISAAC MUSEY

METABOLISM OF FREE AND CONJUGATED
ESTROGEN BY RENAL PREPARATIONS IN VITRO

Renal tissue homogenates of different species: rat, hen, rabbit and dog were used to study the metabolism of ^{3}H-Estrone (E_1), Estradiol 17β (E_2); Estrone Sulfate (E_1S) and Estradiol 17β glucuronide (E_2G) separately. The metabolites were separated by various extraction procedures and the conjugates investigated by chromatography on DEAE-sephadex.

The main steroid found following incubation of E_1 or E_2 in all species was E_1. This agrees with most in vitro studies except where gonadal tissue is concerned. More polar metabolites were virtually absent and there was no evidence of sulfurylation having occurred. Some glucuronide formation was apparent. Incubation of E_1S showed a wide range of sulfatase activities, extremely high in the rat and very low in the dog. The hen, followed by the rabbit showed intermediate values. Kidney preparations from rabbit and dog appeared capable of metabolizing E_1S without prior hydrolysis. Little evidence was obtained for the presence of significant β-glucuronidase activity in these tissues. On occasion it appeared that E_2G could be converted to conjugated metabolites probably the N-acetyl-glucosamine derivative.

METABOLISM OF FREE AND CONJUGATED ESTROGENS BY RENAL PREPARATIONS IN VITRO

by

P. I. MUSEY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY
McGILL UNIVERSITY
MONTREAL, CANADA

JULY, 1969.

ACKNOWLEDGEMENTS

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, Montreal General Hospital.

The financial support of the Canadian International Development Agency is gratefully acknowledged.

In addition, I wish to thank the typist of this manuscript, Miss M. A. Borrelli for her skillful assistance.

The following trivial, abbreviations and systematic names were used:

Estrone (E_1), 3-hydroxy-estra-1,3,5(10)-trien-17-one.

2 Hydroxy Estrone ($2OHE_1$), 2-hydroxy-3-hydroxy-1,3,5(10)-trien-17-one.

2 Methoxy Estrone ($2MeOE_1$), 2-methoxy-3 hydroxy-1,3,5(10)-trien-17-one.

17β-Estradiol ($E_2 17\beta$), Estra-1,3,5(10)-trien, 17β-diol.

17α-Estradiol ($E_2 17\alpha$), Estra-1,3,5(10)-trien-3, 17α-diol.

Estriol (E_3), Estra-1,3,5(10)-trien-3, 16α, 17β-triol.

Estrone Sulfate (E_1S), 3-hydroxy-estra-1,3,5(10)-trien-17-one-3-yl-sulfate.

Estradiol-3-Sulfate (E_2-3S), 3-hydroxy-estra-1,3,5(10)-trien-17β-ol-3-yl sulfate.

Estradiol-17β-Glucuronide ($E_2 17G$), 3-hydroxy-estra-1,3,5(10)-trien-17β-yl glucuronide.

NADPH. - Reduced triphosphopyridine nucleotide.

ATP. - Adenosine triphosphate.

DHAS. - Dehydroepiandrosterone sulfate.

DHA - Dehydroepiandrosterone.

SA. - Specific activity.

M., N. - Molar, Normal.

g., mg., μg - Gram, Milligram, Microgram.

cpm. - Counts per minute.

dpm. - Disintegration per minute.

LIST OF FIGURES

LIST OF TABLES

TO MY FATHER

CONTENTS

A. INTRODUCTION

1. NATURE OF ESTROGENS IN BLOOD AND URINE

There is considerable information on the nature of estrogens in plasma and urine. All works have indicated that the estrogens exist predominantly as their conjugates. They are conjugated with acidic groups, mainly sulfuric and glucuronic acids.

Muhlbock (1, 2) first showed that human and equine blood, especially in the pregnant state contained estrogens in the combined form. Estrogens conjugated or in combination with proteins have also been indicated. Rakoff (3) in 1943 reported that 23-50% of the total estrogens in late pregnant serum was combined with serum proteins in a form that was difficult to hydrolyze. Later, Szego and Roberts (4) in 1946 indicated that as much as two-thirds of the total estrogens in blood was closely associated with proteins, the remainder was conjugated with something other than protein.

As early as 1929, Glimm and Wadehn (5) found that estrogens are excreted via the urine in a form not extractable by organic solvents. They postulated that the unextractable estrogens might be present in combination with an acid. Cohen and Marrian (6) also reported in 1936 that estrogens exist in the urine principally as conjugates, they were the first to isolate Estriol glucuronide as the main estrogen conjugate of late pregnancy urine. These authors, therefore assumed that the conjugates were also present in the blood. This was later confirmed by Sandberg and Slaunwhite (7), Purdy (8) and Migeon (9).

The two principal estrogen conjugates first isolated from urine

were Estrone Sulfate (E_1S) from pregnant mares' urine (10) and from stallions' urine (11) and Estriol Glucuronide (E_3G) from human pregnancy urine (12). Estrone sulfate has also been identified in extracts of human plasma after the administration of Estradiol (^{14}C-E_2) to women and also from late pregnancy plasma (8). From the pooled pregnancy plasma, the concentration of E_1S was found to be approximately four times that of free Estrone (E_1) on a molar basis. These authors (8) concluded that Estrone sulfate is an important circulating form of estrogen in the human. Only small amounts of glucuronides were found either after injecting labeled Estradiol or of endogenous origin in pregnancy plasma. Levitz (13) has also found both Estrone and Estradiol sulfates (E_1S, E_2S) in the umbilical circulation of the human fetus after injecting ^{14}C-E_1 into the mother. Other workers, MacKenna et al (14) and Adlercreutz et al (15) also concluded from their works that the sulfate of Estrone is important in the pregnancy urine. Evidence for Estrone sulfate in the non-pregnant individual has been scanty and inconclusive. The real proof of E_1S in the non-pregnant urine has just been published from our laboratory by Hobkirk et al (16).

Prior to the work of Purdy (8), Roberts and Szego (17) had concluded from their data that Estriol glucuronide (E_3G) was the chief conjugated estrogen in the blood. Large amounts of conjugated estrone has been found in urine (18), and in 1952, Oneson found most of the estrone in pregnancy urine to be present as the glucuronide (19). Since 1936, Marrian and his group (6, 20) have crystallized the Estriol glucuronide from pregnancy urine, as the sodium salt and shown to correspond in composition to an estriol monoglucuronide with a free phenolic group at the steroid C-3 position. The absolute structure of this conjugate

was established by Neeman and Hashimoto (21) by the conversion of the glucuronide into the completely methylated derivative and the subsequent hydrolysis of the latter. The identification of the dimethyl-derivative of estriol, established the constitution of the urinary metabolite as Estriol 16α-glucuronide ($E_3$16αG). In that same year, 1962, Carpenter and Kellie (23) converted the estriol glucuronide fraction of late pregnancy urine by methylation then acetylation into the triacetate methyl ester derivatives. The identification of these derivatives established the presence of Estriol 16α-glucuronide ($E_3$16αG) and Estriol 17β-glucuronide ($E_3$17βG) in pregnancy urine. In subsequent investigation, Kellie (24) could not find convincing evidence of the presence of the E_3-17βG.

Estriol-3-glucuronide has been tentatively identified by many workers. In 1963 Beling (25) detected an estrogen conjugate with similar properties to the 3-glucuronide. Later Goebelsman (26-28) injected labeled E_3-3S, E_3-3G and E_3-16G to pregnant women and found in their urines, a conjugate which they identified as Estriol 3-glucuronide. They therefore concluded that E_3-3G is a major metabolite of estriol. Intestinal synthesis of $E_3$3G has been shown <u>in vitro</u> by Dahm and Breuer (29) and Dahm <u>et al</u> (30). This intestinal synthesis has recently been confirmed <u>in vivo</u> by Støa and Levitz (31) using a human male. The real proof of Estriol 3-glucuronide in the human pregnancy urine has just been reported by Ladany (32) who isolated the metabolite in crystalline form and characterized it by chemical methods as well as by Infra Red Spectroscopy.

All three classical estrogens, namely, Estrone (E_1), Estradiol (E_2) and Estriol (E_3) have been demonstrated in the hen, dog, rat and rabbit either as endogenous products or <u>in vitro</u> metabolites. Estrogen conjugates, sulfates and glucuronides have also been shown <u>in vivo</u> or <u>in vitro</u> in

these animals. In the hen, dog and rabbit, there occurs the epimer of Estradiol-17β, E_2-17α in significant quantities often exceeding $E_2$17β. In the rabbit, particularly, $E_2$17α seems to be the sole endogenous metabolite. This epimer of $E_2$17β has so far not been fully identified in pooled human pregnancy urine. However, Schott and Katzman (33) obtained evidence of its presence in the urine of a single pregnant individual. Adlercreutz and Luukkainen (34) have recently also suggested that $E_2$17α may be present in small amounts in the bile of pregnant women. Rather striking forms of estrogen conjugates have been identified, first in the rabbit and more recently in human. These are the N-acetylglucosamine derivatives. Layne (34-36) has reported that the rabbit excretes Estradiol as Estradiol-3-glucuronide-17α-N-Acetylglucosaminide and that the C-17 α-N-Acetylglucosamine is put on after the C-3 conjugation with glucuronic acid. It is interesting to note that the N-Acetyl-Glucosamine is put only at the 17α position and never β. The conjugation with N-Acetylglucosamine is also seen in the human. After administration of Estrone-^{3}H-Sulfate-^{35}S to a woman, Levitz (37) isolated both Estradiol-3-Sulfate-15αN-Acetyl glucosaminide and 15αhydroxy Estrone 3-Sulfate 15α-N-Acetylglucosaminide (E_2-3S-16αNAcGluNH$_2$ and 15α $OHE_1$3-S-15αN-AcGluNH$_2$). Arcos and Lieberman (38) in reference to progesterone and pregnenolone metabolism reported that about 8% of pregnenolone produced <u>in vivo</u> is excreted in the urine as pregnenolone-3S-20S and Pregnenolone-3S-20-N-AcGluNH$_2$.

Recently, Williamson, Collins and Layne (39) have isolated a novel estrogen conjugate in rabbit urine after injection of Estrone or 17β-Estradiol. This conjugate has glucose at 17α and glucuronic acid at the 3 position; 17α-E_2-3 glucuronic-17α-glucoside. This conjugate was

also isolated, in vitro, with rabbit liver microsome and UDP-Glucose. This finding, as the authors stated, implicates a steroid in a glycosyl transfer reaction and represents the first demonstration of the formation and excretion of a glucoside in mammals.

It is not known whether these new conjugates are peculiar only to the rabbit and human. Other double conjugates with glucuronic and sulfuric acids, as monolithic or mixed are also known to occur in human and the other animals. Estriol-3Sulfo-16αGlucuronide has been identified in the maternal blood during the pregnancy (40). Diczfalusy et al (41) also identified E_3-3S-16αG in the urine of anencaphalic infant and in midterm pregnancy urine after administration of ^{14}C-E_3 (42). Stϕa and Levitz (31) identified E_3-16αG as the predominant bilary metabolite of ^{3}H-E_3. Again, after intravenous administration of ^{3}H-Estriol-3-Sulfate to subjects with bile fistula, Emerman, Twombly and Levitz isolated E3-3S-16G (43). Straw et al (44) detected estriol metabolite containing one molecule of glucuronic acid and one molecule of sulfuric acid in the pregnant human. The possible presence of E_3-3S-16 (17) G or the E_3-3-sulfoglucuronide in the cord blood was also suggested by Troen et al (45). These same authors (45) again suggested the presence of Estriol-triglucuronide in the cord blood, amniotic fluid and urine of newborn. Smith and Kellie (24) have also identified E_3-3S-16αG and 16αOHE_1-3S-16αG in the human late pregnancy urine. The hen excretes estrogens as, almost exclusively, sulfates and a significant portion of these have recently been identified as the disulfate derivative of estradiol ($E_2$3S-17 S) (46). The ratio of estrogen monosulfate to the disulfate in the hen was found to be about 6:1. In the rabbit urine, however, no evidence for the presence of estrogen sulfates has been obtained (35,36,47) though sulfokinase has been detected

in its intestine (48). In 1961, Felger and Katzman (49) tentatively identified a diglucuronide, Estriol-3, 16-diglucuronide in pregnancy urine. The identification was based on the fact that a sample isolated from urine contained double the molar ratio of glucuronic acid as compared to estriol.

2. FUNCTION OF KIDNEY IN STEROID METABOLISM

The kidney can be considered as a clearing house through which the blood passes and is filtered and cleared of all waste metabolites. For the metabolites to be cleared effectively they must be water-soluble. Conjugated estrogens are more water-soluble than their unconjugated forms, hence they are easily excreted via the urine. It appears that conjugation is an essential metabolic process rendering the steroids almost physiologically inactive and relatively water-soluble. This latter property may be a prerequisite for renal excretion of estrogen metabolites. The identification of these conjugates in urine indicates that they existed in plasma before their elimination by the kidney, all available evidence pointing to this fact.

(i) Renal Clearance : It is known that steroid sulfates, whether phenolic or neutral are the predominant circulating forms but in the urine it seems that glucuronides are the major form. It seems therefore that the kidney handles glucuronides differently from sulfates. Bongiovanni et al (50) tried to explain this on the basis that the sulfates are more firmly bound to proteins than glucuronides. The same authors and also Kellie and Smith (50) stated simply that the preponderance of Dehydroisoandrosterone sulfate (DHAS) in human plasma is attributable to its low renal clearance, also that, the renal clearance of DHAS is low because of its rapid tubular

reabsorption in the face of significant filtration by the glomerulus though the tubular reabsorption system was not found to be affected by probenecid, a drug which is known to inhibit tubular reabsorption. After pre-treatment with probenecid, Bongiovanni et al (50) expected to find a higher concentration of DHAS in the urine but results did not confirm the hypothesis. Therefore the renal clearance of the sulfates is more involved than just reabsorption. Gandy and Petersen (52) and also Cohn and Bondy (53) have reported that the metabolic clearance rate of Dehydroisoandrosterone (DHA) is markedly greater than that of its sulfate (DHAS). In 1951, West, Tyler et al (54) reported, after intravenous administration of testosterone to the human, that the conjugated 17-Ketosteroids are excreted by glomerular filtration without significant reabsorption or secretion. They (54) also found very high concentration of conjugated 17-Ketosteroids in the plasma in a case of severe glomerulonephritis. Levitz (55) also obtained results for estrogen clearance which were in agreement with the results reported by others for androgens (Kellie and Smith (51)) that the glucuronides are cleared far more rapidly than the sulfates.

Apart from tubular reabsorption, tubular secretion of conjugates has also been reported. Brown, Saffan, Howard and Preedy (55) measured renal clearance of endogenous estrogen in late pregnancy and stated that some estrogen conjugates and free Estrone and Estradiol are secreted by the renal tubule. They also found a tubular maximum for Estriol and its conjugates, especially E_3G. Tubular secretion for both free and conjugated 17-Ketosteroids has been found by Jacono et al (57) and for glucuronides of 17-Hydroxycorticoids by Daughaday (58). The latter author also reported that in the human, glucuronides of steroids and their metabolites appear in general to have higher

renal clearance rates than the free steroids (58). This finding was later confirmed by Kernel (59).

The differential handling of the conjugates, especially of the sulfates, was also indicated for estrogens by Purdy et al (8) who noticed that although E_1S is the major circulating estrogen, very little was excreted as such by the kidney via the urine.

(ii) Renal Conjugation : Diczfalusy et al (60-62) studied the nature of conjugated estrogens formed by the human fetus in vivo following intraamniotic administration or perfusion of E_2 and E_3. Following E_2 administration, they found E_2S and E_1S in the lung, liver and kidney. Fetuses after E_3 administration were found to contain Estriol-3-sulfate (E_3-3S) in the lung, liver and kidney. Since the kidney was not studied per se, it is not clear whether the metabolites found therein were a part of the general metabolites in transit or were actual metabolic products of the kidney.

One of the earliest in vitro studies involving the kidney was by Arnolt and DeMeio (63, 64) who incubated rat tissues with various phenols in Krebs Ringer solution for two hours. They found phenolic conjugation in the liver, intestine and spleen but none in the adrenals, brain, diaphragm, heart, kidney, ovary, testicles or stomach. In similar experiments with the cat, the liver, kidney and small intestines were involved in the conjugation. Segal (65) used the soluble fraction of rat liver homogenate for incubation in the presence of ATP, Mg^{2+} and ^{14}C-E_1. He isolated ^{14}C-E_1 but could not detect any conjugating activity with the soluble enzymes of the spleen, kidney and heart. Following the work of Diczfalusy, Wengle (66) in 1964 observed that fetal lung and kidney could sulfurylate estrogens and deoxy-

corticosterone (DOC). Renal sulfo-conjugation has so far been demonstrated only in the fetus. This may be expected in view of the generalized high sulfo-conjugation in the fetus.

As far back as 1939, Lipschits and Bueding (67) using rats found the kidney to be involved in glucuronide conjugation. Active glucuronide synthesis in the liver and kidney was observed by Shirai and Ohkuba (68). Cohn and Hume (69) detected androsterone and etiocholanolone glucuronides after perfusing kidney with the free steroids. Luetscher et al (70) studied the conjugation of aldosterone in human liver and kidney by infusion. They found that about 20% of free aldosterone was removed by the kidney but only 1-3% of this appeared in the urine. However, acid-labile conjugates appeared in the urine at a greater rate than could be expected from the renal extraction ratio of this metabolite. They therefore concluded that labile conjugates were being formed in the kidney since conversion of the free aldosterone to conjugated in the kidney accounted for the discrepancy. Sandor and Lanthier (71) also concluded from in vitro studies that kidney slices are capable of forming acid-labile conjugates of aldosterone.

(iii) Renal Metabolism of Steroids Other Than Conjugation : There is very little information on the metabolic potential of the kidney, as an organ. Estrogens are known to increase enzyme content of the proximal tubules of the kidney (72, 73). The metabolic activity of the kidney with respect to the sex hormones was indicated in 1940 when Danby perfused an isolated cow's kidney and concluded that it could inactivate androgens (74). Zondek also reported the loss of biological activity of estrone upon incubation with kidney homogenates (75). An important enzyme of steroid transformation has been found in the kidney of many species (76). This enzyme is the

17β-Dehydrogenase which converts the 17β-OH group to the 17-Keto group. In vitro formation of 16-OXO-E_2-17β and 16-epi-E_3 has been demonstrated by King (77) after incubation of rat kidney homogenates with [16-^{14}C]-E_3. He could not find any 2-Hydroxylase activity in the kidney (78). The interconversion of Estrone and Estradiol has been studied in various human tissue slices including the kidney (79). In all such studies, the equilibrium of interconversion between E_1 and E_2 was heavily in favor of the former. Balikian et al (80) administered E_1 to male dogs and identified $E_3$16α17α in both liver and kidney. The levels tended to be higher in the kidney and they seemed to imply renal metabolism. Chatterton, Chatterton and Hellman (81) found the rabbit kidney to play an important role in the metabolism of progesterone both in vitro and in vivo. Among the enzymes they found present in the kidney were those capable of 20α and 20β-reduction, 5α and 5β-reduction and 6-hydroxylation.

3. β-GLUCURONIDASE AND SULFATASE

β-Glucuronidase is an enzyme which normally catalyzes the hydrolysis of glucuronic acid conjugates, giving an aglycone and glucuronic acid. It is present in high amounts in the liver, spleen and intestine of many species. Some other organs, also contain varying amounts of it. It has been known for a very long time that ingested estrogen glucuronides are hydrolyzed in the intestine prior to their absorption (82-84). The hydrolytic function of β-glucuronidase in vivo other than intestinal hydrolysis is very doubtful. Fishman (85) has reviewed the available data on β-glucuronidase and he prefers to regard its in vivo function as essentially that of a transferase, operating in an integrated multicomponent

cytoplasmic system. Kerr et al (86) and Levvy (87), however believe that β-glucuronidase functions solely to hydrolyze glucuronides in the tissue. Fishman and then Green (88, 89) demonstrated that β-glucuronidase can act as a transferase, transfering glucuronic acid from a number of phenols to different alcohols. Recently, evidence for a glucuronide transconjugation has been found in the intestine (83). β-Glucuronidase was thought to be involved in such a transformation. The optimum pH for hydrolysis by mammalian β-glucuronidase is 4.3 - 4.5 (90). Only the mouse kidney has so far been proved to contain β-glucuronidase (91-93). The same authors also noticed generalized increases in the enzyme content, especially in the kidney following administration of testosterone, estrogen or glucuronidonic substances.

Sulfatases represent a group of hydrolases which catalyze the hydrolysis of sulfate esters of many compounds. These enzymes, unlike β-glucuronidase, do not seem to have any synthetic properties. They have been classified into many groups which include aryl and steroid sulfatases (94). These two groups are distinct from each other but Estrone sulfate (E_1S) and hence estrogen-3-sulfates are hydrolyzed by both of them. The aryl sulfatases are subdivided into Type I (sulfatase C) and Type II (sulfatase A and B).

The type I aryl sulfatases are remarkably insensitive to sulfate or phosphate ions but are strongly inhibitied by sulfite and cyanide (94). The type II enzymes are different from the type I by being strongly inhibited by both phosphates and sulfates. They are also inhibited by sulfite but not cyanide. Dodgson et al have provided much information on the distribution of aryl sulfatase in the rat (96) and the human (97). They showed in general that the liver is the organ richest in aryl sulfatase and that considerable amounts also occur in kidney, pancreas and adrenals. Aryl sulfatases have

also been detected in many species including rabbit, dog and hen (95, 98, 99). Roy (93) did state that there is little to suggest that aryl sulfatase functions *in vivo* especially in the type II, if they are present in the lysosome (100). The only indication that arylsulfatase is active *in vivo* is the claim that E_1S, which is a substrate for the enzyme (101) is rapidly hydrolyzed in the body (102, 103).

The steroid sulfatase catalyzes the hydrolysis of sulfate esters of steroid alcohol. It is present in molluscs (104, 105) and mammalian liver and have been obtained in soluble form (106, 107). The molluscan enzyme is inhibited by sulfate and phosphate but the mammalian variety is inhibited only by phosphate. The steroid sulfatases are highly specific for the 3β-sulfates of 5-α- and 5-ene series (107, 108).

B. PURPOSE OF THE PRESENT INVESTIGATION

The liver plays a central role in detoxification or inactivation of estrogens, and is considered the main site for estrogen conjugation. Since estrogens in the urine and plasma exist mostly as the sulfates and glucuronides, it is of interest to know how they are excreted. The kidney has hitherto been considered solely as a clearing house which filters the blood and eliminates waste metabolites. Exactly how the kidney functions to give a preponderance of glucuronides in the urine whilst sulfates remain quantitatively the most important circulating form is not well understood.

In this study, free estrogens, E_1 and E_2, and estrogen conjugates, E_1S and E_217 G have been incubated with renal homogenates to find out the kidney's metabolic potential. Also, to find out if there is a differential handling of sulfates and glucuronides and how such renal metabolism differs among the species. The rat, hen, dog and rabbit have been used in these studies as test species.

C. MATERIALS AND COUNTING TECHNIQUE

ANIMALS

Dogs, white albino rats and rabbits were obtained through the animal division of The Montreal General Hospital. Hens were obtained through the courtesy of Dr. N. Nikolaiczuk of the Department of Animal Science, MacDonald College of McGill University.

CHEMICALS AND SOLVENTS

Diethyl ether, from Fisher Scientific Co. Canada, was always washed with dilute ferrous sulfate in acid solution then washed 3 times with water then distilled fresh and used within 24 hours.

Bacterial β-Glucuronidase (Type II) and Hyaluronidase were obtained from Sigma Chemicals Co., St. Louis, Mo. Mylase P, containing sulfatase was obtained from Mann Research Laboratories Inc. Ketodase, a β-glucuronidase, was obtained from Warner-Chilcott Div. Morris Plains, N.J. Adenosine Triphosphate (ATP) was purchased as the disodium salt from Nutrional Biochemicals Corp., Cleveland, Ohio.

All unlabeled unconjugates steroids were purchased from Mann Research Laboratories Inc., N.Y. The purity of these were checked by thin-layer chromatography and melting point determination (109).

All other reagents were of suitable chemical grade and purity. All solvents for partition chromatography were made up and allowed to stand overnight to reach phase equilibrium at room temperature. Celite from Anachemia Chemicals Ltd., Montreal and all organic solvents used were processed according to Hobkirk and Metcalfe-Gibson (110).

CHROMATOGRAPHIC MATERIALS

DEAE-Sephadex A-25 ion-exchanger and chromatographic column type K9/60 were purchased from Pharmacia Canada Ltd., Montreal. LKB "Ultrorac" fraction collector Model 7000 with volume-drop siphons was purchased from LKB. Produkter AB. Bromma I. Sweden. Amberlite XAD-2 a synthetic ion-exchange resin was purchased from Rohm and Haas, Philadelphia, Pa.

RADIOACTIVE COMPOUNDS

Estrone-6, 7-^{3}H-3-Sulphate (^{3}H-E_1S) (SA 10 μc/μg) in the ammonium salt was purchased from New England Nuclear Corp. An aqueous solution of this material was extracted with diethyl-ether to remove any free or unconjugated material. Only 1% of the radioactivity was thus extracted. The aqueous fraction was made 3M. with sodium chloride and extracted with ethyl-acetate. The organic phase was evaporated to dryness under pressure. This fraction was found to be 99% pure with respect to Estrone Sulfate by further purification on celite column and thin layer plates (16).

Estrone-6, 7-^{3}H (^{3}H-E_1S) of SA 10 μc per μg and Estradiol-6, 7-^{3}H-17 β(^{3}H-$E_2$17β) of SA 20 μc per μg were purchased from New England Nuclear Corp. Boston, Mass. These materials were chromatographed on a celite partition column (III) and the eluted materials were shown to be 99% pure by crystallization with unlabelled steroids (16) and stored in benzene:methanol (1:1) at -15° C.

Estradiol-6, 7-^{3}H-17β-Glucuronide (^{3}H-$E_2$17G) of SA 10μc/μg was obtained from New England Nuclear Corp. An aqueous solution of this material was extracted with diethylether to remove any free materials, less than 1% of the radioactivity was thus extracted. The aqueous fraction was made

50% $(NH_4)_2SO_4$ and extracted with ether:ethanol (3:1). The organic phase was evaporated under pressure, chromatographed on celite and sephadex-ion-exchanger. The sample was found to be 99% Estradiol glucuronide. This material was stored in methanol at -15° C.

n-Hexadecane-I, 2-^{3}H (SA-2.46 µc/g) was purchased from the Radiochemical Centre, Amersham, Bucks. England. This was used to prepare absolute counting standards.

MEASUREMENT OF RADIOACTIVITY

Counting of the samples were by liquid scintillation spectrometry in 20 ml. screw-cap glass vials form Wheaton Glass Co., N.J. Aqueous samples were mixed with 10 ml. of dioxan scintillation fluid [770 ml. dioxan, 230 ml. absolute ethanol, 143 g. recrystallized naphthalene, 10 g. 2,5-diphenyloxazole (PPO) and 250 mg. 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP]. Methanolic samples in a volume of 0.5 ml. were mixed with 10 ml. of toluene scintillation fluid. The fluid was prepared from concentrated "Spectrafluor" containing butyl PBD, [butyl derivative of 2-phenyl-5-(4-biphenylyl)-oxiadizole-1,3,4. Nuclear Chicago Corp.] by diluting with toluene to give a final phosphor concentration of 7 g. per L.

The counting was performed in a dual channel 'Unilux I' from Nuclear Chicago. The instrument had a ^{3}H-background of about 25 cpm. The efficiency for a ^{3}H-standard was 34% in toluene and 25% in toluene plus 0.5 ml. methanol when butyl PBD was used. Radioactivity in each sample was converted to dpm. by reference to efficiency for standards containing ^{3}H-Hexadecane. Sufficient counts were accumulated to give a statistical error of no more than $\pm$ 3%.

D. METHODS

1. PREPARATION OF TISSUE AND INCUBATION CONDITIONS - TABLE I

The rats were killed by suffocation in ether, the rabbit and hen by a sharp blow at the back of the neck, the dogs were dissected under nembutal.

Kidney tissues from these animals were then removed as quickly as possible and put into a beaker containing Krebs Ringer phosphate solution, pH.7.4 at 4°C. Tissue slices or homogenates were made from representative portions of the kidney. Thin slices, 0.5mm thick were cut with the Stadie-Riggs microtome according to the technique of Stadie and Riggs (112) and Sperry and Brand (113) as modified by Elliott (114). The slices were immediately weighed on a microbalance and put into 50 ml. flasks. Tissue homogenates in the cold buffer were prepared in a Virtis '45' blender for 2 minutes. The final concentration of the homogenate was 100 mg/ml.

Two ml. of the above homogenates or equivalent weight of slices, 200 mg. were added to incubation flasks which contained 3 ml. of the buffer pH 7.4; 4×10^{-2} mM of ATP in 0.2 ml; 2.6×10^{-2} mM of NADPH together with appropriately labelled substrate. Different flasks contained one of the substrates; ^{3}H-Estrone (^{3}H-E_1), ^{3}H-Estradiol 17β(^{3}H-E_2); ^{3}H-Estrone Sulfate (^{3}H-E_1S) and ^{3}H-Estradiol-17β Glucuronide (^{3}H-$E_2$17G). The substrates, in methanol, were added to the flasks, evaporated to dryness before addition of the other constituents. Two drops of propylene glycol were used to solubilize the free steroids. The flasks and constituents were incubated in a metabolic incubator at 37 - 38°C with constant shaking. The incubation

was carried out for two hours with air as the gas-phase. Appropriate control flasks containing buffer, co-factors, substrates and boiled tissue were similarly incubated for one hour.

One ml. samples of the above homogenates were used to study the rate of hydrolysis of Estrone sulfate and Estradiol glucuronide. Such study will be treated later under 'Rate of hydrolysis of E_1S and E_2G'.

2. SOLVENT EXTRACTION OF TISSUE - TABLE II

At the end of the incubation period, the reaction was stopped by addition of 40 ml. of methanol and placed in the deep-freeze overnight to complete precipitation of tissue proteins. The tissue slices were homogenized in methanol in hand homogenizer before placing in the deep-freeze. Each sample was then exhaustively reextracted with fresh methanol several times. The pooled extracts were evaporated to dryness under suction, redissolved in methanol and 0.5% of it was assayed for radioactivity. Drops of concentrated ammonia were periodically added to the samples during evaporation to prevent spontaneous hydrolysis of steroid sulfates.

The extracts were partitioned between 90% methanol and hexane. The hexane or non-steroidal fraction was assayed for radioactivity and discarded. The steroidal fraction (90% methanol) was diluted with water to give 10% methanol and extracted with 3 x 1 volume of diethylether. The ether fraction was designated the "Free" or unconjugated fraction. This fraction was dried over anhydrous sodium sulfate (Na_2SO_4) followed by evaporation to dryness.

3. EXTRACTION OF CONJUGATES- TABLE II

a) Ethyl-Acetate Extraction:

The aqueous fraction from the above stage was made 3M with sodium chloride and extracted with 3 x 1 volume of ethylacetate. The extracts were pooled, washed with 5 ml. water. The extract was then dried over $Na_2SO_4$and evaporated to dryness with additions of ammonia. This fraction was designated - "SULFATE".

b) Ether:Ethanol (3:1) Extraction:

The aqueous solution from the above stage was desalted by passing through a bed of Amberlite XAD-2 resin to absorb steroids conjugates present. Excess salt on the column was washed out with water and the steroid conjugates eluted with methanol (115) followed by evaporation to dryness. The extract was redissolved in water and made 50% with ammonium sulfate $[(NH_4)_2SO_4]$. This was then extracted with 3 x 1 volume with ether:ethanol (3:1) according to Edwards (116) but the pH of the solution was not adjusted.

This fraction was called "GLUCURONIDE" if ethyl acetate extraction had already been carried out or else, 'TOTAL CONJUGATES'.

c) DEAE Chromatography of Extracts:

Chromatography of the total conjugates or the original methanolic extract without further solvent extraction was carried out with DEAE-Sephadex (A-25). The sample was added to the column in 3 x 1 ml. of water and eluted with a linear concentration gradient of NaCl, starting with water. The

gradient was achieved by the use of two identical containers connected at the bottom end by a tube. Five hundred ml. of water was put into the first container (mixer) and equal volume of 0.8M NaCl solution put into the second container (reservoir). Ten ml. aliquots per fraction were collected and 0.2 ml. from each was assayed for radioactivity. The samples were either counted straight in 10 ml. of Dioxane scintillation fluid or evaporated to dryness under a stream of air and counted in 10 ml. Toluene scintillation fluid after dissolving in 0.5 ml. of methanol.

Fractions corresponding to discreet radioactive peaks were pooled and passed through amberlite XAD-2. As always, salts were washed off with water and the conjugated steroids eluted with methanol followed by evaporation to dryness with added ammonia when necessary. Occasionally, if XAD-2 was not used the pooled volume under the peaks was made 50% with $(NH_4)_2SO_4$ and extracted with 3 x 1 volume of ether:ethanol (3:1). Quantitative recoveries were obtained by either method. The only disadvantage of the ether-ethanol extraction is that significant quantities of salts are concomitantly extracted into the organic phase and might require a further desalting procedure.

4. HYDROLYSIS OF CONJUGATES

The appropriate fractions of estrogen conjugates, as sulfates and/or glucuronides, were hydrolyzed with sulfatase or β-glucuronidase. The sulfates were dissolved in 0.1 M acetate buffer, pH 6, and warmed to 38°C. The solution was then mixed with commercial Mylase P (containing a phenol sulfatase) to give a final concentration of 2.5mg/ml (117) and incubated at 38°C for 24 hours. The pH of the solution was then checked

and a further 1.5 mg/ml of enzyme added followed by reincubation for 12 more hours. At the end of the incubation, the solution was cooled to room temperature and the liberated free steroids extracted with 3 x 1 volume of diethylether.

The glucuronides were also taken up in 0.05M phosphate or acetate buffer pH 4.5 and similarly incubated with Bacterial β-Glucuronidase at a concentration of 20 units per ml. (118). A further addition of 10 units per ml. were made after 48 hours incubation. The liberated steroids were similarly extracted with ether. The ethereal solutions were washed with 10% volume of water, dried over anhydrous Na_2SO_4 and evaporated to dryness. Occasionally, it was necessary to do a sequential hydrolysis, starting with Mylase P, extracting the free steroids followed by β-glucuronidase incubation and ether extraction. When deemed necessary, the appropriate fractions were incubated with hyaluronidase (119) and assayed for released steroids.

5. ANALYSIS OF FREE STEROIDS

(i) Girard Reaction and Celite Column Chromatography : The method of Girard (120) as modified by Givner et al (121) was used to separate ketonic from non-ketonic steroids. The two fractions were further analyzed on separate celite partition columns. The samples were applied to the top of the packed columns by absorption onto one gram of dry celite with the aid of minimum mobile phase.

The various solvents used to elute individual steroids were as follows:

Ketonic Column

Benzene:Hexane (2:98)	.....2MeOE$_1$	fraction
Benzene:Hexane (50:50)	.....E$_1$	fraction
Benzene:Hexane (50:50)	.....RD Ketols	fraction

Non Ketonic Column

Benzene:Hexane (55:45)	.....E$_2$17β	fraction
Benzene	.....16 epi E$_3$	fraction
Benzene	.....E$_3$	

(ii) Benzene:Water Partition and Celite Column Chromatography: The method described by Bauld (111, 122) was used to fractionate the free steroids into Estrone and Estradiol. The free estrogens were partitioned between Benzene and water. The aqueous phase was assayed for radioactivity and discarded. The benzene phase containing mostly estrone and estradiol was chromatographed on a 12 cm long celite column with the system Benzene-0.8N NaOH. The column was eluted with benzene for Estrone, then with ethylene dichloride:benzene (3:1) for Estradiol. The eluates were evaporated, and further purified by alkaline treatment and extraction, (N.NaOH and Toluene) (123).

(iii) T.L.C. and Crystallization: The Estrone and Estradiol fractions so obtained from above were chromatographed on thin layer plates, coated with silica gel HF 254, in the system Ethyl Acetate:Cyclohexane: Ethanol (9:9:2). Sections on the plate which contained unlabelled steroid markers were stained by spraying with 2% H_2SO_4 in Methanol or visualized under short wave Ultraviolet lamp. Areas corresponding to the mobility of authentic reference steroids (E_1 and E_2) were scraped and eluted with

methanol. This was assayed for radioactivity and aliquots of it were crystallized in methanol to constant specific activities by reverse isotope dilution following the addition of known weights of unlabelled carriers.

6. RATE OF HYDROLYSIS OF E_1S AND E_2G

Radioactive Estrone sulfate was incubated with 1 ml. (100 mg) of the renal homogenates in 3 ml. of Krebs phospho-saline buffer, pH 7.4 without cofactors for various times and in different flasks at 37°C. The reaction was stopped by addition of methanol. The samples were kept in deep-freeze for about 2 hours and then diluted with water to give a 10% methanolic solution. This solution was extracted with 3 x 1 vol. of diethylether. The ether extracts were evaporated to dryness, redissolved in 10 ml. of methanol and 0.5 ml. of each was assayed for radioactivity. The radioactivity in each sample was taken as percent ^3H-E_1S hydrolyzed. Renal homogenates from each of the species were similarly incubated. Radioactive Estradiol Glucuronide was also similarly incubated with renal homogenates, in phosphate buffers pH 4.5 and 7.4 .

TABLE I

INCUBATION CONDITIONS INCLUDING SPECIFIC SUBSTRATES

RENAL HOMOGENATE	100 mg/ml - 2 ml.
SUBSTRATE	Labelled E_1, E_2, E_1S, E_2G.
NADPH	2.6×10^{-2} mM 0.2 ml.
ATP	4×10^{-2} mM 0.2 ml.
BUFFER	KREBS PHOSPHATE pH 7.4
GAS PHASE	OXYGEN/AIR
TIME/TEMPERATURE	2 HRS. 37 - 38°C.

TABLE II

EXTRACTION AND SEPARATION OF METABOLITES

1 SOLVENT EXTRACTION: Hexane, Ether, Ethyl Acetate, Ether/Ethanol.

2 EXTRACTION OF CONJUGATES: Ether/Ethanol, Amberlite XAD-2; DEAE-Sephadex.

3 EXTRACTION OF PEAKS: Ether/Ethanol, XAD followed by hydrolysis.

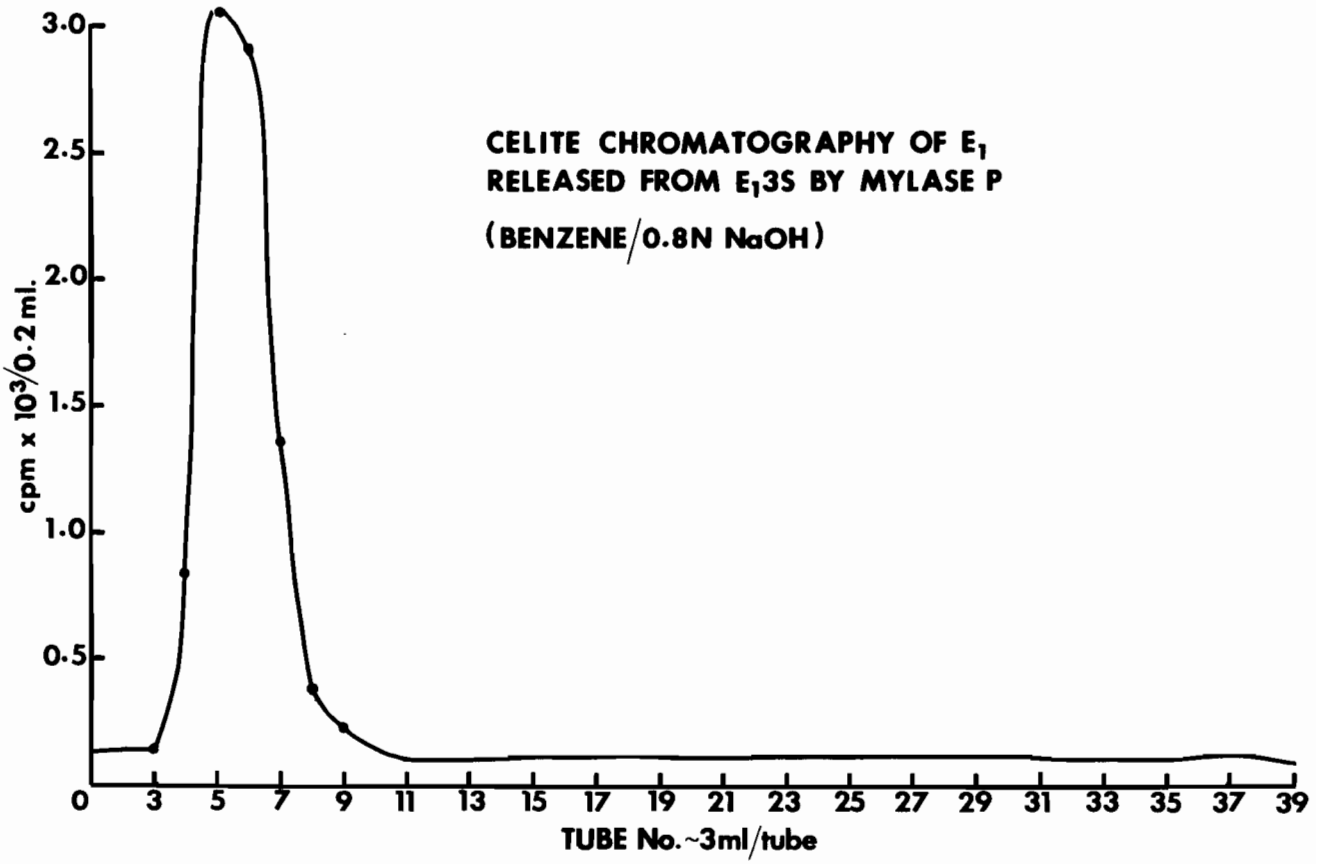
4 ANALYSIS OF FREE STEROIDS:

i Girard - Celite Chromatography

ii Celite Column Chromatography; Benzene/0.8N NaOH, Benzene/Ethylene Dichloride.

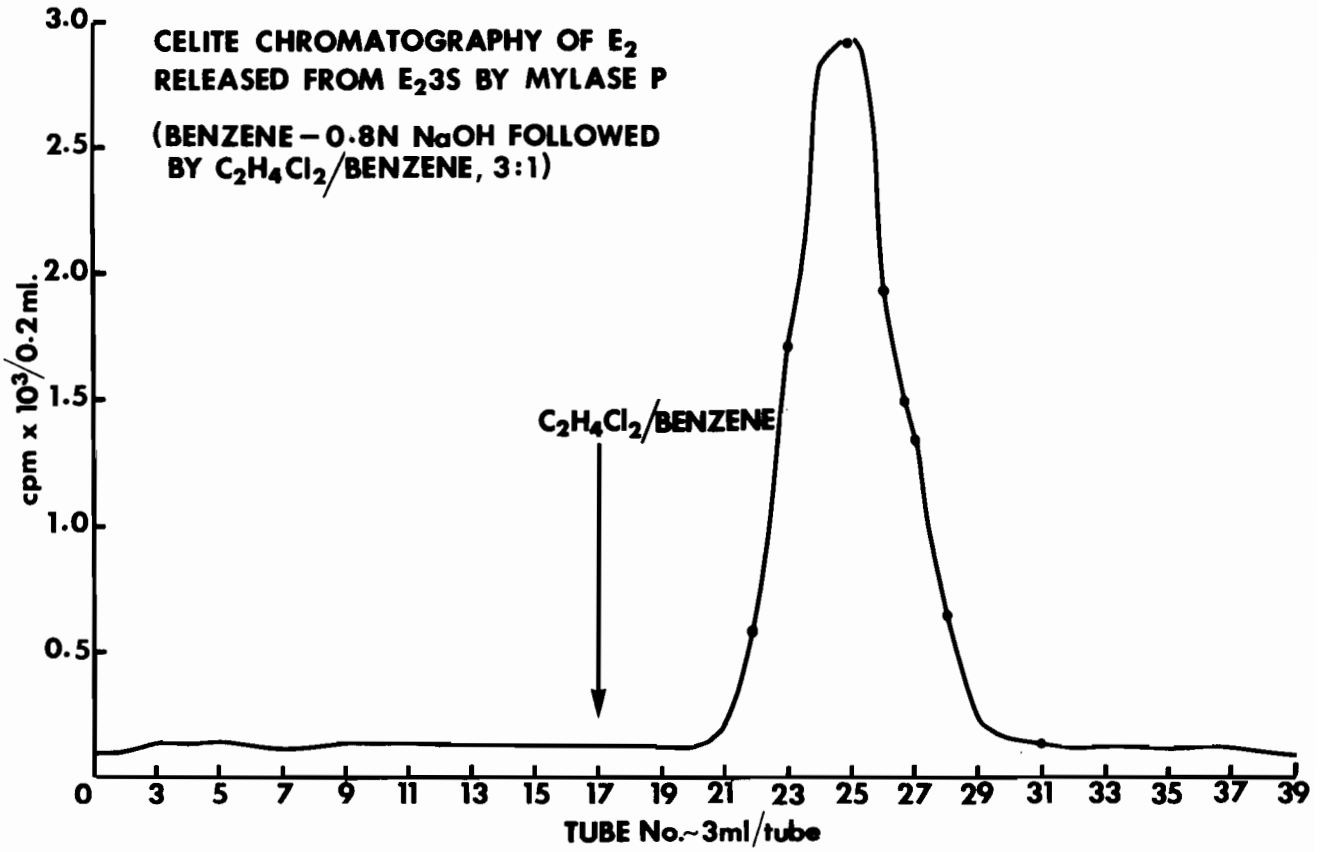
iii TLC and Crystallization

FIGURE I



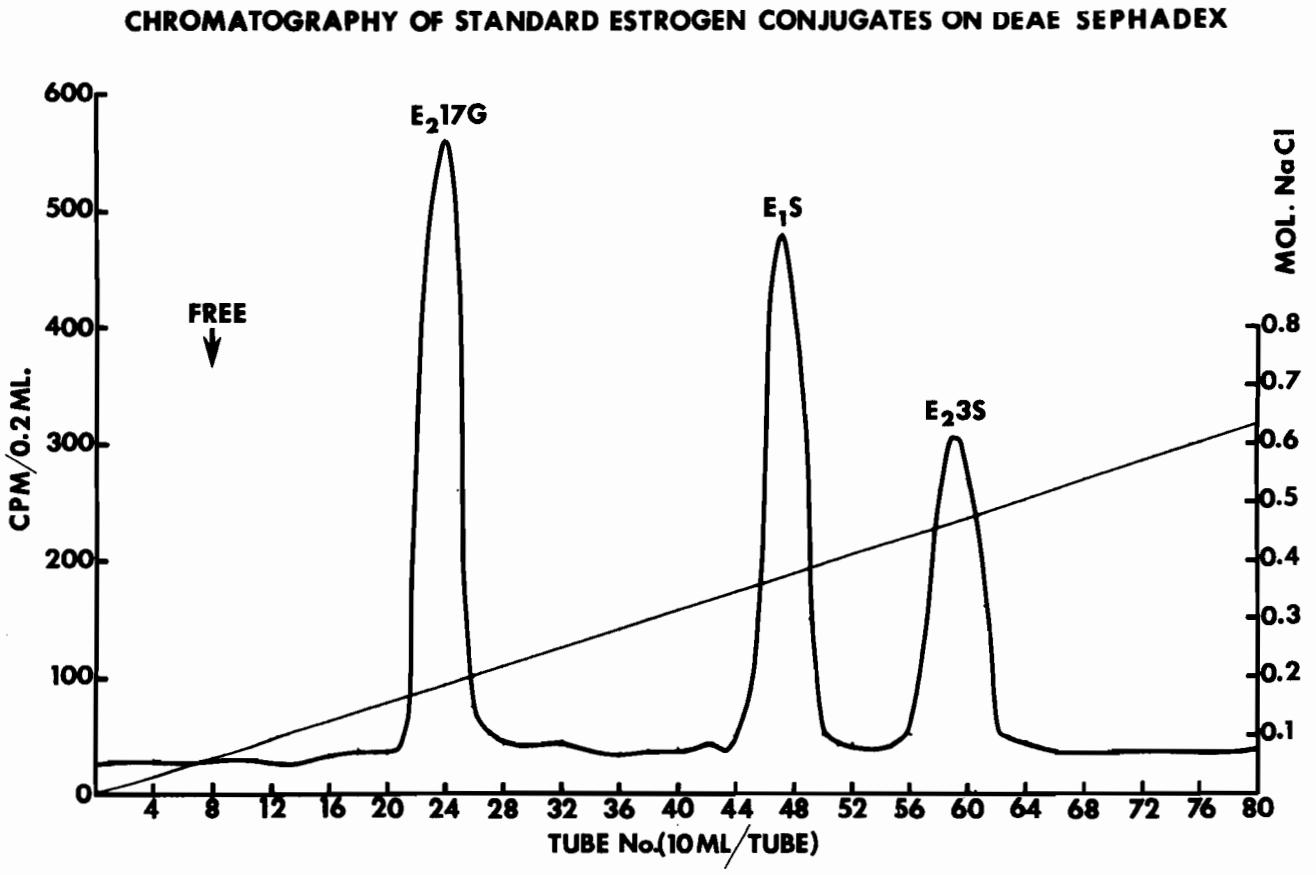
Celite partition chromatography of Estrone (Bauld 111, 122) with Benzene as the mobile phase and 0.8N NaOH as stationary phase. Rate of elution 10ml/hr. Recovery from column 86%. (See methods)

FIGURE II



Celite partition chromatography of Estradiol (Bauld 111, 122) with system Benzene/0.8N NaOH. The mobile phase, Benzene, was changed to Ethylene dichloride - Benzene (3:1) at tube N° 17. Rate of elution 10 ml/hr. Recovery from the column 86%. (See methods)

FIGURE III



DEAE-Sephadex-(A25) chromatography of Estrogen Conjugates by linear gradient elution with NaCl. Free estrogens when present elute about tube N° 7. Note complete resolution of Estrone and Estradiol sulfates. Rate of elution 50 ml/hr. Total recovery from the ion-exchanges is over 98%. Details of the chromatography are explained in text.

E. RESULTS AND DISCUSSION

1. RESULTS AND DISCUSSION - RAT

(i) Distribution of Radioactivity Following Incubation :

The results of the various extraction procedures, following incubation of rat kidney tissue with the indicated estrogens are given in Tables III and IV. The kidney is quite different from the liver[1] in that the recoveries, after incubation with the estrogens in all species studied were quantitative. This might probably be due to less binding of the steroids to particulate matter. The hexane extractable fraction, that is, non-steroidal material, was quite considerable in all the species for free estrogen incubation. In cases where significant conjugate hydrolysis occured, - rat ^{3}H-Estrone sulfate incubation, the hexane extracts contained radioactivity comparable in amount to that found in the free steroid incubation. Control incubations with either free or conjugated estrogens did not yield significant hexane extractable material, Table XIX.

It appears rather unlikely that estrogens would yield non-steroidal metabolites but it is possible that free estrogens on incubation might complex with lipid present, in which case they would be hexane extractable from 90% methanol. Such hexane extracts were not processed any further.

The very high ether extractable (FREE) material indicates limited conjugation upon ^{3}H-Estrone or ^{3}H-Estradiol incubation and a highly efficient ^{3}H-Estrone sulfate hydrolysis. The ether extractable material from ^{3}H-Estradiol 17β-glucuronide incubation, Table III might be due to experimental

[1]Personal observation following incubations with liver tissue.

TABLE III

Distribution of Radioactivity
Following Incubation of Estrogens
With Male Rat Kidney Slices

LABELLED FRACTIONS RECOVERED	SUBSTRATE INCUBATED dpm					
	^{3}H-Estradiol-17β 16.09 x 10^6		^{3}H-Estrone-Sulfate 2.45 x 10^6		^{3}H-Estradiol-17β-GLU. 1.96 x 10^6	
Recovery	DPM x 10^6	%	DPM x 10^6	%	DPM x 10^6	%
	15.88	98.7	2.45	100	1.91	99.0
Hexane	0.62	4.0	0.08	4.0	0.009	0.5
Ether (Free)	12.86	81.0	1.95	80.0	0.08	4.0
Ethyl-Acetate (Sulfate)	0.42	2.6	0.11	4.7	0.02	1.3
Ether:Ethanol (Glucuronide)	1.73	10.8	0.34	14.0	1.45	76.2
FRACTION OF RECOVERY ACCOUNTED FOR	15.63	98.4	2.48	102.0	1.559	82.0

Percentages relate to original amount incubated.

inaccuracies but not glucuronide hydrolysis since a different extraction procedure, Table IV, by DEAE-sephadex did not show such hydrolysis. If, however, the hydrolysis was real, then the β-glucuronidase activity in the kidney should be very low indeed. There was no evidence in either of the rat experiments to indicate sulfurylation, though the 4.7% "sulfate" fraction Table IV seems to indicate the contrary. This is thought to be an artifact, and will be explained later under analysis of conjugates.

The only form of conjugation noticed was glucuronide synthesis as evident from the ether-ethanol glucuronide extracts. These extracts were indicated to be glucuronides by β-glucuronidase incubation - to be presented later. Experimental losses from all these procedures were minimal judging from the total material accounted for.

(ii) Analysis of Ether Extracts : The "free" extracts (ether) from male rat were separated into Ketonic and non-Ketonic fractions followed by celite column chromatography, Table V. The Estriol, 16 epiEstriol, 2-Methoxy Estrone and Ring Dα Ketol fractions were not further purified or identified. They only represent the radioactivity eluted from the columns with the polarity of E_3, $16E_3\beta$, $2MeOE_1$ and RD Ketols. This experiment was designed with emphasis on Estrone-Estradiol conversions since preliminary studies did not reveal any other metabolite in quantities worth identifying. The ^3H-E_1 and ^3H-E_2 eluted from the columns were further purified on celite columns (111), thin layer chromatography and crystallized from methanol to constant specific activity. Often, it was observed that further processing did not increase their purity but only caused losses. The ^3H-E_1 and ^3H-E_2 obtained from the columns could be directly crystallized to constant specific activity without any difficulty.

TABLE IV.

Distribution of Radioactivity
Following Incubation of Estrogens
With Female Rat Kidney Homogenates

LABELLED FRACTIONS EXTRACTED	SUBSTRATE INCUBATED dpm							
	^{3}H-Estrone 0.94×10^6		^{3}H-Estradiol-17β 3.76×10^6		^{3}H-Estrone-Sulfate 2.82×10^6		^{3}H-Estradiol-17β-GLU. 1.97×10^6	
Recovery	DPM $\times 10^6$	%	DPM $\times 10^6$	%	DPM $\times 10^6$	%	DPM $\times 10^6$	%
	0.91	97	3.76	100	2.77	98	1.97	100
Hexane	0.05	6.0	0.21	6.0	0.15	5	0.007	0.4
Ether (Free)	0.73	82.0	3.11	88.0	2.21	80.0	0.002	1.2
Ethyl-Acetate (Sulfate)	0.002	2.0	0.002	0.7	0.003	1.0	0.003	1.4
Ether-Ethanol (Glucuronide)	0.004	4.0	0.009	2.6	0.09	4.0	1.79	91.0
FRACTION OF RECOVERY ACCOUNTED FOR	0.786	94.0	3.331	97.3	2.453	90.0	1.802	94.0

TABLE V

Analysis of Ether Extract (Free)

Following Incubation of Substrates

With Male Rat Kidney Slices

LABELLED EXTRACT AND METABOLITES	SUBSTRATE INCUBATED dpm					
	^{3}H-Estradiol-17β 16.09 x 10^6		^{3}H-Estrone-Sulfate 2.45 x 10^6		^{3}H-Estradiol-17β-GLU. 1.96 x 10^6	
Ether Extract (Free)	DPM x 10^5	%	DPM x 10^5	%	DPM x 10^5	%
	128.6	81.0	19.5	80	0.8	4.0
Estrone (^{3}H-E_1)	54.6	42.4	8.1	41.2	0.3	40.3
Estradiol 17β(^{3}H-E_2)	24.1	18.7	3.4	17.1	0.2	23.8
Estriol (^{3}H-E_2)*	1.5	1.2	0.3	1.6	0.01	1.8
16-EpiEstriol (^{3}H-$E_3$16β)*	1.2	0.9	0.3	1.6	0.01	1.6
2 Methoxy Estrone (^{3}HMeOE_1)*	2.2	1.7	0.5	2.7	0.02	2.4
RD Ketols*	1.1	0.8	0.2	0.9	0.02	2.8

*Not rigorously identified. The metabolites are expressed as percentages of the ether extract.

TABLE VI

Analysis* of Ether Extracts (Free)

Following Incubation of Estrogens

With Female Rat Kidney Homogenates

LABELLED FRACTIONS AND METABOLITES	SUBSTRATE INCUBATED dpm					
	^{3}H-Estrone 0.94×10^6		^{3}H-Estradiol 17β 3.76×10^6		^{3}H-Estrone-3-Sulfate 2.82×10^6	
	DPM x 10^5	%	DPM x 10^5	%	DPM x 10^5	%
Ether Extract (Free)	7.3	82.0	31.1	88.0	22.1	80.0
Benzene Phase of Benzene:H_2O Partition	7.2	98.6	31.0	98.5	19.8	90.2
^{3}H-E_1	5.4	76.6	24.3	78.0	15.1	77.4
^{3}H-E_2	0.84	12.0	3.8	12	2.1	10.2

* Analyzed by Benzene/H_2O Partition then celite chromatography (Bauld's 1956) E_1 and E_2 are expressed as percent of the benzene extract.

The ether extract from the female rat was analyzed directly on celite columns (111), without prior Girard reaction. The results are given in Table VI and as stated above the ^{3}H-E_1 and ^{3}H-E_2 were crystallized (see below) with unlabelled carrier without any complications. Not withstanding the nature of the starting substrate, Estrone emerged as quantitatively the most important metabolite in the free fraction.

(iii) Analysis of Conjugates : The crude glucuronide and sulfate pool from male rat are analyzed in Table VII. Following chromatography of the crude glucuronides on the ion-exchange sephadex, peaks 1 and 2 were designated 3- and 16 (17) glucuronides resprectively (124). Such categorization was abandoned following further analysis of the peaks. It is desirable to consider them as a single peak since Peak 2 is actually the tailing end of Peak 1. Thus, 91, 82 and 87% were recovered from the column. The figures in the brackets represent percentages of the above extracts (Peaks) released following Bacterial-β-glucuronidase hydrolysis. These released free materials were further purified and analyzed on celite columns; crystallization of these fractions will be considered later.

The crude sulfate fractions, Table VII (ethylacetate) obtained seem to indicate that sulfurylation had occurred during the incubation. This, however, is misleading, since in all the incubations and in all species no real evidence of sulfurylation was obtained. Phenolsulfatase (Mylase P) hydrolysis in our hands has yielded over 90% of free estrogens from pure estrogens sulfates. If the 19, 30 and 11% hydrolysis quoted in the table were to represent the fraction of sulfate conjugates actually present then the ethylacetate fractions become 0.5, 1.4 and 0.1% respectively. Such percentages of <1 may be disregarded. It is for these reasons that

TABLE VII

Enzymatic Hydrolysis of Conjugates
From DEAE-Sephadex and/or Solvent Extraction
Following Male Rat Kidney Incubation

LABELLED FRACTIONS RECOVERED	SUBSTRATE INCUBATED dpm		
	^{3}H-Estradiol-17β 16.09×10^{6}	^{3}H-Estrone Sulfate 2.45×10^{6}	^{3}H-Estradiol-17β-GLU 1.96×10^{6}
Glucuronide	1.73×10^{6}	0.34×10^{6}	1.45×10^{6}
(% of Incubated)	10.8	14.0	76.0
Sephadex (% of glucuronide)			
PEAK 1	81 (79)*	82 (79)	87 (86)
PEAK 2	10 (80)		
SULFATE	4.2×10^{5}	1.2×10^{5}	0.25×10^{5}
Ethyl-Acetate (% incubated)	2.6	4.7	1.3
Sulfatase Hydrolysis %	19	30	11

* Figures in brackets ( ), are explained in text.

sulfurylation by the kidney preparations under the conditions of the experiment has been discounted.

Table VIII shows the analysis of conjugates from the ^{3}H-Estradiol-17-Glucuronide incubation. The bulk of the conjugate was a glucuronide of estradiol, most likely representing unchanged substrate.

(iv) Characterization of Metabolites : The estrogen metabolites were characterized by reverse isotope dilution and crystallized to constant specific activity (SA). Table IX gives the SA for the metabolites, ^{3}H-E_1 and ^{3}H-E_1 obtained after incubation of the specified substrates. The crystallizations were made in methanol and they represent the free (ether)fraction. Table X also indicates the characterization of glucuronide metabolites. The glucuronides from various incubations were hydrolysed with β-glucuronidase as given in Table VII, and fractioned into ^{3}H-Estrone and ^{3}H-Estradiol.

The very close agreement between the SA of two successive crystallizations and between crystals, mother liquor and the expected values proved the reliability of the processing procedures and identification of ^{3}H-E_1 and ^{3}H-E_1 as metabolites. These crystallizations were taken as representatives and no further crystallizations of E_1, E_2 metabolites were carried out in subsequent experiments.

In general, and as already stated, rat kidney preparations do not seem to sulfurylate E_1 or E_2 or to hydrolyze E_2-17-glucuronides. The sulfatase activity is high even in the Krebs phospho-saline buffer though phosphates and chlorides are known to inhibit steroid sulfatase in vitro (94, 96). No significant metabolites (free), more polar than Estradiol were found and Estrone - Estradiol metabolism favoured the former.

TABLE VIII

Analysis of Conjugates Chromatographed On
And Extracted from DEAE-Sephadex
Following Incubation of ^{3}H-Estradiol-17β-Glucuronide
With Female Rat Kidney

CONJUGATE FRACTION			β-GLUCURONIDASE TREATMENT		^{3}H-E_1		^{3}H-E_2	
	DPM x 10^5	%	DPM x 10^5	%*	DPM x 10^5	%*	DPM x 10^5	%*
Peak 1	0.63	3.2	0.57	91	0.02	2.6	0.07	11.6
Peak 2	17.25	87.7	17.5	102	3.03	18.5	11.0	66

The conjugate fraction is % incubated. *As percent of the conjugate fraction.

TABLE IX

Crystallization of Ether-Soluble Estrogens (Free)

From Female Rat Kidney Incubations

SPECIFIC ACTIVITY cpm/mg

Estrogen Metabolite	Substrate Incubated	Crys. N°	Crystals	Mother Liquor	Theoretical
Estrone (^{3}H-E_1)	^{3}H-Estrone	1 2	879 912	900 948	925 880
	^{3}H-Estradiol-17β	1 2	929 919	1150 940	1077 929
	^{3}H-Estrone-Sulfate	1 2	1250 1244	1361 1262	1340 1250
Estradiol-17β (^{3}H-E_2)	^{3}H-Estrone	1 2	699 680	1235 705	900 699
	^{3}H-Estradiol-17β	1 2	1087 1091	1305 1119	1200 1087
	^{3}H-Estrone-Sulfate	1 2	1080 1050	1241 1030	1120 1080

TABLE X

Crystallization of Glucosiduronate Fractions, Male Rat.

SPECIFIC ACTIVITY cpm/mg

Estrogen Metabolite	Substrate Incubated	Crys. N°	Crystals	Mother Liquor	Theoretical
^{3}H-Estrone-Glucuronide as E_1	^{3}H-Estrone	1 2	913 890	721 910	903
	^{3}H-Estradiol-17β	1 2	786 796	681 780	835
	^{3}H-Estrone-Sulfate	1 2	1430 1392	1390 1380	1288
^{3}H-Estradiol-Glucuronide as E_2	^{3}H-Estrone	1 2	1017 1040	1248 1025	1083
	^{3}H-Estradiol-17β	1 2	650 630	715 660	720
	^{3}H-Estrone-Sulfate	1 2	599 510	1043 540	745

2. RESULTS AND DISCUSSION - HEN

(i) Distribution of Radioactivity : The distribution of radioactivity obtained from laying hen kidney is given in Table XI. The glucuronide fraction shown represents the total radioactivity remaining in the aqueous fraction after ethylacetate extraction. It was recovered through a bed of Amberlite XAD-2. In the ^{3}H-Estrone and ^{3}H-Estrone-Sulfate incubation, such XAD-2 processing was not carried out, because there was practically no radioactivity left in the aqueous fraction at that stage. It has been observed that ethylacetate extraction from 3M NaCl solution will not only remove sulfates but also some other forms of conjugates present particularly glucuronides and N-Acetylglucosamine derivatives (36).

For these reasons and others previously given (Section on Rat) the ethyl acetate extracts were not further processed, except the 21% from the ^{3}H-Estrone-sulfate incubation.

(ii) Analysis of Free and Conjugate Fractions : The free or unconjugated metabolites in the ethereal fractions were analyzed as before, the results appear in Table XII. Using this type of method, Estrone-Estradiol interconversion favoured the former and it seems Estrone as a metabolite was about ten times (10x) greater than Estradiol, irrespective of whether E_1 or E_2 was incubated.

The 21% sulfate extract, Table XI, was completely hydrolyzable by phenolsulfatase (Mylase P). The released steroid extracted into ether was identified as Estrone. This represented unmetabolized substrate. The glucuronides were hydrolyzed with Bacterial-β-glucuronidase or Ketodase (a β-glucuronidase), Table XIII, and ether - extracted. The hydrolyzed

TABLE XI

Distribution of Radioactivity
Following Incubation of Estrogens
With Laying Hen's Kidney Homogenates

LABELLED FRACTIONS EXTRACTED	SUBSTRATE INCUBATED dpm							
	^{3}H-Estrone 0.89 x 10^6		^{3}H-Estradiol-17β 4.59 x 10^6		^{3}H-Estrone-Sulfate 2.56 x 10^6		^{3}H-Estradiol-17β-GLU. 1.70 x 10^6	
	DPM x 10^6	%	DPM x 10^6	%	DPM x 10^6	%	DPM x 10^6	%
Recovery	0.84	94.3	4.48	97.6	2.48	96.8	1.63	95.8
Hexane	0.05	6	0.20	5	0.10	4	0.009	0.5
Ether (Free)	0.77	92.3	3.99	89.0	1.91	77.0	0.02	1.5
Ethyl-Acetate (Sulfate)	0.03	3.9	0.07	1.6	0.51	21.0	0.07	4.3
Glucuronide (XAD-2)	*		0.12	2.6	*		1.50	92.0
FRACTION ACCOUNTED FOR	0.85	102.2	4.38	98.2	2.52	102	1.59	98.3

*Amberlite XAD-2 processing was not carried out.

TABLE XII

Analysis of Ether Extracts (Free)
Following Incubation of Estrogens
With Laying Hen Kidney Homogenates

FRACTIONS AND METABOLITES	SUBSTRATE INCUBATED dpm					
	^{3}H-Estrone 0.89 x 10^6		^{3}H-Estradiol-17β 4.59 x 10^6		^{3}H-Estrone-Sulfate 2.56 x 10^6	
	DPM x 10^5	%*	DPM x 10^5	%*	DPM x 10^5	%*
Ether-Extract (Free)	7.77	92.3	39.91	89.0	19.06	76.7
Benzene Layer from Benzene/H_2O Partition	7.61	90.3	39.60	88.4	18.22	73.4
Estrone	6.61	73.2	29.12	65.0	13.23	54.0
Estradiol	0.62	7.3	3.57	7.9	1.28	5.1

* Percentages quoted are with reference to original incubation recoveries.

TABLE XIII

Analysis of Glucosiduronate Fraction
Following Incubation of Estrogens
With Laying Hen's Kidney

FRACTIONS AND METABOLITES	SUBSTRATE INCUBATED dpm			
	^{3}H-Estradiol-17β 4.59×10^6		^{3}H-Estradiol-17β-GLUCURONIDE 1.70×10^6	
	DPM x 10^6	%	DPM x 10^6	%
Glucuronide (XAD-2 Extract)	1.2	2.6	15.0	92.0
β-Glucuronidase Hydrolysis	0.7	60.0	14.7	98.0
Benzene Layer from benzene/H_2O Partition	0.68	97.0*	14.3	97.0*
^{3}H-E_1	0.26	40.0*	0.1	1.2*
^{3}H-E_2	0.22	34.1*	12.1	82.0*

* Based on ether extractable material from β-Glucuronidase hydrolysis.

estrogens were similarly fractionated into Estrone and Estradiol. The glucuronide from the ^{3}H-$E_2$17G incubation was entirely Estradiol indicating non-metabolism of Estradiol glucuronide by the hen kidney. There was approximately 1:1 ratio of E_1:E_2 glucuronides from the ^{3}H-Estradiol incubation Table XIII. Since E_1, E_2 interconversion has been found to be about 10 times in favour of E_1, it seems glucuronide synthesis proceeds equally well with either E_1 or E_2. It is also possible that Estradiol was preferentially glucuronidated followed by dehydrogenation to Estrone glucuronide. The glucuronides formed from the E_1 and E_2 incubations were very small and as such their significance becomes even doubtful.

3. RESULTS AND DISCUSSION - DOG

(i) Distribution of Radioactivity : Table XIV gives the distribution of radioactivity extracted after incubating dog kidney with various estrogen substrates. Amount of radioactivity extracted into hexane is again quite considerable for the free steroids but for the conjugates it gives an indication of the degree of hydrolysis especially of E_1S. The dog's kidney like the rat and hen tissues did not yield much conjugation, but the total conjugate pool from Estradiol incubation was slightly higher than that obtained from the Estrone conjugation. The total conjugates from the free steroids incubations were hydrolysed with Ketodase but those obtained from the conjugate incubations were chromatographed on DEAE-sephadex followed by extraction and hydrolysis of radioactive peaks.

The analysis of ether - soluble fractions is given in Table XV. The very low radioactivity in the aqueous phase of the Benzene/H_2O partition was indicative of the kidney's inability to form more polar unconjugated metabolites. The aqueous phase would have contained Estriol or E_3 -like metabolites. Estriol and particularly its epimer $E_3 16\alpha 17\alpha$ have been found to be major estrogen metabolites in the dog.(125). The benzene phase was analyzed as usual by chromatography on celite columns. The Estradiol produced by incubation of either free Estrone or Estrone sulfate was about one ninth of the estrone so produced.

(ii) Analysis of Conjugates : The total conjugates from the incubation of ^{3}H-Estrone and ^{3}H-Estradiol were separately incubated with Ketodase, 500 units/ml. in acetate or phosphate buffer pH 5 for 30 hours. Upon extraction with ether, 55% and 49% hydrolysis respectively were obtained.

TABLE XIV

Distribution of Radioactivity
Following Incubation of Estrogens
With Female Dog Kidney Homogenates

LABELLED FRACTIONS EXTRACTED	SUBSTRATE INCUBATED dpm							
	^{3}H-Estrone 0.99×10^6		^{3}H-Estradiol-17β 3.36×10^6		^{3}H-Estrone-Sulfate 2.66×10^6		^{3}H-Estradiol-17β-GLU. 1.90×10^6	
	DPM $\times 10^6$	%	DPM $\times 10^6$	%	DPM $\times 10^6$	%	DPM $\times 10^6$	%
Recovery	0.98	99	3.33	99	2.63	99	1.90	100
Hexane	0.06	6	0.20	6	0.02	0.9	0.01	0.5
Ether (Free	0.89	91	2.79	84	0.25	10	0.03	1.3
Total Conjugate (XAD-2)	0.04	4	0.20	6	2.28	87	1.89	99
FRACTION ACCOUNTED FOR	0.99	101	3.19	96	2.55	97.9	1.93	100.8

TABLE XV

Analysis of Ether Extract (Free)
Following Incubation of Estrogens
With Dog Kidney

FRACTIONS AND METABOLITES	SUBSTRATE INCUBATED dpm					
	^{3}H-Estrone 0.99×10^6		^{3}H-Estradiol-17β 3.36×10^6		^{3}H-Estrone-Sulfate 2.66×10^6	
	DPM x 10^5	%	DPM x 10^5	%	DPM x 10^5	%
Ether Extract (Free)	8.9	91	27.9	84	2.5	10
Aqueous Phase	0.2	2.2	0.3	1.2	0.04	1.5
Benzene Phase	8.1	91	27.7	99.2	2.4	94.3
Estrone*	6.5	73	17.4	63	1.9	76
Estradiol*	0.8	9	3.9	16	0.2	8

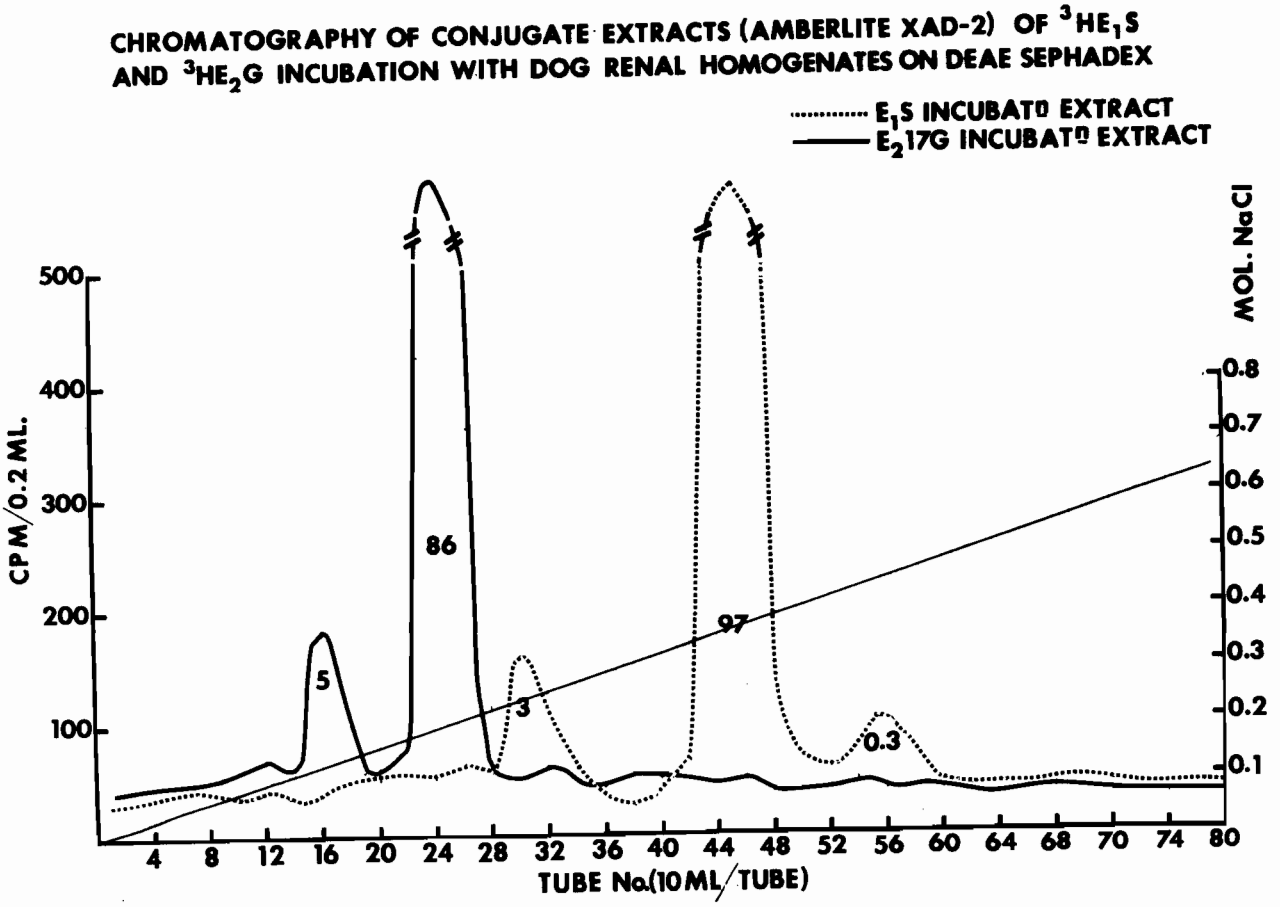
*Percentages based on original ether extract.

Further incubation of the residual aqueous fraction with Mylase P did not release additional ether soluble steroids. Thus, as mentioned elsewhere, glucuronidation seems to be the only significant form of conjugation encountered in the kidneys under the experimental conditions.

Fig. IV shows the DEAE sephadex chromatographic pattern of the conjugates from the estrogen conjugates (^{3}H-E_1S; ^{3}H-E_2G) incubated. The percentages of the various components are written into the peaks.

^{3}H-E_2G Incubation : Peaks 1 and 2 were extracted through XAD-2 and incubated with 500 units/ml. of Ketodase in phosphate buffer pH 5 and recovery into ether was quantitative. Further analysis of the hydrolyzed Peak 2 by celite chromatography yielded 1.7% of E_1 and 86% of E_2. Thus, this fraction represents residual substrate. Peak 1 is therefore also a glucuronide but a metabolite of $E_2$17G. This was initially suspected to be the 3-Glucuronide of Hahnel (124) but further consideration suggested that this was not likely since this would imply hydrolysis of the 17-Glucuronic acid followed by reconjugation at the C-3 position. β-Glucuronidase has been implicated in transconjugation (83, 84), if so, it could account for a C-3 conjugation and the resulting steroid would be either E_1G or $E_2$3-G. However, β-glucuronidase activity has been discounted in these experiments. It is interesting to note that further analysis of the free steroid from this metabolite (Peak 1) yielded only radioactivity of 6 and 4% with the mobility of Estrone and Estradiol respectively. With such low values, the urge, to conclude that the steroid molecule might be metabolized to a form hitherto unrealized, is very strong. The only steroid metabolite less polar than E_1 and E_2, is 2MeOE_1 but Balikian, Southerland et al (125) could not detect 2 hydroxy- or 2 methoxylation in the dog. In a repeat of this

FIGURE IV



DEAE-Sephadex-(A25) chromatography of conjugates from dog renal incubations. The peaks in each plot are refered to in text in sequential order from the left. Peak 1 and 2 refer to the solid line $E_2$17G incubation extract, and Peak 1s, 2s, and 3s refer to the dotted line E_1S incubation extract. The inscribed figures represent the incubation percentages of the amount chromatographed.

experiment, then analysis of the conjugates by Girard reaction followed by celite chromatography, no evidence for 2 Methoxy Estrone was obtained. Further evidence for a similar metabolite will be give for E_1S metabolism.

^{3}H-E1S Incubation: Peaks 2s and 3s from the E_1S incubation extract were completely hydrolyzable with Mylase P (a phenolsulphatase). They were identified as Estrone and Estradiol respectively by solvent partition and celite chromatography. Peak 2s therefore represents residual substrate, E_1S. Peak 3s, however respresents E_2S, a metabolite of the Estrone sulfate. Thus, the dog kidney can convert $E_1S \rightarrow E_2S$ without prior hydrolysis. This pathway is however minor, for it accounts for only 0.3% of the substrate. Peak 1s defied logical analysis, presenting the same problems as Peak 1 from the glucuronide fraction. Judging from its behaviour on the ion-exchanger, it was suspected to be a glucuronide. The Peak was therefore incubated with Bacterial β-glucuronidase, for 36 hours but yielded only 1% ether-soluble radioactivity. The aqueous fraction was reincubated with Ketodase (1000 units of β-glucuronidase) for a further 48 hours but again yielded $< 1\%$ ether-soluble material.

It was therefore reincubated with phenolsulfatase for 24 hours and this time, over 70% ether-soluble material was extracted. This Peak 1s is therefore a sulfate conjugate metabolite of ^3H-E_1S. Exactly what metabolite of E_1S which elutes earlier than E_1S and is not a 2-Methoxy derivative is unknown at present. Again, as has been pointed out above, a repeat of the experiment to isolate $2MeOE_1$ from the total conjugate metabolite of E_1S yielded no result. Celite column chromatography (111) of the Peak 1s extract gave a distribution of 15 and 30% fractions with the polarity of E_1 and E_2 respectively.

It is therefore concluded that the Peaks 1 and 1s metabolites from the two conjugate incubations (E_1S, E_2G) do not simply contain E_1 or E_2 but probably a new metabolite less polar than the parent E_1 or E_2 and not 2 Methoxy derivatives. Could they possible be polymethoxylated metabolites or in conjugation with other unknown less polar moieties? Answers to these questions must await further investigation. It is the belief of the author that these metabolites are due to similar transformation of the steroid moieties of E_1S and E_2G.

4. RESULTS AND DISCUSSION - RABBIT

(i) Distribution of Radioactivity: The distribution of radioactivity from the rabbit kidney incubations is given in Table XVI. A remarkable feature in the table is the very high hexane extract after Estrone incubation. It is difficult to account for this since the hexane layers were not further analyzed. Again, since Estradiol was converted preferentially to Estrone, one would have expected the Estradiol incubation also to yield a high hexane extract. The total conjugate from the same (E_1) incubation is also remarkably high. All other extracts in the table conform to expected pattern with very high experimental recoveries.

Analysis of the free extracts (ether) gave a pattern similar to other species studied, Table XVII. The complete extraction into the benzene phase confirmed the absence of Estriol or Estriol-like metabolites. The Estrone - Estradiol interconversion pattern was also evident here, i.e. heavily in favour of Estrone.

(ii) Analysis of Conjugates: The conjugate extract (33%) from the ^{3}H-Estrone incubation was divided equally into two fractions. These were enzymatically hydrolyzed. One half was incubated with β-glucuronidase (Ketodase) and the other half with Hyaluronidase. The Ketodase incubation yielded 99% of the radioactivity into ether but the Hyaluronidase resulted in only 40% of the steroid being extracted into ether. The residual activity in the aqueous phase (hyaluronidase incubation) could not be released by a repeat incubation of hyaluronidase but rather Ketodase. Rabbits are known to excrete estrogens, ($E_2$17α) as the N-Acetyl-glucosamine derivative in a mixed conjugate with a molecule of glucuronic acid (35, 36). Layne

TABLE XVI

Distribution of Radioactivity
Following Incubation of Estrogens
With Female Rabbit Kidney Homogenates

LABELLED FRACTIONS EXTRACTED	SUBSTRATE INCUBATED dpm							
	^{3}H-Estrone 0.73×10^6		^{3}H-Estradiol-17β 3.03×10^6		^{3}H-Estrone-Sulfate 2.46×10^6		^{3}H-Estradiol-17β-GLU. 1.38×10^6	
	DPM $\times 10^6$	%	DPM $\times 10^6$	%	DPM $\times 10^6$	%	DPM $\times 10^6$	%
Recovery	0.66	90	2.88	95	2.29	93	1.34	97
Hexane	0.12	18	0.17	6	0.11	5	0.02	1.9
Ether (Free)	0.24	36	2.21	76	0.34	15	0.01	1.0
Total Conjugate (XAD-2)	0.22	33	0.28	9	1.61	70	1.21	91.1
FRACTION ACCOUNTED FOR	0.58	87	2.66	91	2.06	90	1.24	94

TABLE XVII

Analysis of Ether Extracts (Free)
Following Incubation of Estrogens
With Rabbit Kidney

EXTRACTS AND METABOLITES	SUBSTRATE INCUBATED dpm					
	^{3}H-Estrone		^{3}H-Estradiol-17β		^{3}H-Estrone-Sulfate	
Ether Extract (Free)	DPM x 10^5	%	DPM x 10^5	%	DPM x 10^5	%
	2.40	36	22.18	76	3.36	15
Benzene Phase	2.35	97.9	22.01	99.2	3.25	96.7
Estrone	0.83	60*	14.66	67*	2.08	62*
Estradiol	0.24	10*	3.08	14*	0.24	7*

*Based on ether extract.

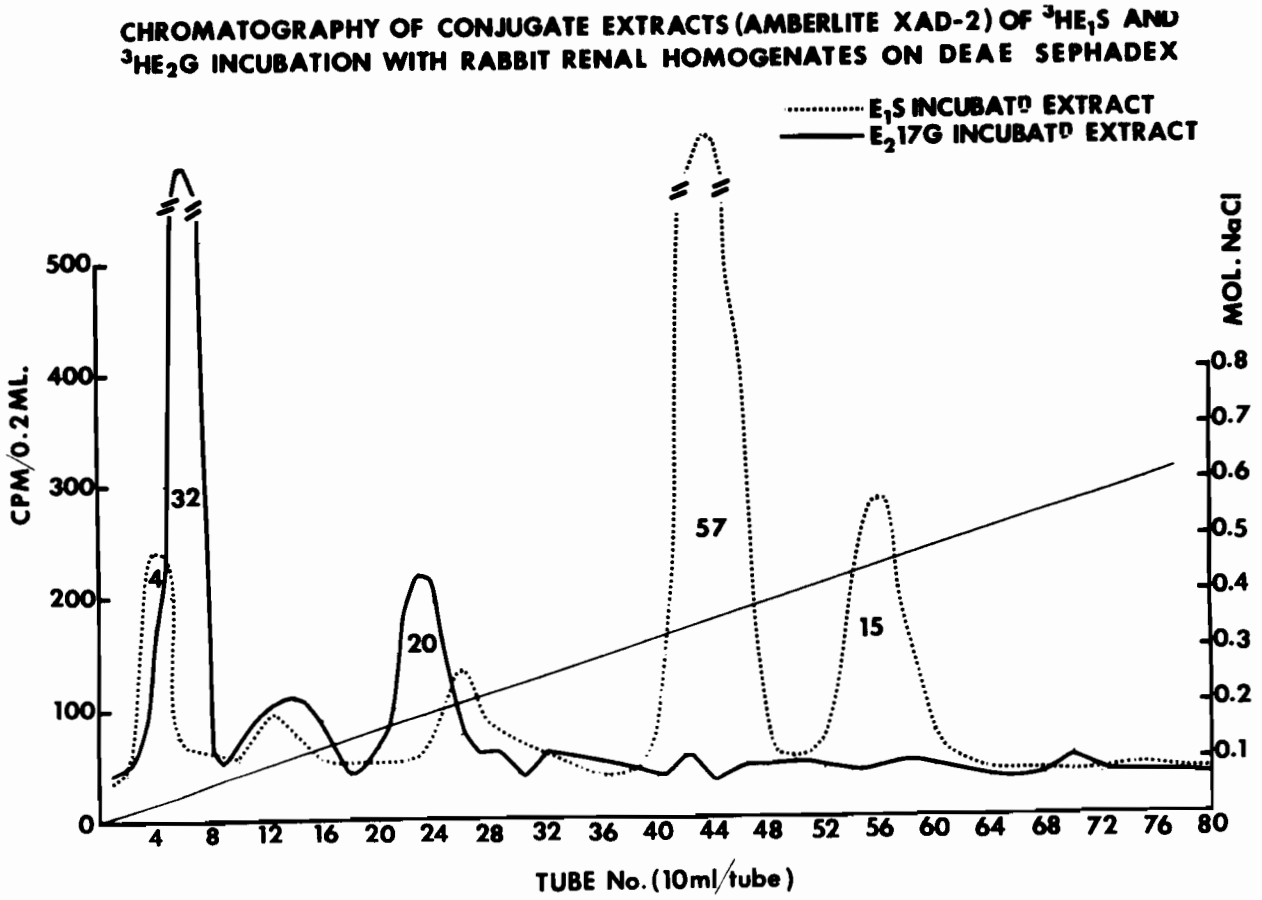
has reported that Ketodase hydrolyzes about 25% of the N-acetylglucosamine linkage but the hyaluronidase which hydrolyzes N-AcGluNH$_2$ linkage almost completely does not have any β-glucuronidase activity (35). Therefore it seems, from the two enzymatic treatments, that the greater part of the conjugate extract was a glucuronide and perhaps less than 40% of it was an N-AcGluNH$_2$ conjugate. Such an N-AcGluNH$_2$ complex should be a mono-conjugate without an additional glucuronic acid moiety or else, it would not have been ether-soluble following hyaluronidase treatment alone. The recent finding of estrogen glucoside in the rabbit (39) puts the real nature of this Peak 1 into a new perspective; that, if this Peak 1 was an estrogen glucoside, it would account for its complete hydrolysis by the Ketodase. The effect of hyaluronidase on estrogen glucoside is not known. It might have a partial hydrolytic effect; thus the 40% hydrolysis obtained from the hyaluronidase incubation.

The total conjugate from the ^{3}H-Estradiol incubation was chromatographed on DEAE-sephadex. Its elution pattern (not shown in diagram) coincides with Peaks 1, 2 and 3 Fig. V. The corresponding percentages to these three minor peaks were 23, 20 and 6 respectively. These three peaks were not further processed because of insufficient radioactivity.

The chromatographic pattern of conjugates from ^{3}H-E$_1$S and ^{3}H-E$_2$17G incubation are shown in Fig. V. The peaks are numbered in sequential order from the left. The inscribed figures represent the percentages of the amount chromatographed.

^{3}H-E$_2$G Incubation: Chromatography of the ^{3}H-E$_2$G metabolites on the ion-exchange sephadex yielded only three peaks. The absence of a peak in the region of the known sulfates, Fig. III, in this and in the case

FIGURE V



DEAE-Sephadex-(A25) chromatography of conjugates from rabbit renal incubations. The peaks in each plot are numbered in text in sequential order from the left. The inscribed figures represent the percentages of the amount chromatographed. Rate of elution 50 ml/hr.

of the ^3H-E_2 conjugates, above, is a further negative proof for sulfo-conjugation in the kidney. It is significant that the three peaks here correspond to those from the ^3H-E_2 incubation.

Only Peaks 1 and 3 were extracted and further processed. Peak 2 (8%) was considered of too low activity to warrant further handling. Peaks 1 and 3 could not be hydrolyzed by phenolsulfatase. Peak 3 was completely hydrolyzed by β-glucuronidase and was identified as Estradiol, thus it represents residual substrate. Only 52% of Peak 1 was released by β-glucuronidase and ethylacetate extraction of the aqueous residue removed only 10% (Ethylacetate is used to extract the mono-N-AcGluNH$_2$ conjugate (35)). This ethylacetate extract was returned to the aqueous phase and then pooled with the corresponding aqueous phase (see below - Peak 1 E_1S incubation) after β-glucuronidase treatment. The pool was then incubated with hyaluronidase following which less than 22% of the activity present was released into ether. This free material was not further processed. Analysis of the original 52% yielded 40% E_1-like and less than 5% of E_2-like. Owing to the already noted comments on renal β-glucuronidase, this 40% is less likely to be Estrone. Further evidence against the Peak 1 fraction being just Estrone is given below.

^3H-E_1S Incubation : Peak 1 (4%) from the chromatography of ^3H-E_1S incubation conjugates, Fig. V, was found to be only 35% hydrolyzable by β-glucuronidase. Further analysis of this released material, on celite yielded only 22% E_1-like and less than 1% E_2-like. This very low recovery as estrone from E_1S incubation raises some doubt about the real nature of the steroid metabolite. Peak 2 (3%) like the corresponding Peak 2, above, was not processed further. Peak 3 (6%), in the diagram, from its elution profile seemed

F. RATE OF HYDROLYSIS OF E_1S AND E_2G BY RENAL HOMOGENATES

1. INTRODUCTION

From the above incubations, it seems that β-glucuronidase activity in these species is very small or absent. Sulfatase activity, however, is quite considerable especially in the rat. The lowest value noticed was in the dog, Table XVIII. The 4.7% recovery of incubated E_1S in one instance (rat) has been accounted for previously and it is felt that the value is high probably due to extraction error. Steroid glucuronides are said to be excreted by glomerular filtration without significant reabsorption (54) and are also secreted by the tubules (56). Steroid sulfates on the other hand may have a rapid tubular reabsorption (50). From the high sulfatase activity found in some of these animals, it was considered desirable to investigate the sulfatase further.

2. RELATIVE POTENCIES OF RENAL SULFATASE IN THE SPECIES

Fig. VI gives the time course of the H-E_1S hydrolysis for each species. The diagram confirms the range of sulfatase activities seen in Table XVIII, i.e. the activity is extremely high in the rat and very low in the dog. The hen followed by the rabbit showed intermediate values. The kidney preparations from the laying hen showed a higher sulfatase activity than the non-laying hen. It was difficult to assess the significance of this since only two different hens were used.

Since chlorides and phosphate are known to inhibit sulfatase, the rat experiment was repeated in TRIS buffer pH 7.4. The TRIS plot Fig. VI

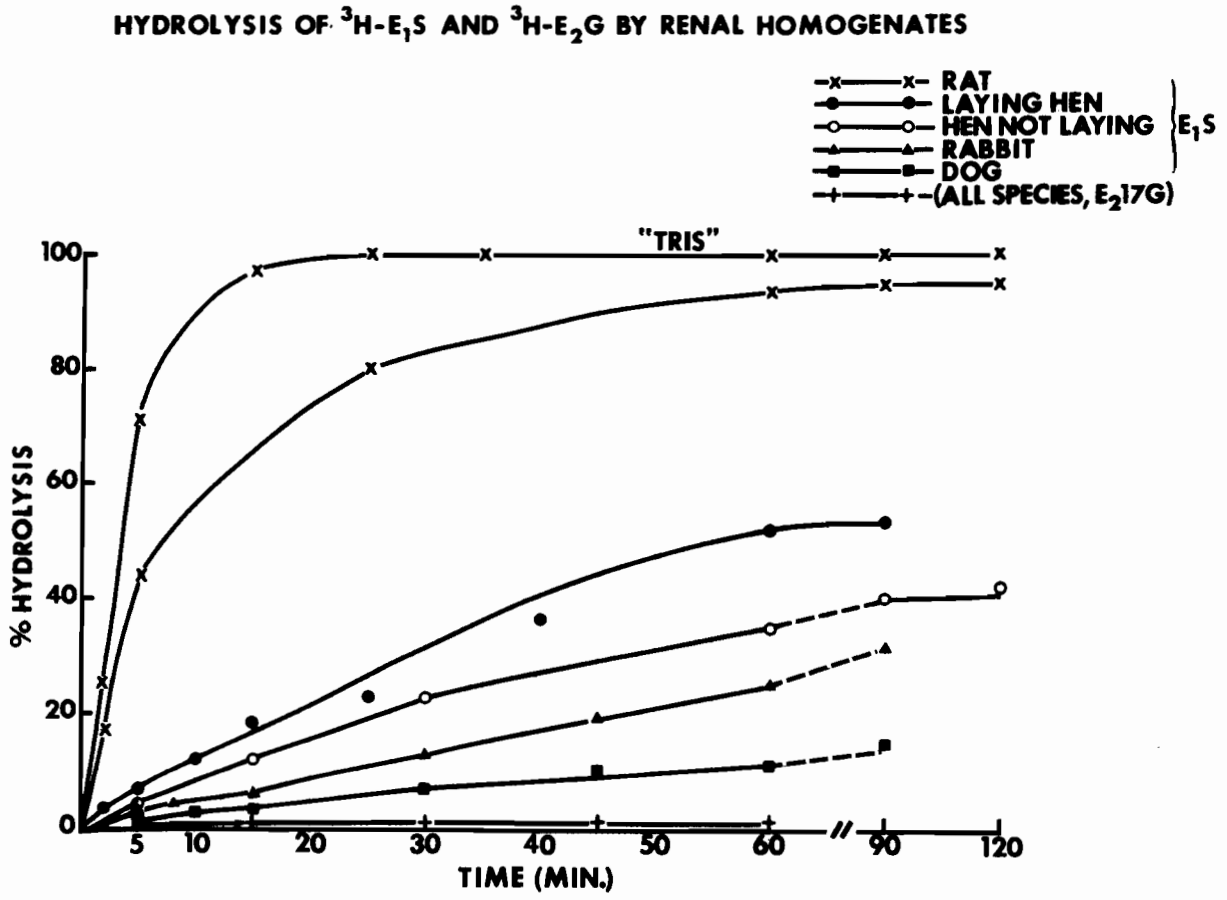
TABLE XVIII

RECOVERY OF ESTROGEN SULFATES AFTER INCUBATING ^{3}H-E_1S WITH RENAL HOMOGENATES

SPECIES	AMT. INCUBATED DPM x 10^6	RECOVERY DPM x 10^6	RECOVERY %
Rat M	1.96	0.12	4.7*
F	2.77	0.025	1.0
Hen	2.50	0.51	20.5
Rabbit	2.30	1.24	78.0
Dog	2.63	2.28	86.8

* Higher value, probably due to extraction procedure.

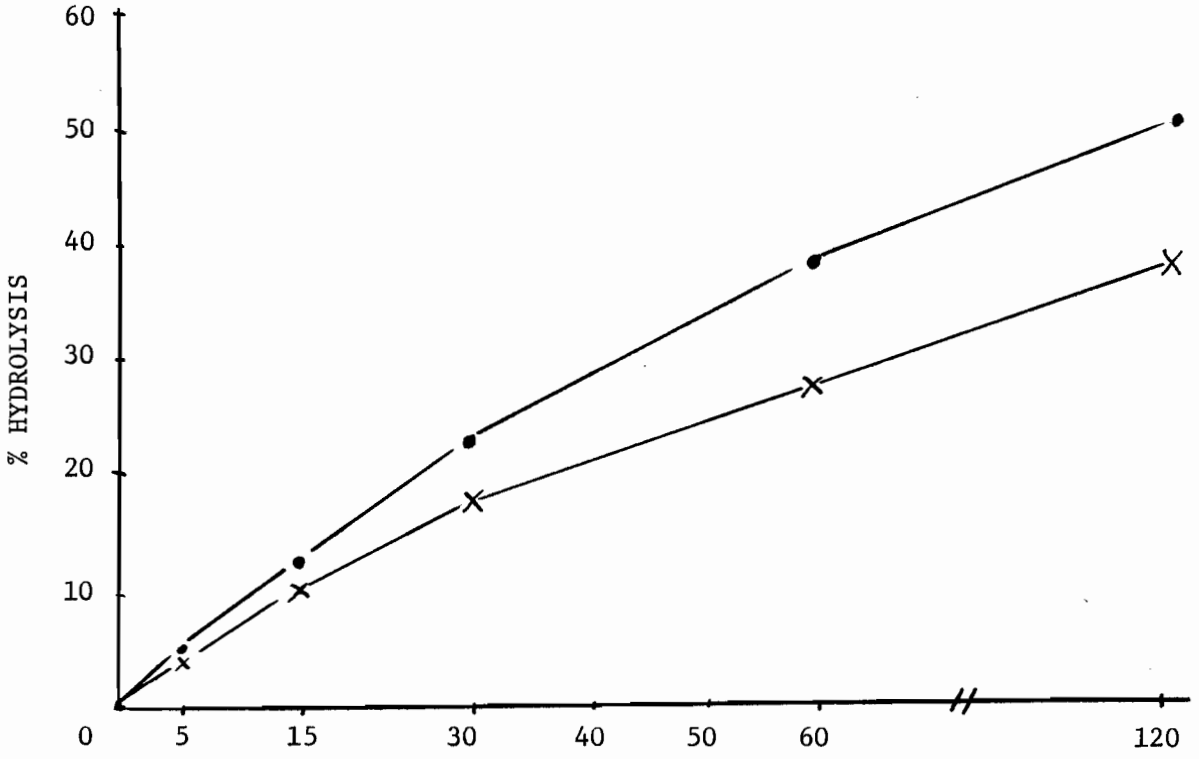
FIGURE VI



Rate of hydrolysis of E_1S and E_217G by renal homogenates. Phosphate buffer pH 7.4 was used in all the E_1S hydrolysis except one labelled "TRIS". The single plot for E_217G (hydrolysis) is typical for all species and phosphate buffers pH 4.5 and 7.4 .

FIGURE VII

HYDROLYSIS OF ^{3}H-E_1S BY HEN KIDNEY HOMOGENATES



Rate of hydrolysis of Estrone Sulfate, (. - . - .) in Krebs Phospho-saline buffer pH 7.4, (x - x - x) in 'TRIS' buffer pH 7.4 . Note the high Sulfatase activity in the phosphate buffer and inhibition in the TRIS buffer.

shows that inhibition of sulfatase in the phospho-saline buffer was minimal in that the terminal percent hydrolysis was not significantly different from the phosphosaline plot. The only difference seemed to be in the initial velocity, as it were, lowering the Tm.[1] In a similar TRIS experiment with the laying hen kidney, Fig. VII, the sulfatase was actually inhibited. This was also true for the non-laying hen. These findings about renal sulfatase make an investigation into its physiological role imperative. Perfusion studies would reveal very interesting findings.

3. RENAL β-GLUCURONIDASE

The optimum condition for mammalian β-glucuronidase activity is pH 4.5 in acetate or phosphate buffer (90, 93, 126). Renal homogenates were prepared as for sulfatase studies in phosphate buffers, pH 4.5 and 7.4 and incubated with ^{3}H-$E_2$17G. The incubation media were then processed as above. The single plot in Fig. VI represents the type of results obtained at both pH's and in all species. It was also thought that the C-17 Glucuronide might be resistant to the cellular enzyme. The experiment was therefore repeated with ^{3}H-$E_2$3G [2] but the results were not different. The findings are self evident, i.e., that the renal tissue from these species does not appear to contain β-glucuronidase. Fishman (91) and Varma et al (93) have demonstrated β-glucuronidase in the mouse kidney but it seems to be species specific to the mouse (88, 89, 92).

---

[1] Tm here is used to refer to time to attain 50% hydrolysis.

[2] Supplied as the 17α epimer by D.S. Layne, University of Ottawa.

TABLE XIX

*Distribution of Radioactivity
Following Incubation of Estrogens
With Boiled Rat Kidney Tissue (Blank)

LABELLED FRACTIONS EXTRACTED	SUBSTRATE INCUBATED dpm							
	^{3}H-Estrone 3.80×10^5		^{3}H-Estradiol-17β 6.81×10^5		^{3}H-Estrone-Sulfate 5.81×10^5		^{3}H-Estradiol-17β-GLU 4.30×10^5	
Recovery	DPM x 10^5	%	DPM x 10^5	%	DPM x 10^5	%	DPM x 10^5	%
	3.77	99	6.80	100	5.80	100	4.30	100
Hexane†	0.03	1	0.05	0.08	0.02	0.4	0.008	0.2
Ether (Free)	3.6	98	6.59	97	0.05	1.0	0.03	0.9
Ethyl-Acetate (Sulfate)	0.03	0.9	0.06	1.0	5.68	98	0.04	1.0
Ether-Ethanol (Glucuronide)	0.01	0.3	0.06	0.9	0.04	0.8	4.2	98

*These results are taken to present the blanks of all species since very similar distributions were obtained in each case.

†Fraction percentages relate to amount recovered after incubation.

G. GENERAL DISCUSSION

The results of the preceeding investigation indicate that, the kidney, like other organs and tissues can metabolize both free and conjugated estrogens. It is interesting to note that the recoveries of radioactivity from these incubations were almost quantitative indicating little strong binding or absorption of the estrogens to the kidney tissue. This contrasts with observations for other organs especially the liver. In preliminary studies by the author, using liver homogenates, low recoveries were obtained. A number of other workers Szego (127), Reigel and Mueller (128), and Sandberg et al (129) have shown that Estrone and 17β-Estradiol can be irreversibly bound to liver proteins, hence the low recoveries. The kidney tissues of all the animals metabolize Estrone and Estradiol preferentially to Estrone and this agrees with the results of other workers using various animal tissues (79, 130-132) except in the case of the testis (130, 133), where the equilibrium between Estrone and Estradiol is shifted in favour of Estradiol. These results are also in agreement with those found in human (134, 135). Thus,human as well as the animals involved in the present studies metabolize administered Estrone in almost exactly the same manner as 17β-Estradiol and the equilibrium between E_1 and E_2 must be reached very quickly in the body. Beer and Gallagher (136, 137) have furnished extensive quantitative information on the interconversion of Estrone and 17β-Estradiol in man.

All the species seemed capable of forming estrogen glucuronides, these were, however, small but are of some significance in view of the fact that the incubation mixtures were not fortified with UDPGA (uridine di-

phospho-glucuronic acid). As far as has been reported, renal conjugation has been confined to only glucuronide both *in vivo* and *in vitro* (63-64, 67-70). It therefore seems that the kidney tissue can only form glucuronides since no evidence of sulfurylation was obtained in any of the species. However, no attempt was made to influence sulfurylation with co-factors. The rat and hen did not metabolize either Estrone sulfate or Estradiol glucuronide to other conjugated products. Apart from hydrolyzing the estrogen sulfate, they did not appear to alter the glucuronide in any way not even to hydrolyze it.

The apparent absence of β-glucuronidase activity can be seen in all the four animals studies though other workers, Fishman (91), Varma (93), and Toshiyoshi (138), have detected β-glucuronidase in the kidneys of rats and mice. The assay for their enzyme was dependent upon the hydrolysis of the non-physiological substrate, phenolphthalein glucuronide. The dog and rabbit, apart from exhibiting sulfatase activity, were also capable of transforming Estrone sulfate and Estradiol glucuronide to other conjugated products. Some of these metabolites, as noted earlier, were of unknown nature. Though evidence from these studies is inconclusive, it should be noted that, in view of the recent findings of Layne *et al* (34-36, 39), these metabolites formed in the rabbit tissue might well be glucosides and/or N-acetylglucosaminides. Only the dog and rabbit showed a steroid sulfate interconversion pathway, in this case $E_1S \rightarrow E_2S$. This pathway was more quantitatively important in the rabbit (15% conversion) than in the dog (0.3% conversion). The rabbit alone seemed capable of metabolizing E_1S and E_2G to a number of other metabolites.

The Estradiol metabolite obtained in these studies, especially

from hen, dog and rabbit could conceivably be a mixture of both epimers, $E_2 17\alpha$ and $E_2 17\beta$, since these animals are all known to secrete both forms. Attempts were made to detect the $E_2 17\alpha$ in the Estradiol by radioautography but the tritium emission was too weak to be picked up.

None of the species tested here showed any sign of sulfo-conjugation, it is therefore felt that these renal tissues can not sulfurylate, at least, under the conditions of the incubations. The variation of sulfatase activities was very striking in the preparations. The activity in the rabbit was comparable to that in the dog but it was significantly lower than those of the rat and hen. It is interesting that the renal sulfatase was active in the Krebs phospho-saline buffer although 0.05M chloride, 0.05M phosphate and 0.05M sulfate are reported to inhibit sulfatases (96, 108). The actual physiological significance of these sulfatases will be of great interest because they appear to be lysosomal and according to DeDuve (100) they cannot function in the intact cell since they would become active only when the lysosomes are ruptured. Further, even if they were liberated into cell fluids, they would be virtually completely inhibited by the ions present therein. If these renal tissues and, particularly the rat tissue, could overcome this inhibition in the Krebs phospho-saline buffer, it is possible that the enzyme might have real physiological function. The fact that the placenta is very rich in sulfatases which are extremely active <u>in vivo</u> lends great support to the reappraisal of intracellular localization and physiological significance of sulfatases. It may be premature to speculate, but if the renal sulfatase was functional <u>in vivo</u> it could explain much of the uncertainty about steroid sulfate excretion; that the renal excretion of steroid sulfates is very low because of this cellular hydrolysis. In

the same hypothesis, the apparent absence of β-glucuronidase activity might be related to the almost quantitative renal excretion of steroid glucuronides.

H. SUMMARY

Incubations of free and conjugated estrogens with kidney preparations from rat, hen, dog and rabbit indicate that:

i) The equilibrium between the interconversion of Estrone and Estradiol-17β is greatly in favor of Estrone.

ii) The kidney tissue cannot sulfo-conjugate, at least, under the conditions of the experiment; and does not exhibit β-glucuronidase activity. Some glucuronide formation was evident.

iii) There is a wide range of sulfatase activity, very high in the rat and very low in the dog.

iv) The dog and rabbit can metabolize E_1S without prior hydrolysis and that the conversion of $E_1S \rightarrow E_2S$ is a major pathway in the rabbit kidney.

v) Both dog and rabbit can metabolize E_1S and E_2G to other products of unknown nature.

BIBLIOGRAPHY

1. MUHLBOCH, O., Z. Physiol. Chem. 250: 139, 1937.
2. MUHLBOCH, O., Lancet, 236: 634, 1939.
3. RAKOFF, A.E., PASCHKIS, K.E., and CANTAROW, A., Am. J. Obstet. Gynecol. 46: 856, 1943.
4. SZEGO, C.M. and ROBERTS, S., Proc. Soc. Exptl. Biol. Med. 61: 161, 1946.
5. GLIMM, E. and WADEHN, F., Biochem. Z. 207: 361, 1929.
6. COHEN, S.L., MARRIAN, G.F., and ODELL, A.D., Biochem. J. 30: 2250, 1936.
7. SANDBERG, A.A. and SLAUNWHITE, W.R., J. Clin. Invest. 36: 1043, 1957.
8. PURDY, R.H., ENGEL, L.L., and ONCLEY, J.L., J. Biol. Chem. 236: 1043, 1961.
9. MIGEON, C.J., WALL, P.E., and BERTRAND, J., J. Clin. Invest. 38: 619, 1959.
10. SCHACHTER, B. and MARRIAN, G.F., J. Biol. Chem. 126: 663, 1938.
11. JENSEN, H., LARIVIERE, M., and ELIE, J.J., Rev. Can. Biol. 4: 535, 1945.
12. COHEN, S.L. and MARRIAN, G.F., Biochem. J. 30: 57, 1936.
13. LEVITZ, M., CONDON, G.P., MONEY, W.L. and DANCIS, J., J. Biol. Chem. 235: 973, 1960.
14. MACKENNA, J., MENINI, E., and NORYMBERSKI, J.K., Biochem. J. 79: IX, 1961.
15. ADLERCREUTZ, H. and BELING, C.G.: Vit. and Hormones. 20: 285, 1962.
16. HOBKIRK, R., NILSEN, M., and BLAHEY, P.R., J. Clin. Endocr. 29: 328, 1969.
17. ROBERTS, S., and SZEGO, C.M., Endocrinology, 40: 73, 1947.
18. COHEN, H. and BATES, R.W., Endocrinology, 45: 86, 1949.
19. ONESEN, I.B. and COHEN, S.L., Endocrinology, 51: 173, 1962.
20. GRANT, J.K. and MARRIAN, G.F., Biochem. J. 47: 1, 1950.
21. NEEMAN, M. and HASHIMOTO, Y., J. Amer. Chem. Soc. 84: 2972, 1962.
22. VIALA, R. and GIANETTO, R., Can. J. Biochem. Physiol. 33: 839, 1955.
23. CARPENTER, J.G.D. and KELLIE, A.E., Biochem. J. 84: 303, 1962.

24. SMITH, E.R. and KELLIE, A.E., Biochem. J. 104: 83, 1967.

25. BELING, C.G., Acta Endocr. Suppl. 79: 1963.

26. GOEBELSMAN, U., SJOBERG, K., WIQVIST, N., and DICZFALUSY, E., Acta Endocr. 50: 261, 1965.

27. GOEBELSMAN, U., ERIKSSON, G., WIQVIST, N., and DICZFALUSY, E., Acta Endocr. 50: 273, 1965.

28. GOEBELSMAN, U., COOKE, I., WIQVIST, N., and DICZFALUSY, E., Acta Endocr. 52: 30, 1966.

29. DAHM, K. and BREUER, H., Z. Klin. Chem., 4: 153, 1966.

30. DAHM, K., LINDLAU, M. and BREUER, H., Acta Endocr. 56: 403, 1967.

31. STØA, K.F. and LEVITZ, M., Acta Endocr. 57: 657, 1968.

32. LADANY, S., Steroids 12: 717, 1968.

33. SCHOTT, E.W. and KATZMAN, P.A., Endocrinology 74: 870, 1964.

34. ADLERCREUTZ, H. and LUUKKAINEN, T., Biophys. Biochem. Acta 97: 134, 1965.

35. LAYNE, D.S., SHETH, N.A., and KIRDANI, J., J. Biol. Chem. 239: 3321, 1964.

36. LAYNE, D.S., Endocrinology, 76: 600, 1965.

37. JIRKU, H. and LEVITZ, M., J. Clin. Endocr. 29: 615, 1969.

38. ARCOS, M. and LIEBERMAN, S., Biochemistry 6: 2032, 1967.

39. WILLIAMSON, D.G., COLLINS, D.C., and LAYNE, D.G., 51st Meeting of Endocrine Soc. Abs. No. 339, N.Y. 1969.

40. TOUCHSTONE, J.C., GREEN, J.W., MCELROY, R.C., and MURAWEC, T., Biochemistry 2: 653, 1963.

41. DICZFALUSY, E., BARR, M., and LIND, J., Acta Endocr. (Copenhagen) 46: 511, 1964.

42. WILSON, R., ERIKSSON, G., and DICZFALUSY, E., Acta Endocr. (Copenhagen). 46: 525, 1964.

43. EMERMAN, S., TWOMBLY, G.H., and LEVITZ, M., J. Clin. Endocr. 27: 539, 1967.

44. STRAW, R.F., KATZMAN, P.A. and DOISY, E.A., Endocrinology 57: 87, 1955.

45. TROEN, P., NILSON, B., WIQVIST, N., and DICZFALUSY, E., Acta Endocr. 38: 361, 1961.

46. MATHUR, R.S., COMMON, R.H., COLLINS, D.C., and LAYNE, D.S., Biochem. Biophys. Acta. 176: 394, 1969.

47. LAYNE, D.S. and COLLINS, D.C., Can. J. Biochem. 46: 1089, 1968.

48. NOSE, Y. and LIPMAN, F., J. Biol. Chem. 233: 1348, 1958.

49. FELGER, C.B. and KATZMAN, P., Fed. Proc. 20: 199, 1961.

50. BONGIOVANNI, A.M. and EBERLEIN, W.R., J. Clin. Endocr. Metab. 17: 238, 1957.

51. KELLIE, A.E. and SMITH, E.R., Biochem. J. 66: 490, 1957.

52. GANDY, M. and PETERSEN, R.E., Endocrine Soc. 46th Ann. Meeting Abs. 63: 1964.

53. COHN, G.L., BONDY, P.K., and CASTIGLIONE, C., J. Clin. Endocr. Metab. 23: 671, 1961.

54. WEST, C.D., TYLER, F.H., BROWN, H., and SAMUELS, L.T., J. Clin. Endo. II: 897, 1951.

55. LEVITZ, M., Estrogen Assays in Clin. Med. (Ed. Alvin Paulsen) Washington Univ. Press. 157, 1964.

56. BROWN, C.H., SAFFAN, B.D., HOWARD, C.M., and PREEDY, J.R.K., J. Clin. Invest. 43: 295, 1964.

57. JACONO, G., BRANCACCIO, A., D'ALESSANDRO, B., and DELUCA, R., Endocrinology 69: 231, 1961.

58. DAUGHADAY, W.H., J. Clin. Invest. 35: 1428, 1956.

59. KERNEL, L., J. Clin. Invest. 35: 1428, 1956.

60. DICZFALUSY, E., CASSMER, O., ALONSO, C., and DE MIGUEL, Acta Endocr. 37: 353, 1961.

61. DICZFALUSY, E., CASSMER, O., ALONSO, C., and DE MIGUEL, Acta Endocr. 38: 31, 1961.

62. DICZFALUSY, E., TILLINGER, K.G., WIQVIST, N., LEVITZ, M., CONDON, G.P., and DANCIS, J., J. Clin. Endocr. 23: 503, 1963.

63. ALNOLT, R.J. and DEMEIO, R.H., Rev. Soc. Argt. Biol. 17: 570, 1941.

64. ALNOLT, R.J. and DEMEIO, R.H., J. Biol. Chem. 156: 577, 1944.

65. SEGAL, H.L., J. Biol. Chem. 213: 161, 1955.

66. WENGLE, B., Acta Soc. Med. Upsalien, 69: 105, 1964.

67. LIPSCHITS, W.I. and BUEDING, E., J. Biol. Chem. 126: 663, 1939.

68. OHIRAI, Y. and OHKUBA, T., Biochem. J. 41: 341, 1954.

69. COHN, G.L. and HUME, M., J. Clin. Invest. 39: 1584, 1960.

70. LUETSCHER, J.A., HANCOCK, E.W., CAMARGO, C.A., DOWDY, A.J., and NOKES, G.W., J. Clin. Endocr. 25: 628, 1965.

71. SANDOR, T. and LANTHIER, A., Acta Endocr. (Kobenhavn) 39: 87, 1962.

72. SOULAVIAC, A., DESCLAUX, P., and TEYSSEYRE, J., Ann. Endocrinol. 10: 535, 1949.

73. PHILLIPS, W.E.J., COMMON, R.H., and MAW, W.A., Can. J. Zool. 30: 201, 1952.

74. DANBY, M., Endocrinology 27: 236, 1940.

75. ZONDEK, B., Scand. Arch. Physiol. 70: 133, 1934.

76. SAMUELS, L.T., Rec. Prog. Hor. Res. 4: 65, 1949.

77. KING, R., Biochem. J. 76: 7p, 1960.

78. KING, R., Biochem. J. 79: 361, 1961.

79. RYAN, K.J., and ENGEL, L.L., Endocrinology 52: 287, 1953.

80. BALIKIAN, H., SOUTHERLAND, J., HOWARD, C.M., and PREEDY, J.R.K., Endocrinol. 82: 500, 1968.

81. CHATTERTON, R.T., CHATTERTON, J.A., and HELLMAN, L., Endocrinology 84: 1089, 1969.

82. ODELL, L.D., SKILL, D.I., and MARRIAN, G.F., J. Pharmacol. Exptl. Therap. 60: 420, 1937.

83. INONE, N., SANDBERG, A.A., GRAHAM, J.B., and SLAUNWHITE, W.R., J. Clin. Invest. 48: 380, 1969.

84. INONE, N., SANDBERG, A.A., GRAHAM, J.B., and SLAUNWHITE, W.R., J. Clin. Invest. 48: 390, 1969.

85. FISHMAN, W.H., The Enzymes. I: 1950. J. Sumner, ed. Academic Press.

86. KERR, L.M.H., and LEVVY, G.A., Nature 160: 463, 1947.

87. LEVVY, G.A., KERR, L.M.H., and CAMBELL, L.G., Biochem. J. 42: 462, 1948.

88. FISHMAN, W.H., and GREEN, S., J. Amer. Chem. Soc. 78: 880, 1956.

89. FISHMAN, W.H., and GREEN, S., J. Biol. Chem. 225: 435, 1957.

90. FISHMAN, W.H., J. Biol. Chem. 131: 225, 1939.

91. FISHMAN, W.H., J. Biol. Chem. 136: 229, 1940.

92. FISHMAN, W.H., J. Biol. Chem. 169: 716, 1947.

93. VARMA, T.N.R., BRODIE, H.J., KWASS, G., HAYANO, M., and DORFMAN, R.I., Acta Endocr. 45: 40, 1964.

94. ROY, A.B., Advances in Enzymology (Ed. F.F. Nord) 22: 205, 1960.

95. ROY, A.B., Biochem. J. 68: 519, 1958.

96. DODGSON, K.S., SPENCER, B., and THOMAS, J., Biochem. J. 53:452, 1953.

97. DODGSON, K.S., SPENCER, B., and WYNN, C.H., Biochem. J. 62: 500, 1956.

98. NEY, K.H., and LIPMANN, F., J. Biol. Chem. 233: 1348, 1958.

99. RUTENBURG, A.M., and SELIGMAN, A.M., Arch. Biochem. Biophys. 60: 198, 1956.

100. DEDUVE, C., and BERTHET, J., Intern. Rev. Cytol. 3: 225, 1954.

101. BUTENANDT, A., and HOFSTETTER, H., Z. Physiol. Chem. 259: 222, 1939.

102. DAVIS, M.E., KELSEY, F.E., FUGO, N.W., LUCKS, J.E., and VOSKUIL, P., Proc. Soc. Exptl. Biol. Med. 74: 501, 1950.

103. HANAHAN, D.J., and EVERETT, N.B., J. Biol. Chem. 185: 919, 1950.

104. HENRY, R., THEVENEL, M.,and JARRIGE, P., Bull. Soc. Chem. Biol. 34: 897, 1952.

105. SANDBERG, M., and HOLLY, O.M., J. Biol. Chem. 96: 443, 1932.

106. GIBIAN, H., and BRATFISCH, G., Z. Physiol. Chem. 305: 266, 1956.

107. ROY, A.B., Biochem. J. 66: 700, 1957.

108. RAY, A.B., Biochem. J. 62: 41, 1955.

109. LISBOA, B.P., and DICZFALUSY, E., Acta Endocr. (Copenhagen) 40: 60, 1962.

110. HOBKIRK, R., and METCALF-GIBSON, A., Standard Methods Clinical Chemistry (Ed. D. Seligson) 4: 65, 1963. (Academic Press)

111. BAULD, W.S., Biochem. J. 63: 488, 1956.

112. STADIE, W.C., and RIGGS, B.C., J. Biochem. Chem. 154: 687, 1944.

113. SPERRY, W.M., and BRAND, F.C., Proc. Soc. Exptl. Biol. Med. 42: 147, 1939.

114. ELLIOTT, K.A.C., Methods in Enzymology (ed. Colowick and Kaplan) I: 3, 1955. (Academic Press)

115. BRADLOW, L.H., Steroids II: 265, 1968.

116. EDWARDS, R.W.H., KELLIE, A.E., and WADE, A.P., Mem. Soc. Endocrinol. 2: 53, 1953.

117. EMERMAN, S., TWOMBLY, G.H., and LEVITZ, M., J. Clin. Endocr. 27: 539, 1967.

118. BUGGE, S., NILSEN, M., METCALFE-GIBSON, and HOBKIRK, R., Can. J. Biochem. 39: 1501, 1961.

119. COLLINS, D.C., and LAYNE, D.S., Can. J. Biochem. 46: 1089, 1968.

120. GIRARD, A., and SANDULESCO, G., Helv. Chem. Acta. 19: 1095, 1936.

121. GIVNER, M.L., BAULD, W.S., and VAGI, K., Biochem. J. 77: 400, 1960.

122. BAULD, W.S., and GREENWAY, R.M., Methods Biochem. Anal. 5: 337, 1957.

123. HOBKIRK, R., and NILSEN, M., J. Clin. Endocr. 26: 625, 1966.

124. HAHNEL, R., and ABDUL RAHMAN, G., Biochem. J. 105: 1047, 1967.

125. BALIKIAN, H., PREEDY, J.R.K., SOUTHERLAND, J., and HOWARD, C.M. Endocrinology 82: 500, 1968.

126. ALFSEN, A., Bull. Soc. Chim, Biol. 11: 1469, 1959.

127. SZEGO, C.M., Endocrinology, 54: 649, 1953.

128. REIGEL, I.L., and MUELLER, G.C., J. Biol. Chem. 210: 249, 1957,

129. SANDBERG, A.A., SLAUNWHITE, W.R., and ANTENIADES, H.N., Res. Progr. Hor. Res. 13: 209, 1957,

130. RYAN, K.J., and ENGEL, L.L., Endocrinology 52: 277, 1953.

131. MIGEON, C.J., LESCURE, O.L., ZINKHAM, W.H., and SIDBURY, J.B., J. Clin. Invest. 41: 2025, 1962.

132. JACOBSON, G.M., and HOCHBERG, R.B., J. Biol. Chem. 243: 2985, 1968.

133. NYMAN, M.A., GEIGER, J., GOLDZIEHER, J.W., J. Biol. Chem. 234: 16, 1959.

134. BROWN, J.B., J. Endocrinology 16: 202, 1957.

135. FISHMAN, J., BRADLOW, H.L., and GALLAGHER, T.F., J. Biol. Chem. 235: 3104, 1960.

136. BEER, C.T. and GALLAGHER, T.F., J. Biol. Chem. 214: 335, 1955.

137. BEER, C.T. and GALLAGHER, T.F., J. Biol. Chem. 214: 351, 1955.

138. TOSHIYOSHI, S., Kumamoto Daigaku Taishitsu Igaku Kenkyusho Hokoku 11: 60, 1960.