COMPARATIVE ASPECTS OF ESTROGEN DETOXIFICATION. THE CONJUGATION AND EXCRETION OF ESTROGENS IN FOUR SPECIES OF LABORATORY ANIMAL.

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TABLE OF CONTENTS

CHAPTER IV

CHAPTER V

GLOSSARY

The following compounds are referred to in this thesis by their trivial names.

Trivial Name	Compound
Estrone	Estra-1,3,5(10)-trien-3-ol-17-one
17β-Estradiol	Estra-1,3,5(10)-trien-3,17β-diol
17α-Estradiol	Estra-1,3,5(10)-trien-3,17α-diol
Estriol	Estra-1,3,5(10)-trien-3,16α, 17β-triol
16-Epiestriol	Estra-1,3,5(10)-trien-3,16β, 17β-triol
Diethylstilbestrol	3,4-Bis (p-hydroxyphenyl)-3-hexene
Estrone sulfate	Estra-1,3,5(10)-trien-3-01-17-one potassium sulfate
17β-Estradiol 17-sulfate	Estra-1,3,5(10)-trien-3,17β-diol 17-sodium sulfate
17β-Estradiol 3-sulfate	Estra-1,3,5(10)-trien-3,17β-diol 3-sodium sulfate
17β-Estradiol disulfate	Estra-1,3,5(10)-trien-3,17β-diol disodium sulfate
17β-Estradiol-3-glucuronide	Estra-1,3,5(10)-trien-3,17β-diol-3-yl-β-D-glucopyranosiduronic acid
17α-Estradiol-3-glucuronide-17-N-acetylglucosaminide	Estra-1,3,5(10)-trien-17α-yl-2'-acetamido-2'-deoxy-β-D-gluco-pyranosid-3-yl-β-D-gluco-pyranosiduronic acid
17α-Estradiol-17β-D-glucoside	Estra-1,3,5(10)-trien-3-ol-17α-yl-β-D-glucopyranosiduronic acid

Tris Buffer	Tris (hydroxymethyl) amino-methane
ATP	Adenosine-5'-triphosphate, disodium
UDPGA	Uridine-5'-diphosphoglucopyra-noside, trisodium

C H A P T E R I

I. GENERAL INTRODUCTION - MECHANISMS OF DETOXIFICATION

Detoxification mechanisms include a variety of metabolic changes. Compounds, whether endogenous or exogenous, which undergo these changes are converted into products which are usually less toxic and more rapidly excreted than their precursors. These metabolic changes consist of oxidations, reductions, hydrolyses and syntheses, or any combination thereof (1).

Conjugation may be defined as a coupling of a compound with a group to change the characteristics of that compound, usually with regard to its solubility in aqueous media. Table I shows the main conjugation reactions which occur in animals (2).

TABLE I

Common Conjugation Reactions

a) Mechanisms of Wide Species Occurrence	Conjugating Agent
Glucuronide synthesis	Glucuronic acid
Ethereal sulfate synthesis	Sulfate
Hippuric acid synthesis	Glycine
Mercapturic acid synthesis	N-Acetylcysteine
Methylation	Methyl group
Acetylation	Acetyl group
Thiocyanate synthesis	Thiol group

TABLE I

b) Mechanisms of Limited Occurrence	Conjugating Agent
Ornithuric acid (certain birds and reptiles)	Ornithine
Glutamine conjugation (man and higher apes)	Glutamine
Glucoside synthesis (insects and mollusks)	Glucose

The most common detoxification conjugations in the animal kingdom are with glucuronic and sulfuric acids (2).

Animals excrete steroids in the conjugated form. Exceptions to this are the lower aquatic animals, which possess excretory mechanisms that allow rapid removal of unchanged steroid from the body, so that conjugating mechanisms are unnecessary (3). Steroids usually undergo some degree of hydroxylation prior to conjugation (4) and are excreted in animals mainly as glucurono- or sulfoconjugates. Other steroid conjugates have, however, been found, such as phosphates (5), N-acetylglucosaminides (6) and glucosides (7).

The main effect of conjugation of a substance with glucuronic acid or sulfuric acid is to produce a strongly acidic compound which is more water-soluble at physiological pH values than the precursor. Water solubility facilitates excretion from the body via any of the excretory routes such as hepatic,

renal, salivary, or intestinal (8). Such a procedure is essential in removal of the estrogens, which are water insoluble, and little free estrogen is observed in the excretory products. An exception may be the guinea pig where kidney clearance of the free phenolic steroid is apparently high (9) (10). The rat is known to oxidize the A ring of the steroid nucleus, to form a more water soluble metabolite, which may be excreted without conjugation (11).

Boyland states that conjugation processes are an adaptation to terrestrial life (12). Fish and aquatic amphibia do not have the ability to form glucuronides and this process only occurs in higher forms of the animal kingdom. Sulfuric acid conjugation appears to be the usual form of metabolism of un-ionized compounds, including phenolic steroids, in reptiles and birds, while glucuronic acid conjugation is the predominant means of estrogen excretion in mammals.

II. GLUCURONIDE FORMATION

Dutton gives an excellent review of the mechanism of glucuronic acid conjugation and a historical approach to the elucidation of this mechanism (13). Glucuronic acid is able to form glycosides, which are termed glucuronides or glucosiduronic acids. Conjugation with steroids is of the "ether" type in which the carbon atom in position one of the glucuronic acid is joined to a hydroxyl group of the aglycone by a relatively stable glycosidic bond. A high energy intermediate is required

for this transfer of the glucuronic acid moiety to the aglycone. This intermediate is a nucleotide, uridine diphosphate glucuronic acid (UDPGA), which is in effect an active form of glucuronic acid. This transfer of the glucuronic acid from the nucleotide to the aglycone is effected by an enzyme, glucuronyl transferase (see figure I). Many aglycones are handled in this way eg. thyroxine, bilirubin, foreign drugs, and steroids. The substrates in which we are interested in this treatise are the phenolic steroid estrogens.

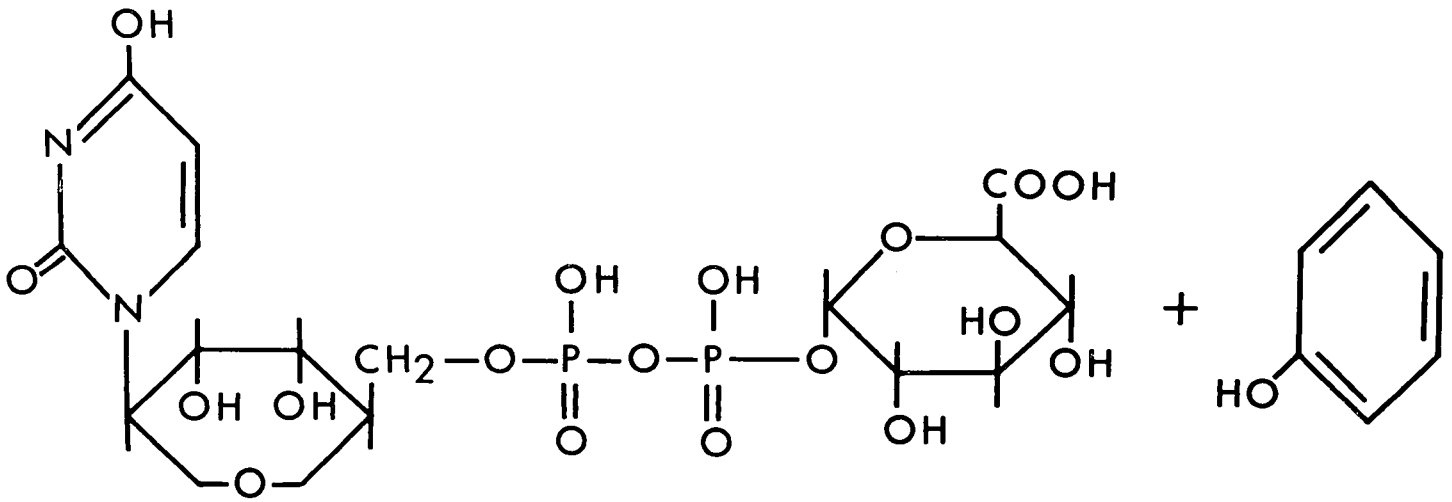
Dutton has extensively reviewed the natural sources of UDPGA and UDP-glucuronyl transferase both as to tissue distribution and species distribution (13). Liver, kidney, alimentary tract and skin, in that order, appear to be the major organs capable of conjugating steroids with glucuronic acid. The liver is by far the most active tissue.

III. SULFATE FORMATION

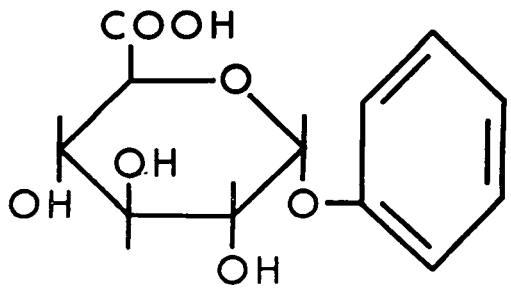
Boström et al have reviewed the mechanism of sulfate conjugation (14). The sulfate is first activated with ATP to form 3'-phosphoadenosine-5'-phosphosulfate (PAPS) in a two step reaction, and is then transferred to the hydroxyl group of the acceptor molecule. This transfer is effected by a sulfokinase (see figure II).

Sulfokinase activity appears to be of wide occurrence in animal tissues (15). It is greatest in liver, adrenals, intestinal mucosa and kidney but has been found in spleen,

FIGURE I. SCHEMATIC OUTLINE OF GLUCURONYL TRANSFER FROM UDP GLUCURONIC ACID TO AN ACCEPTOR SUBSTRATE



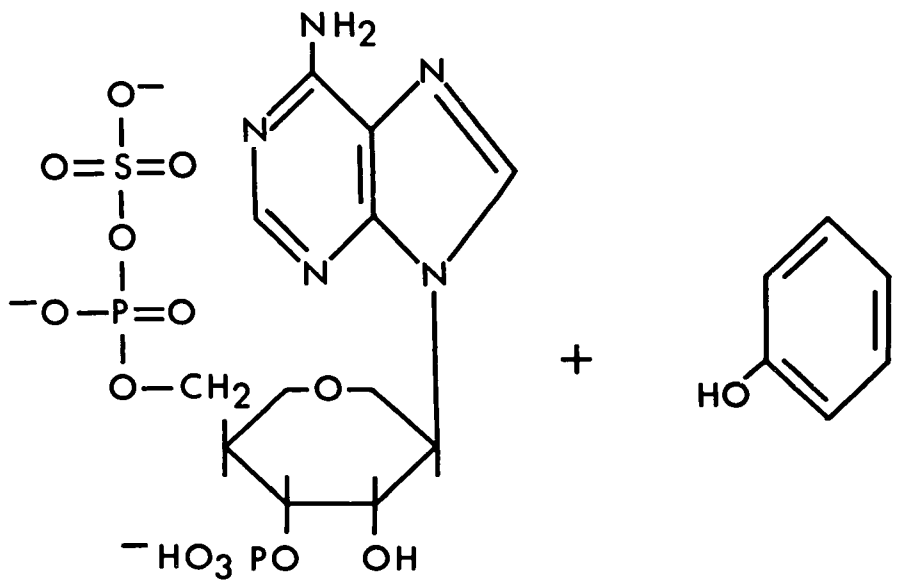
UDP GLUCURONYL TRANSFERASE



GLUCURONIDE CONJUGATE

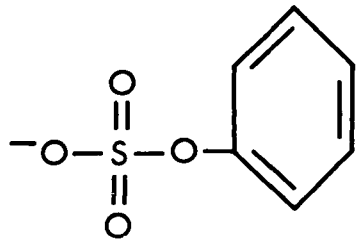
+ UDP

FIGURE II. SCHEMATIC OUTLINE OF SULFATE TRANSFER FROM PAPS TO AN ACCEPTOR SUBSTRATE



PAPS ACCEPTOR

SULFOKINASE



SULFATE CONJUGATE

+3′—PHOSPHOADENOSINE
— 5′—PHOSPHATE

heart, lung and testes. The sulfokinase in the latter tissue appears to be important in preventing feminization in boars and stallions which have a high testicular estrogen production (16). The liver, in comparison to other tissues, possesses a high capacity to conjugate steroids with sulfuric acid (17). The steroid sulfokinase appear to be a large family of enzymes with quite sharply defined substrate specificity (18).

IV. COMPARATIVE ASPECTS OF ESTROGEN DETOXIFICATION

There has been very little comparative work published on steroid conjugation among various species of the animal kingdom, and the present work was undertaken to provide information on this subject, but was limited to a study of the phenolic steroid estrogens. Many other steroids and foreign molecules possessing phenolic or alcoholic groups may undergo similar detoxification steps, but mention of these will be made only in the light of estrogen conjugation. Interest in this subject arises from the fact that such isolated work as has been done indicates that 1) different animals may employ different conjugation reactions in dealing with the estrogens, and 2) evidence that liver or other tissues from a given animal can form specific conjugates under <u>in vitro</u> conditions gives no reliable indication that these are the types of conjugates finally excreted. For example, while rabbit liver is known to make estrogen sulfates <u>in vitro</u> (18), the only estrogen conjugates excreted in rabbit urine are glucuronides, N-acetyl-glucosaminides and glucosides (6) (7). Conversely, the

urinary estrogen conjugates of the hen are entirely sulfates (19), although hen liver can readily form glucuronides in the presence of UDPGA (13). These differences between the in vitro ability to form conjugates and the type of conjugate excreted in urine or bile are particularly interesting when it is remembered that while the formation of conjugates is an important pathway in the elimination of foreign compounds as well as endogenous metabolites, the possibility exists that conjugates, in particular those of steroids, may have a biochemical role in the body. It is known that endogenous steroids are present in human blood as glucuronides (20), and in human fetal blood as sulfates (21) as well as being excreted in these forms (22). It has been postulated that the conjugates are inactive forms of steroid hormones, and may in fact be transport forms. Steroids may be carried in the blood as inactive conjugates; being released at target tissues rich in β-glucuronidase or sulfatase. The active hormone could be released locally to perform its biological effect. This would become important in the localization of an estrogen in various intercellular and intracellular compartments, thus limiting its effects and making it a specialized hormone. Although such a mechanism is possible there is no proof that it actually occurs. It is known that estrogen conjugates may be metabolized as such without removal of the acid moiety (23) (24) (25) (26). This would tend to disprove the theory that steroid conjugates

are only detoxification products. However, it is widely accepted that conjugated estrogens are biologically less active than the free form (2). Two factors which might be responsible for this difference are: 1) the effect of conjugate formation on lipid-solubility, and 2) the influence of conjugate formation on the biochemical reactivity of an active substance. The former would affect the ease with which lipid soluble compounds traverse biological membranes; the latter would alter the molecular structure and might prevent interaction with a receptor in tissues.

V. APPROACH TAKEN IN THE PRESENT WORK

Four species were used in this work; namely, the lapine, feline, cavia and avian species. The rabbit was chosen because of the extensive research previously done on this animal and because its conjugation of the estrogens resembles in some ways that found in the human. The chicken was studied because of its phylogenetic position, in closer relationship to the reptile family than to the mammalian family. The guinea pig is a typical rodent. The cat was selected because it appears to conjugate foreign molecules poorly, and has a high susceptibility to many toxicological conditions not observed in other animals.

The approach taken was 1) to obtain an *in vitro* estimate of the relative potential of tissues from each species to form estrogen glucuronides and sulfates, 2) to investigate *in vivo*

estrogen excretion patterns of each of the species, 3) to attempt to elucidate the metabolic pathways by using an isolated organ perfusion technique. This was done for three species.

C H A P T E R II

I. INTRODUCTION - IN VITRO EXPERIMENTS

The transfer of glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to phenolic steroids takes place in liver microsomal systems (27) (28) (29). The UDP glucuronyl transferase is located in the endoplasmic reticulum (13). Glucuronic acid conjugation occurs in the washed microsomal fraction if UDPGA is added. In contrast, phenol sulfokinase is located in the microsome-free supernatant of tissue homogenates (30) (31). Conjugation with sulfuric acid occurs in a system consisting of this supernatant, ATP, and magnesium and sulfate ions.

In the present work, tissue homogenates were used as the enzyme source in the glucuronic acid conjugation system and microsome-free supernatant for sulfuric acid conjugation. The reason for this was that the homogenate undoubtedly contains a vast assortment of endogenous factors, and for comparative work the use of homogenates rather than washed microsomes would give a better indication of the ability of different species to conjugate estrogen substrates. The high speed supernatant was found, in preliminary experiments, to form sulfates readily, and the possibility of endogenous glucuronide formation was thus avoided by its use in sulfation experiments.

Estrone, estradiol-17β and estradiol-17α are the major phenolic estrogens in many animals (32). These estrogens have all been found in either blood or urine of the four species used in this work, (6) (10) (19) (33) (34) (35) and were therefore used as the substrates in in vitro experiments. In addition it was of interest to investigate the metabolism of a synthetic non-steroidal estrogen for comparison. Diethylstilbestrol is a synthetic molecule which lacks the steroid nucleus but possesses three times the biological potency of the naturally occurring estrogenic hormones. The difference in structure could result in a difference in detoxification mechanism. Since diethylstilbestrol is widely used in clinical therapeutics and as a growth promoting agent in animals it was decided to use this compound as an example of a synthetic hormone.

II. REVIEW OF SPECIES EXAMINED

As mentioned earlier, the rabbit was selected as a comparative animal because of the extensive research that has been done on estrogen conjugation in this species (36). Rabbit liver slices have been shown by Crépy to conjugate estradiol with glucuronic acid (37). Smith and Breuer (29) and Isselbacher (38) showed that UDP-glucuronyl transferase was localized in the microsomal fraction. De Meio et al found that rabbit liver supernatant formed sulfates of estradiol and estrone (39). The ability of the guinea pig to form estrogen sulfates in vitro

has not previously been investigated. The laying hen excretes estrogens as sulfates and is capable of conjugating estrogens with sulfuric acid (40), but there is no definitive published work on the *in vitro* conjugation of estrogens with glucuronic acid in the avian species. Dutton has reported that developing chick liver homogenate does conjugate o-aminophenol with glucuronic acid *in vitro* (41) (42). This has also been demonstrated in the pigeon, indicating that glucuronide synthesis with some aglycones can occur in birds (43).

All adult mammals investigated possess UDP glucuronyl transferase (13), but an idiosyncrasy appears in the feline species. Hartiala demonstrated a lack of o-aminophenol conjugation with glucuronic acid in slices of cat liver (44), while Lathe *et al*, found only traces of glucuronide synthesis in a homogenate of cat liver (45). Dutton showed that these results were due to a very low level of UDP-glucuronyl transferase activity toward o-aminophenol. UDP-glucose dehydrogenase and UDP-glucuronic acid were at normal levels (46) (47). No work has been done on the ability of the cat to conjugate steroids with glucuronic acid or sulfuric acid *in vitro*.

The rabbit kidney synthesizes glucuronides from estrogens *in vitro* (29), but the kidney appears to contain much less glucuronyl transferase activity than the liver (48). This holds true for the guinea pig and the chicken, although in these animals only o-aminophenol has been utilized as a substrate

(42) (47) (49). There has been no published report of the ability of the cat kidney to conjugate steroids *in vitro* with sulfuric acid or glucuronic acid. Hartiala found only a trace of glucuronic acid conjugation of phenols by cat kidney slices (44). No attempt has previously been made to compare these differences in a quantitative manner using different species under the same experimental conditions.

The experiments which follow were designed to compare the four animal species with regard to the ability of their liver and kidney tissues to form estrogen sulfates and glucuronides under standard conditions of assay.

III. EXPERIMENTAL PROCEDURE

1. Materials

The substrates used were estrone, estradiol-17β, estradiol-17α and diethylstilbestrol. Solutions of estrone, 6,7-^{3}H (48 ci/mM) and estradiol-17β-6, 7-^{3}H (5.6 ci/mM) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. 17α-Estradiol-6, 7-^{3}H (5.6 ci/mM) was prepared as described by Layne *et al* (50). Diethylstilbestrol monoethyl-1-^{14}C (54 mci/mM) was obtained from Amersham Searle Corp., Des Plaines, Ill., U.S.A. Pure crystalline estrone, estradiol-17β, estradiol-17α and diethylstilbestrol were supplied by Mann Research Laboratories. These substrates were judged pure by thin-layer chromatography prior to use. Solutions containing 10^{-2} μmole/.225 μci were made up of each of these substrates by the addition of carrier

steroids to the labelled estrogen. ATP and UDPGA were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

2. Radioactivity Measurements

Radioactivity was measured by liquid scintillation counting in a Nuclear Chicago Unilux II spectrophotometer. Measurement procedures were those described by Arai et al (51) and by Flood et al (52). The scintillation medium was 10 ml toluene, 0.4 mg 2,5-diphenyloxazole (PPO) for non polar samples. Hydrophilic samples were counted in a medium of 9.9 ml toluene, 0.4 mg PPO and 0.1 ml Bio-Solv, BBS-3 (Beckman Instruments Inc., Cal., U.S.A.).

3. Tissue Preparation

The animals were New Zealand white rabbits, Cornish-cross hens, English short-hair guinea pigs and domestic short-hair cats. All animals were mature females, and were killed by cervical dislocation. The cats, which were first anesthetized with pentobarbital sodium, had undergone a surgical operation (bile cannulation, see chapter II of this thesis) twenty-four hours prior to death. The abdominal viscera were checked for macroscopic normality prior to each experiment.

The liver and kidneys were immediately excised following death, stripped of all adhering connective tissue, and homogenized for one minute with 4 volumes of 0.15 M potassium chloride in a Sorval tissue blender. When microsome free

supernatant was required, the homogenate was initially centrifuged at 12,000 x g for 45 minutes and finally at 105,000 x g for 60 minutes. This fractionation method was based on that of Schneider and Lewbart (31). The tissue fractions were maintained in an ice bath at all times and used immediately following preparation. It was noted that stability of the glucuronyl transferase and sulfokinase enzymes varied between species and even between individuals within a species if the preparations were subjected to frozen storage. For comparative work it was therefore essential to use only freshly prepared tissue fractions.

4. Incubation Procedure

The incubation procedure for glucuronide conjugation was that of Jirku and Layne (53) with some technical modifications, as described below. A methanol solution containing $10^{-2}\mu M$ moles (.225 μci) of estrogen was added to dry 15 ml conical incubation tubes and the methanol was removed under a nitrogen stream before addition of Tris buffer (Fisher Scientific Co., Fairlawn, N.J.,U.S.A.). Each incubation mixture was composed of 2 ml Tris buffer (18.15 mg/ml, pH 7.6), and 0.01 to 0.5 ml of homogenate, with the final volume made up to 3 ml with 0.15 M KCl. Each incubation tube contained 0.5 μmoles of UDPGA. Blank tubes were prepared as above, but without the UDPGA. The incubation procedure for sulfate formation detailed below is essentially that of Schneider and Lewbart (31) as

modified by Raud and Hobkirk (54). 10^{-2} μmole (.225 μci) estrogen was added to the dry incubation tubes, and the methanol was removed under nitrogen. The incubation medium added to each tube was 2 ml of Tris buffer (18.15 mg/ml, pH 7.6) and 0.01 to 0.5 ml of microsome-free supernatant fluid. The total volume was made up to 3 ml with a solution of $MgCl_2$ (0.013 mg/ml) and K_2SO_4 (0.008mg/ml). Each incubation tube contained 7.0 μmoles of ATP. Blank tubes were prepared as above, but without the ATP.

All incubations were performed in a water bath at 37°C for 60 minutes with continuous shaking. Each incubation was carried out in duplicate for each of two animals. Glucuronide and sulfate formation were measured simultaneously for each tissue.

Preliminary experiments were carried out in both systems prior to the recorded experiments to determine the volume of enzyme necessary to provide maximum conjugation. Neither glucuronic acid conjugation (13) nor sulfuric acid conjugation (40) is very sensitive to minor changes of pH and temperature in the conditions described above (31)(53).Raud and Hobkirk (40) have found that variation of incubation conditions in the case of the liver system did not result in any increased sulfurylation of dehydroandrosterone as compared with that of estrone and estradiol-17β. The conditions described are optimal for sulfate synthesis (40) and for glucuronide synthesis (13). The minimum enzyme concentration to conjugate 10^{-2} μmole of estrogen was determined for each species in the

present work before comparative experiments were done.

5. Product Fractionation

Following each incubation, the reaction was stopped by the addition of excess benzene in the case of the steroids and ethyl acetate in the case of diethylstilbestrol. This was because diethylstilbestrol was poorly soluble in benzene. The tubes were stoppered and shaken vigorously. The amount of radioactivity in the solvent extracts and in the aqueous layer was determined by liquid scintillation counting. The glucuronide and sulfate conjugates were not extracted from the buffer by benzene or ethyl acetate at pH 7.6 (48). The percentage of the added radioactivity which remained in the aqueous phase following solvent extraction, corrected for the value obtained with a blank sample, was used as a preliminary measure of the extent of conjugation. Very little conjugation of estrogen by the liver was observed in the absence of added UDPGA. The blanks were higher (5-20%) in the kidney incubations, probably due to absorption of the estrogen by precipitated protein, since the amounts of tissue needed for an adequate concentration of enzyme were much greater for the kidney than the liver.

The aqueous phase was adjusted to pH 2 by the addition of 0.25 ml of 1.0 N HCl. Extraction with ethyl acetate resulted in the quantitative removal of the monoconjugates but not of double conjugates (42). This partition pattern has proven to be independent of the nature of the steroid. The ethyl acetate

phase which contained the monoconjugates was removed, evaporated to dryness in a Buchi Rotavapor and examined by thin-layer chromatography. Excess ethanol was added to the aqueous phase to precipitate the protein. After centrifugation, the ethanol extract which contained the diconjugates was taken to dryness and then subjected to chromatography. To avoid the rish of solvolysis of sulfate conjugates, an alkaline pH was maintained. Solvolysis was observed to occur on ethyl acetate extraction of diethyl stilbestrol. This was minimized by maintaining alkaline conditions and examining the conjugates immediately after the incubations.

6. Characterization of Estrogen Conjugates

The characterization of the estrogen conjugates was carried out by a combination of two procedures, namely thin-layer chromatography and hydrolysis under specific conditions.

A. Thin-layer Chromatography:

Thin-layer chromatographic examination was accomplished on silica Gel H (EM Reagents Div. Brinkmann Instruments Inc., Westbury, N.Y.) in chloroform: isopropyl alcohol: formic acid, 5:3:1 (55). This system provides a means of separating monoglucuronides from both diconjugates and monosulfates (56).

The second thin-layer chromatographic solvent system used was benzene: ethyl methyl ketone: ethanol: water, 3:3:3:1

(57). This provided an excellent separation of monosulfate from both disulfate and mono- or diglucuronide. These latter three types of conjugates have the same R_F in this system.

By the use of both the above solvent systems with appropriate standards a reliable preliminary characterization of the conjugates in incubation mixtures was achieved. Estrogen and estrogen conjugates were visualized on the chromatoplates by spraying with 2% (V/V) sulfuric acid in ethanol and then heating the plates to 110°.

B. Hydrolytic Procedures:

After chromatography, further evidence for the nature of the conjugating groups was provided by the effect of specific hydrolytic procedures on the conjugates examined. These were enzyme hydrolysis and solvolytic hydrolysis.

β-glucuronidase cleaves the glucuronic acid moiety from the estrogen nucleus, and saccharo(1→4)-lactone is a specific inhibitor of this enzymatic cleavage. Ketodase (Warner-Chilcott Laboratories Co., Toronto) was used as the enzyme source. A reaction mixture of 10 ml/0.1 M sodium acetate buffer at pH 5.0, 1.0 ml of Ketodase and an aliquot of the fraction to be examined was incubated for 24 hours at 37°C. Control tubes consisted of the above plus 10 mg of saccharo (1→4)-lactone (Calbiochem Co.).

Burstein and Lieberman have shown that hydrogen sulfates can be selectively solvolysed (58), and this provides a good qualitative means of identifying sulfate conjugates. The

solvolytic procedure used was that of Burstein and Lieberman (59) as applied by Segal et al (60). The aqueous phase was brought to pH 1.0 with HCl and saturated with sodium chloride. Excess ethyl acetate was added and the mixture was incubated for 24 hours at 37°C. Burstein showed that these conditions will cleave sulfates, but do not appreciably hydrolyse steroid glucuronides (61). These findings have been verified in this laboratory using radioactive labelled conjugates.

The alkyl hydrogen sulfates are relatively stable to hydrolysis in comparison with the more labile phenolic hydrogen sulfates (58). Hydrolytic cleavage thus provides a means of differentiating between sulfate conjugates which could be formed from estradiols in these experiments, namely estradiol-3 monosulfate and estradiol-17 monosulfate. Estradiol-3, 17β disulfate can be hydrolyzed under mild conditions, namely 12 hours at pH 2.0, to form one product (62). The ultraviolet absorption and infrared spectra of this compound revealed that it was estradiol-17 monosulfate as described by Kirdani (63). Payne and Mason have obtained similar results (64). Estradiol-17 monosulfate could then be solvolysed completely under the more rigorous conditions of Burstein and Lieberman (59).

Using the above techniques, a study was carried out of the relative ability of liver and kidney tissues from the four animal species to form sulfates and glucuronides of steroid estrogens and of diethylstilbestrol. The incubation conditions

were as described. The amounts of homogenate used varied with the species, and the relative conjugate formation was expressed as μmoles of substrate conjugated per mg. of tissue.

IV. RESULTS AND DISCUSSION

The conjugate formation by the liver and kidney tissues of the four species are shown in Tables II and III and the hepatic conjugate formation is presented graphically in Figures III, IV and V. The results show that major differences exist between the species in relative ability to form conjugates (Figure V). The rabbit and chicken provide a particularly interesting contrast. In the rabbit liver, sulfate synthesis is poor, and is about two orders of magnitude lower than glucuronide synthesis under the conditions of these experiments. The chicken shows a better ability to make estrogen sulfates than glucuronides, although the two synthetic abilities are more nearly equal in this animal than in the rabbit. This result is in accord with published in vivo data which indicate that the rabbit excretes no estrogen sulfates in the urine (6), while the urinary products in the chicken are predominantly, if not entirely, sulfates (19).

The guinea pig liver has a high capacity to form both sulfate and glucuronide conjugates of the estrogens examined. Sa'at and Slaunwhite (65) have shown the rabbit and guinea pig are superior to the mouse and human in their ability to conjugate estrone and estradiol with glucuronic acid. This advantage

TABLE II

The ability of liver preparations of various species to form estrogen sulfates and glucuronides. Conditions of assay as recorded in text. Results are 10^5 x μmoles of substrate conjugated per mg. of wet tissue.

	SULFATE SYNTHESIS				GLUCURONIDE SYNTHESIS			
Substrates	Rabbit	Chicken	Guinea Pig	Cat	Rabbit	Chicken	Guinea Pig	Cat
Estrone	0.87	48.2	79	39.8	400	44.0	233	n
17β-Estradiol	3.29	91.8	227	46.9	428	8.3	549	n
17α-Estradiol	6.91	96.7	14.9	42.5	479	5.2	406	n
Diethylstilbestrol	2.49	97.1	198	19.4	459	17.8	252	5.6

n - no detectable conjugate formation (level of detection 2.0×10^{-6} μmole/mg wet tissue).

TABLE III

The ability of kidney preparations of various species to form estrogen sulfates and glucuronides. Conditions of assay as recorded in text. Results are 10^6 x μmoles of substrate conjugated per mg. of wet tissue.

	SULFATE SYNTHESIS				GLUCURONIDE SYNTHESIS			
Substrates	Rabbit	Chicken	Guinea Pig	Cat	Rabbit	Chicken	Guinea Pig	Cat
Estrone	0.51	*	3.3	n	n	*	n	n
17β-Estradiol	*	*	46.5	13.3	*	*	5.8	n
17α-Estradiol	0.29	3.3	8.7	6.4	0.23	1.04	9.2	n
Diethylstilbestrol	3.9	0.35	1.2	16.2	22.0	0.18	1.2	n

n - no conjugate detected (less than 2.0×10^{-7} μmole/mg.)

* - sample not done.

FIGURE III. GLUCURONIDE SYNTHESIS OF ESTROGENS BY LIVER PREPARATIONS IN VARIOUS SPECIES. RESULTS ARE μ MOLES OF SUBSTRATE CONJUGATED PER MG OF WET TISSUE.

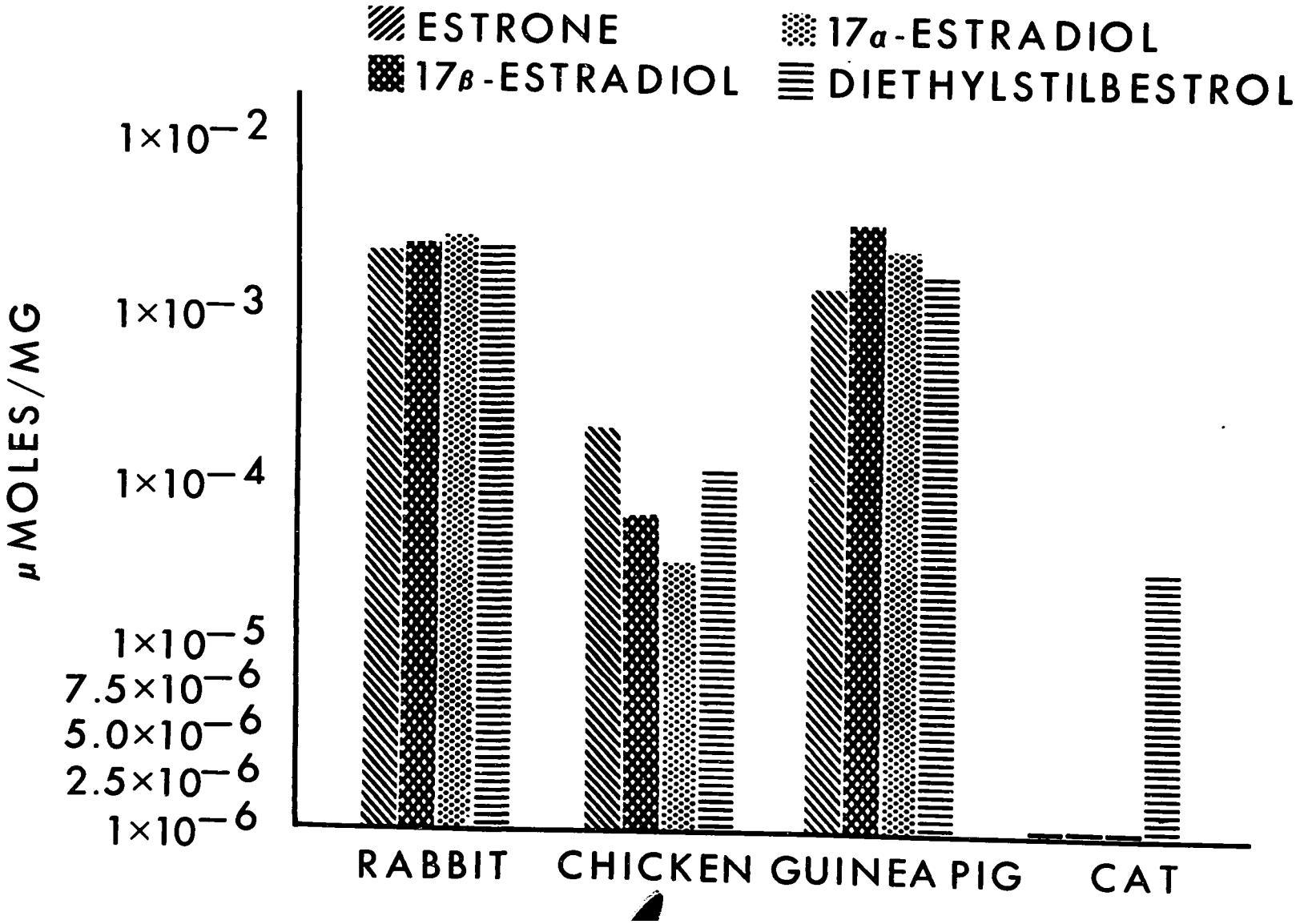


FIGURE IV. SULFATE SYNTHESIS OF ESTROGENS BY LIVER PREPARATIONS IN VARIOUS SPECIES. RESULTS ARE μ MOLES OF SUBSTRATE CONJUGATED PER MG OF WET TISSUE.

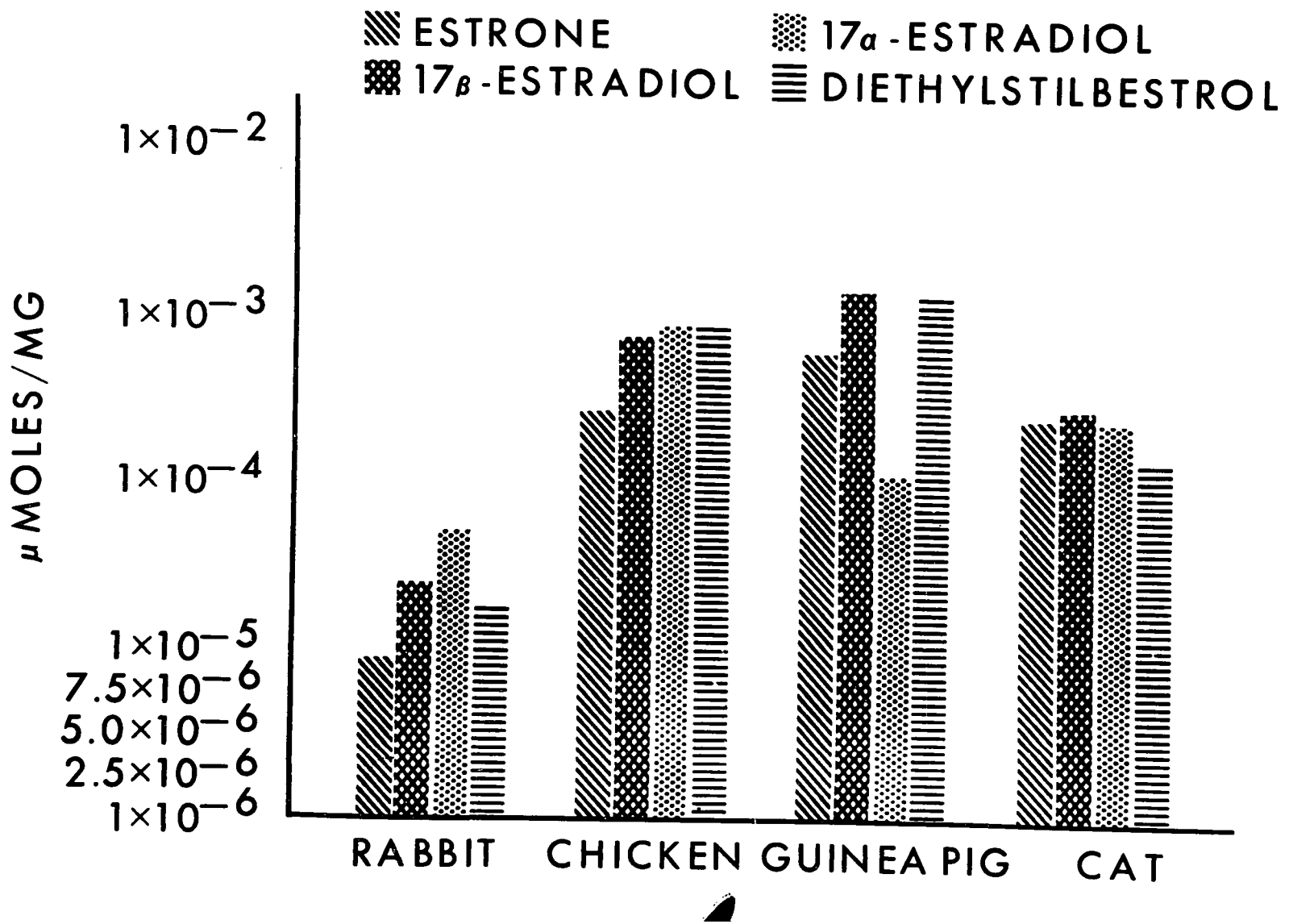
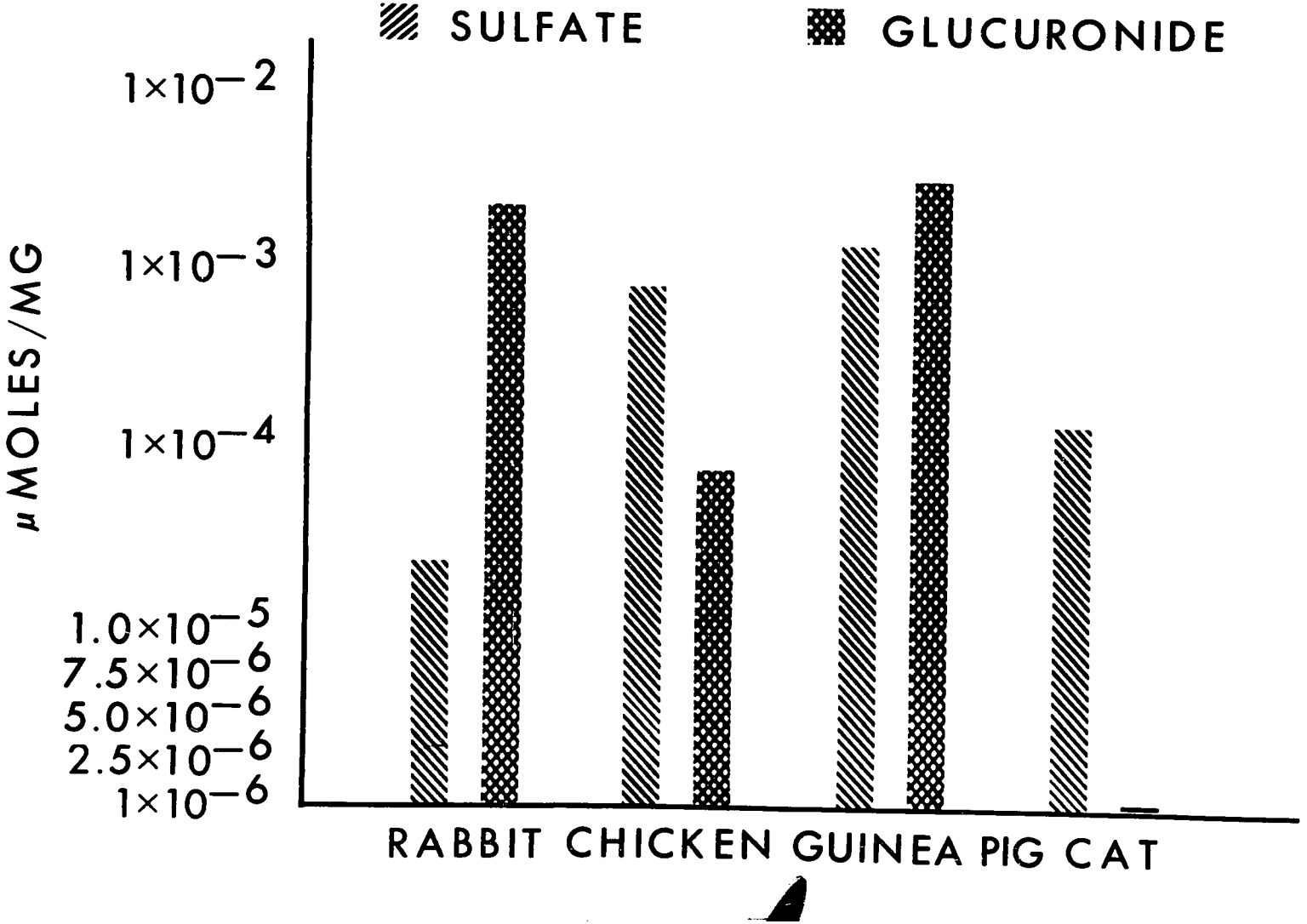


FIGURE V. COMPARISON OF SULPHATE AND GLUCURONIDE SYNTHESIS OF A REPRESENTATIVE ESTROGEN, 17 β - ESTRADIOL, BY LIVER PREPARATIONS IN VARIOUS SPECIES. RESULTS ARE μ MOLES OF SUBSTRATE CONJUGATED PER MG OF WET TISSUE.



was approximately four-fold. These results are comparable with those given here, but constant assay conditions were not used in each case in the previous work (65). From Table II, it might be expected that the mouse and human possess a high capability to detoxify steroids via sulfate formation.

The feline species, in contrast, appears to be deficient in both detoxification mechanisms but less so in the ability to form sulfates. Although no glucuronic acid conjugation was found at the present enzyme levels, traces of glucuronide formation have been reported with nonsteroid substrates (37) (46). The cat appears to possess only the sulfate mechanism for detoxification; and it is measureably lower than either the chicken or guinea pig. This low ability of the cat to form conjugates may partially explain the known sensitivity of this animal to various toxicological conditions.

Some minor differences in the ability of a given species to conjugate various substrates can be discerned in the results in Tables I and II. Estradiol appears to be more readily conjugated with both sulfuric acid and glucuronic acid than is estrone in the rabbit. The guinea pig sulfates 17α-estradiol less readily than estrone or the 17β isomer of estradiol. Both the liver and kidney tissues of the rabbit and the chicken sulfated 17α-estradiol to a greater extent than the 17β epimer. This is in accord with the investigations of Wengle and Boström in rat liver (66). In contrast, the guinea pig appears to conjugate 17β-estradiol more readily in both tissues.

The synthetic estrogen diethylstilbestrol is conjugated to about the same extent as the natural steroidal compounds, with the exception that the cat appears to form stilbestrol glucuronide but not phenolic steroid glucuronides.

No chromatographic or partition evidence for the formation of diglucuronide was observed in these experiments for any of the species examined. This confirms the results of Jirku and Layne (53) who reported that rabbit liver homogenate formed principally estradiol 17β-3-glucuronide when it was incubated with estradiol-17β and UDPGA for 1 hour.

Disulfate formation of all compounds was always extremely small. This was in accord with Wengle and Bostrom (66). Species differences were observed, however, in the ability of the different animals to form disulfates. The rabbit, guinea pig and chicken had ratios of approximately, 6:1, monosulfate to disulfate, for the four estrogens examined, as judged by thin-layer chromatography. The monosulfate appeared to be the phenolic 3-sulfate. This is in contrast to the results obtained in the cat. Only monosulfate was observed and this appeared to be the alkyl or 17-sulfate. The cat formed a trace of disulfate of diethylstilbestrol in the kidney preparation. If the observations that the alkyl position is more favorable for sulfate conjugation, are accurate one would expect that diethylstilbestrol would be poorly conjugated with sulfuric acid relative to the steroids. This was in fact our experimental result (see figure IV) and is interesting for two reasons. 1) The inter-conversion of estrone to estradiol

does not seem to be the rate limiting step in diconjugate formation, because estrone would demonstrate different mono to diconjugate ratios than does estradiol. 2) The cat appears to attach the sulfate group first to the 17-hydroxyl position, then to the 3-hydroxyl position. The opposite is true for the rest of the animal species. The observation that the 3-OH position is the most favorable one for sulfate formation is a common one for many substrates (31) (40) (64) (67) (68). The idea that the cat contains a different sulfokinase enzyme than other animals is an appealing one from the data obtained.

The limited experiments on kidney tissue show that although the conjugate synthesizing ability of the kidney is much lower than that of the liver in all the species examined, no real qualitative differences in conjugating pattern between the two tissues is detectable under these <u>in vitro</u> conditions.

V. SUMMARY

1. A comparison of phenolic steroid sulfokinase and glucuronyl transferase activity in four selected species was carried out using a standard assay procedure.

2. The rabbit was found to have high glucuronyl transferase activity but a low sulfokinase activity towards phenolic steroids and diethylstilbestrol.

3. The chicken possesses a high sulfokinase activity but a low glucuronyl transferase activity.

4. In the guinea pig both sulfokinase and glucuronyl transferase activity were high, and of roughly the same level.

5. The cat showed no ability to conjugate phenolic steroids with glucuronic acid and a relatively poor ability to conjugate stilbestrol. Sulfate synthesis in this animal, while appreciable, was of a low order compared to the chicken or guinea pig.

6. The liver is a much better source of conjugating enzymes than is the kidney in all species examined.

C H A P T E R III

I. INTRODUCTION - IN VIVO EXPERIMENTS

The in vivo excretion of estrogen metabolites occurs principally in bile and urine. Other excretory routes such as the mammary, salivary, gastro-intestinal and sebaceous secretions, and the expired air, have not been examined completely, but they appear to be very minor pathways for conjugated steroids (69).

The biliary excretion of steroids permits their metabolism in the intestine, from which they may either be reabsorbed or eliminated in the feces. The reabsorbed estrogen may be recycled through the intestine or excreted in the urine by the kidney.

II. REVIEW OF SPECIES EXAMINED

The excretory patterns of the estrogenic hormones differ among animal species. The intact rabbit and guinea pig excrete the majority of the natural estrogens in the urine (70) (71). The guinea pig excretes estrogens in the free form and as glucuronide conjugates (72) (73) (74) (75). The aglycones are mainly estradiol-17β with estrone as a minor metabolite. In the rabbit, estrogen metabolites are excreted in the urine as a diglycoside, estradiol-3-glucuronide-17α-N-acetyl-glucosaminide (6) (76) (77). The biliary excretion products have not been described.

Estrogen conjugation and urinary excretion by the domestic hen has been well documented by Mathur et al (19). This animal, when injected with radioactive 17β-estradiol, excreted approximately 32% of the radioactivity in the urine and 51% in the feces. The estrogen metabolites were exclusively monosulfate and disulfate conjugates, in a ratio of 6:1. The aglycones were estradiol-17β and estradiol-17α with minor amounts of estrone, 16-epiestriol and estriol. Hopwood and Gassner have described the retention of diethylstilbestrol by various tissues in the chicken, but diethylstilbestrol conjugates have not been fully investigated in this species (78) (79). The biliary excretion products of the natural estrogens and stilbestrol have not been described in the chicken, although this appears to be quantitatively the more important pathway.

Taylor et al have reported that anesthetized cats injected with steroids excrete the metabolites almost completely in the bile (80) (81) (82). The conjugates of the phenolic steroids appear to be sulfates, although 30% of the conjugated material was unidentified (82). The experiment described below was done prior to the above publication, and all the estrogen conjugates have been identified.

In the present thesis estrogen metabolites were examined in both the urine and feces of the guinea pig. The detoxification of the synthetic estrogen, diethylstilbestrol, and of

the natural phenolic steroids were examined in the hen. The estrogen conjugates in the urine and bile were compared in both the rabbit and the cat.

III. EXPERIMENTAL PROCEDURE

1. Materials and Methods

Radioactive estrogens were those listed in Chapter II.

Two mature, virgin female rabbits were used. The animals were maintained in metabolic cages on an *ad libitum* diet of commercial rabbit cubes, lettuce and a bile salt replacement solution (83). The rabbits were anesthetized with pentobarbital sodium. Gall bladder intubation and bile collection were carried out as described by Boegli *et al* (83). The rabbits were allowed to recover for 18 hours following the surgical procedure. Each rabbit was then injected intravenously with 22.8 μci of 17β-estradiol-6, 7-^{3}H (specific activity 5.6 ci/mM) dissolved in 0.25 ml ethanol and 1.0 ml saline. Bile and urine samples were collected at six hour intervals, diluted with water, assayed for radioactivity, and immediately frozen.

Two female domestic cats were placed in metabolic cages and maintained on a diet of commercial cat food and bile salt replacement solution (83). The cats were starved for 24 hours prior to the surgical operation. The bile duct was cannulated with Intramedic PE 190 tubing (Becton Dickson Co., N.J., U.S.A.). This cannula was connected to a 100 ml Nalgene

bottle, taped to the back of the cat. After recovery from anesthesia, each cat was injected in the femoral vein with 9.7 μci of estrone-6, 7-^{3}H (specific activity 48.0 ci/mM), dissolved in 0.25 ml ethanol and 5 ml saline. Bile and urine samples were collected at 6 hour intervals, and were handled as described for the rabbits. The bile samples were analysed individually, but the urine from the two cats was pooled and analysed as a single sample.

Mature, female guinea pigs were maintained in separate metabolic cages, and were fed a commercial guinea pig ration, lettuce and water, ad libitum. Guinea pigs were injected intracardially, intraperitoneally or subcutaneously with 3.5 μci of estrone-6, 7-^{3}H (specific activity 48.0 ci/mM). Urine and feces were collected over 12 hour intervals. These were handled by the procedure described above for rabbit excreta.

Four mature Cornish cross hens were used. Since hen urine and bile collections involved two separate surgical operations, it was decided to obtain bile and urine from separate birds.

A procedure similar to that used for bile collection in the rabbit was followed. Two laying hens were anesthetized with pentobarbital sodium. A four inch incision parallel to the keel and extending dorsally from the ventral midline to the posterior ribs on the right side was made. The abdominal cavity was opened and the two bile ducts were located and ligated. The ducts were more easily located if the bird was

starved for 24 hours prior to the operation. The gall bladder was intubated with PE 190 polyethylene tubing (Becton Dickson Co., N.J., U.S.A.), which was led out of the ventral incision to a Nalgene bottle taped to the side of the bird. The abdominal incision was closed and the bird was allowed to recover from anesthesia. The chickens were maintained in a restraint box which prevented access to the surgical site, and had free access to a commercial poultry layer mash and bile salt replacement solution (83).

Each bird was injected intravenously with a mixture of 2.3 μci of diethylstilbestrol (monoethyl-1-^{14}C) (specific activity 54 mci/mM), and 10.0 μci estrone-6, 7-^{3}H (specific activity 48.0 ci/mM). The injection vehicle was 0.25 ml ethanol and 1.0 ml saline. Bile was collected over 6 hour intervals, and the individual collections were handled as described above for the rabbit.

Two hens were used for urine and feces collections. Since it was desirable to obtain uncontaminated urine and feces, it was necessary to surgically separate the ureteral openings from the rectum. The ureters were transposed from the cloaca to a position dorsal to the vent on the pygostyle. The surgical procedure followed was similar to that of Ainsworth (84), except for the following modifications. A purse string suture was put in to maintain the integrity of the mucosal-skin junction and a vertical mattress suture pattern was used to avoid everted skin. The urine was collected

by means of a finger cot clipped to the skin, surrounding the ureteral openings, by the use of Michel clips. The end of the finger cot was cut off and a small polyethylene bag was attached to the finger cot by Michel clips. This was a very convenient procedure for collection of large volumes of urine and for replacing the collection bag. The finger cot was maintained in place throughout the experiment and proved very reliable. Obstruction by high concentrations of urates, which is characteristic of avian urine during periods of low urine flow, was prevented by regular flushing with water. The clipped skin was regularly anesthetized locally with subcutaneous injections of Xylocaine (Xylocaine HCl, 2%, Astra Pharmaceutical Company, Mississauga, Ontario).

The chickens were maintained in a restraint box which prevented turning, thus they were not allowed access to the surgical operation site or excreta collection. The diet was a commercial poultry layer mash (15% protein). The hens had free access to this mash and water supply and they were also force fed, with an esophageal tube twice a day to ensure adequate feed and water intake. The chickens were maintained this way for two weeks to allow surgical healing and to accustom them to the environmental conditions. The hens were laying up to the time of the experiment at which time one hen stopped and the other continued to lay until the experiments were near completion.

Each chicken was used for two experiments. One week was

allowed between these experiments to insure complete elimination of the radioactive tracer. The surgically transposed ureteral area of one of the birds had contracted previous to the second experiment. This made collection of the feces, without urine contamination, difficult, and it was decided to discontinue the experiment with this bird.

Each hen was injected by means of the median vein with 15.6 μci (specific activity 54 mci/mM) of diethylstilbestrol (monoethyl-1-^{14}C). The vehicle was 0.25 ml ethanol in 1.0 ml saline. No hematoma was formed on withdrawal of the needle, and there was no loss of steroid to the subcutaneous tissue.

Urine and feces collections were obtained at 6 hour intervals. The ureteral area was carefully flushed with distilled water at the time of collections to prevent accumulation of solid urate material. The urine sample was assayed for radioactivity by counting a 0.1 ml aliquot, and the remaining volume was frozen immediately. The fecal samples were frozen at each collection interval and maintained at -20°C.

At the end of all experiments the collection devices, restraint boxes, and metabolic cages were carefully washed with distilled water and the washings were added to the respective samples.

The urine samples for each animal were pooled. Each of the fecal samples was homogenized in a Sorval Omnimix (Ivan Sorvall Inc.) in four volumes of methanol per gram of wet weight.

The radioactivity in a 0.1 ml aliquot of each methanol extract was counted, and the extracts were then pooled, evaporated to dryness at 40^{o}, and taken up in water.

2. Fractionation Procedure

The individual collections of urine, feces and bile described above were each processed as follows. The aqueous fraction was applied to a column of Amberlite XAD-2 resin (Rohm and Haas, Toronto). The procedure of preparation and elution of the column were as described by Bradlow (85). The methanol eluate contained essentially all of the total radioactivity applied to the column. This processing of the samples on the Amberlite column produced a preliminary separation of estrogens and estrogen conjugates in a methanol solution, considerably purified from salts and pigments. The procedure was particularly useful for the heavily pigmented bile fractions.

An aliquot of each of the methanol eluates was subjected to solvent partitions as shown schematically in Figure VI. The methanol eluate was taken to dryness in a Rotovapor (Buchi) and dissolved in water. The pH of these solutions was between 5 and 8, and two extractions with equal volumes of benzene were performed to remove the steroid present in the free form, which was quantitated by counting the radioactivity in the benzene extract.

Figure VI. Procedure for fractionation and characterization of estrogen metabolites in excreta of the rabbit, guinea pig, cat and chicken. See text for details.

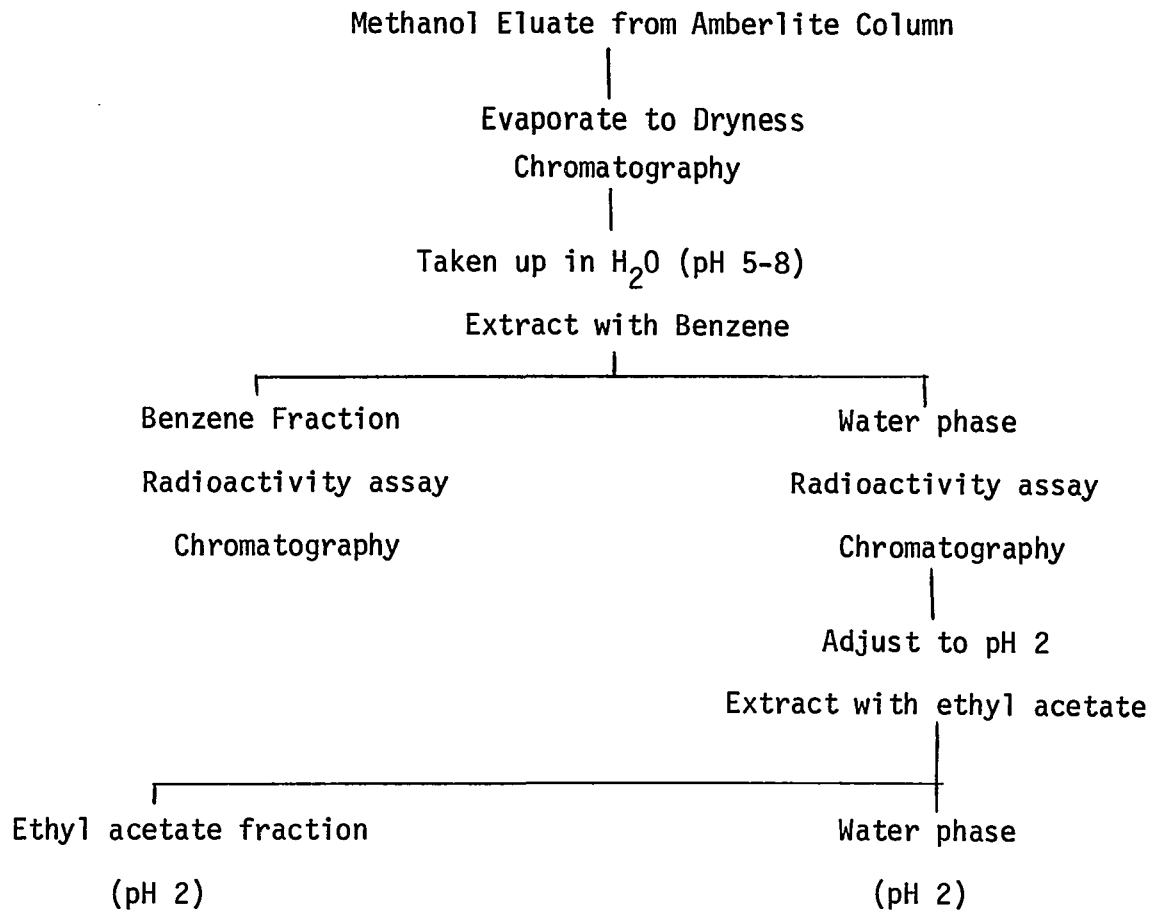
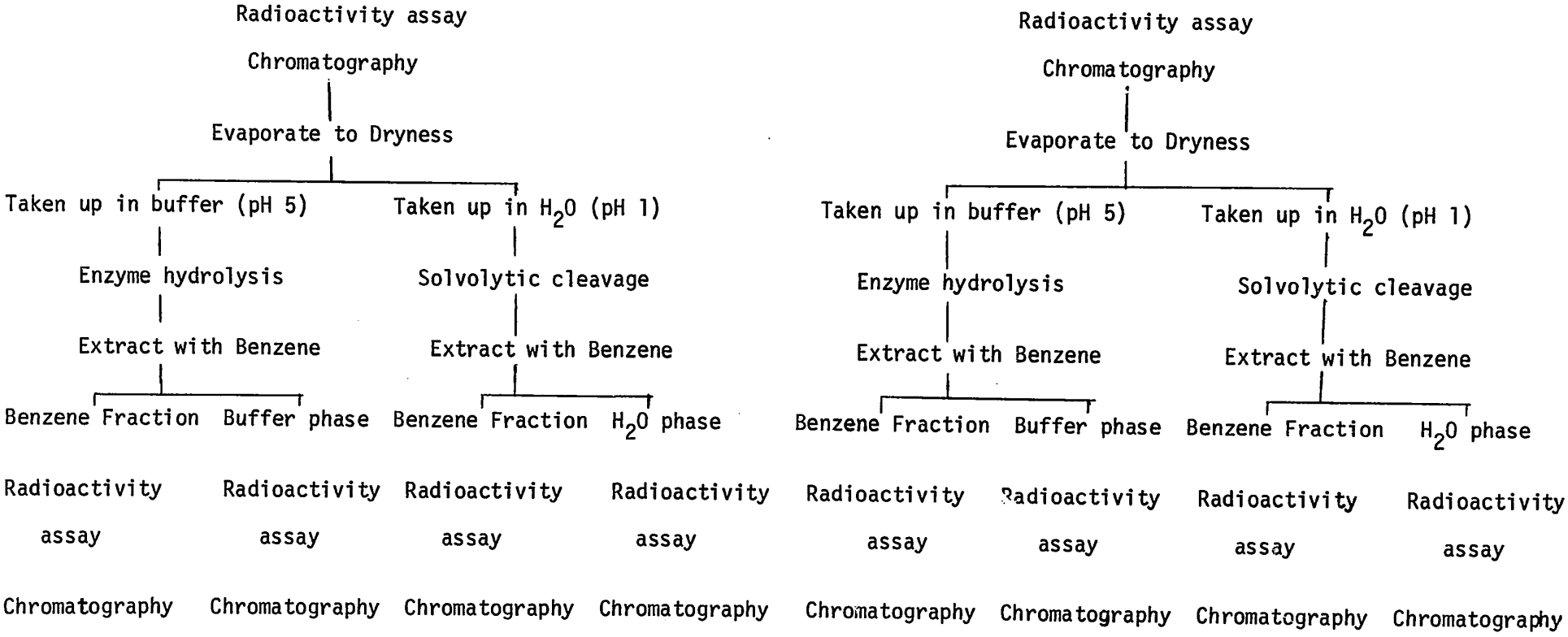


Figure VI. (continued)



The water phase was then adjusted to pH 2.0 with 1.0 N HCl, and extracted twice with equal volumes of ethyl acetate. This partition removed the monosulfate and monoglucuronide conjugates. The radioactivity in this phase was determined, and the nature of the radioactive conjugates was investigated chromatographically. The fraction was then subjected to hydrolytic procedures followed by solvent extraction, radioactivity assay and chromatography to characterize and quantitate the steroid aglycones.

The water phase (pH 2.0) was taken to dryness on the rotary evaporator with repeated additions of ethanol to aid the evaporation. Hydrolytic procedures to cleave the diconjugates were then carried out, followed by extraction and chromatographic examination of the aglycones.

The above procedure has proven to give a reliable estimation of free and conjugated estrogen and was used routinely as a preliminary estimate of the type and extent of conjugation which took place in these experiments (48).

Minor modifications of this typical flow pattern (figure VI) were used depending on the results of preliminary examination. When sulfate and glucuronide conjugates were present, an estimate of the proportion of sulfate to glucuronide conjugation was accomplished by initially submitting the conjugate fraction to solvolysis and then separating the free estrogens which arose from cleavage of sulfates from the glucuronides. This was carried out on both the mono and diconjugate fractions.

3. Assay of Radioactivity

Radioactivity was determined as described in Chapter II. All samples were counted with an efficiency greater than 40%. The bile samples were first diluted with water to minimize the chromagen error and then counted in duplicate, at intervals twenty-four hours apart. The doubly labelled tracers in the chicken bile samples were counted by the use of two channels and a barium external standard.

4. Chromatography

Thin-layer chromatography was carried out on Silica Gel H (Merck). The solvent systems used were:

1) Benzene: ethyl methyl ketone: ethanol: water, 3:3:3:1 (57).
2) Chloroform: isopropanol: formic acid, 5:3:1 (55).
3) Chloroform: ethanol, 7:3 (56).
4) Benzene: ethyl acetate, 7:3 (56).

Appropriate reference standards were used in all systems as described by Collins and Layne (56).

5. Hydrolytic Procedures

Solvolytic cleavage and glucuronidase hydrolysis were carried out as described in Chapter II. The presence of N-acetyl glucosaminide conjugates was confirmed by enzyme hydrolysis using bovine testicular hyaluronidase (86). The sample to be tested was dissolved in 10 ml of 0.1M sodium

citrate buffer at pH 4.3, and incubated at 37°C with 1 mg of bovine testicular hyaluronidase (Sigma, Type IV). Extraction of the aglycone and subsequent chromatographic identification was carried out as described for glucuronidase hydrolysis.

IV. RESULTS AND DISCUSSION

Table IV shows the excretion pattern of radioactivity in the bile urine and feces of the various species following the injection of radioactive estrogens. Although there are considerable differences in excretion rate, in all cases a large amount of the radioactivity had been excreted within 12 hours. This is in accord with many previous observations to the effect that estrogens are rapidly metabolized in the body. Bile collection experiments were not carried beyond 48 hours because it was not possible to do this humanely.

Velle (87) has pointed out that the route of excretion of steroids appears to be independent of the phylogenetic position of the animal, the endogenous steroids present in the body, the conjugate type and the conjugating tissue. In general it may be stated that if the liver forms the conjugate, it may be excreted by the biliary route or put into the blood for renal excretion. If other organs form the conjugate, it is entirely cleared by the kidney. Since the liver is the major site of conjugation, hepatic cell function would seem to determine whether the conjugated estrogen is placed in the bile or back into the blood circulation. These speculations

TABLE IV

Excretion of radioactivity in the chicken, rabbit, guinea pig and cat following injection of ^{14}C-diethylstilbestrol, ^{3}H-estrone and/or 17β-estradiol-^{3}H as shown. All figures are average percentages of total injected estrogen of two animals except chicken urine and feces which are average of three birds.

Time	Chicken		Chicken		Rabbit		Guinea Pig		Cat	
	Urine	Feces	Bile	Bile	Urine	Bile	Urine	Feces	Urine	Bile
Estrogen Injected	Diethylstil-bestrol		Estrone	Diethylstil-bestrol	17β-Estradiol		Estrone		Estrone	
6	10.3	9.6	-	-	-	19.3	-	-	-	-
12	0.6	14.5	42.8	39.8	70.8	2.6	53.8	-	-	-
24	0.7	7.6	5.8	4.3	0.8	1.3	3.4	5.8	1.2	64.1
36	0.6	3.2	-	-	-	0.1	-	-	-	-
48	0.4	6.9	-	-	-	-	-	-	-	-
60	0.5	2.8	-	-	-	-	-	-	-	-
72	0.3	5.3	-	-	-	-	-	-	-	-
84	0.2	0.4	-	-	-	-	-	-	-	-
Total	13.6	50.3	48.6	44.1	71.6	23.3	57.2	5.8	1.2	64.1

-- samples not taken

are in accord with the results of the present work. The rabbit and guinea pig excrete a major portion of the estrogens via the urine and a small amount in the bile. This is in contrast to the cat which excretes estrogens almost exclusively via the biliary route.

In the chicken, a larger proportion of the diethylstilbestrol was excreted in the bile (44%) or in the feces (50%) than by the renal pathway in the urine (14%). This ratio (3/1) is similar to that found for the excretion of steroid estrogens in this animal (19) (88) (89).

The numerical results of estrogen metabolite excretion are presented in Tables V and VI.

The proportions of free and conjugated estrogen in the material from the two excretory routes of the hen are shown in Table V. The bile sample contained about 5% of the labelled estrogen in the free form while 95% was conjugated. This is in contrast to the fecal samples in which the ratio of free to conjugated diethylstilbestrol was approximately one to one. This could be explained by conjugate breakdown by bacterial or endogenous glucuronidase and sulfatase found in the intestinal environment (90) (91) (92) (93).

Solvolytic cleavage of the estrogen conjugate isolated from hen excreta released 40-50% of the radioactivity. Thus the glucuronic acid excretory mechanism is important in diethylstilbestrol excretion in the chicken <u>in vivo</u>, despite the fact that, under the <u>in vitro</u> assay conditions used in Chapter II there is more

TABLE V

Comparative estrogen conjugates found in the excretory products of the four selected species. ^{14}C-Diethylstilbestrol, ^{3}H-estrone and/or 17β-estradiol-^{3}H was injected as shown. All figures are average percentages of total tracer recovered from the separate excretory routes for two animals except urine and feces of the chicken which are averages of three birds.

Conjugate	Chicken Urine	Chicken Feces	Chicken Bile	Chicken Bile	Rabbit Urine	Rabbit Bile	Guinea Pig Urine	Cat Urine	Cat Bile
	Diethylstil-bestrol		Estrone	Diethylstil-bestrol	17β-Estradiol		Estrone	Estrone	
Free Estrogen	10.0	50.0	2.5	7.9	-	0.9	82.8	2.8	19
Monoconjugate	90.0	50.0	62.6	71.7	-	16.8	16.2	16.8	40.4
Monosulfate	32.8	16.7	15.1	52.5	-	0.0	-	16.8	35.4
Monoglucuronide	58.2	33.3	42.5	19.2	-	16.8	16.2	-	5.0
Diconjugate	Trace*	Trace*	34.9	20.4	100.0	82.3	1.0	80.4	40.6
Disulfate	-	-	*	*	-	-	-	75.6	40.6
Diglucuronide	-	-	*	*	-	-	1.0	-	-
Sulfate-glucuronide	Trace*	Trace*	*	*	-	-	-	4.8	Trace*
Estradiol-3-glucuronide-17-N-acetyl-glucosaminide	-	-	-	-	100.0	82.3	-	-	-

* Located on thin-layer chromatography and proven by hydrolysis.

TABLE VI

Estrogen aglycones of the major monoconjugate fraction of the four selected species. All figures are percentages of total monoconjugate fraction.

Aglycone	Chicken Bile	Rabbit Urine	Rabbit Bile	Guinea Pig Urine	Guinea Pig Feces	Cat Urine	Cat Bile
Estrogen injected	Estrone	17β-Estradiol		Estrone		Estrone	
Estrone	18	-	-	83*		41	41
17α-Estradiol	-	-	50	-		-	-
17β-Estradiol	63	-	50	17*		59	59
Estriol	19	-	-	-		-	-

* Ratios varied with route of tracer administration.

sulfokinase activity in both liver or kidney than there is glucuronyl transferase activity (see page 23).

The hen is capable of excreting the natural estrogens and the synthetic diethylstilbestrol as a glucuronide and sulfate conjugate in the bile. The feces were not examined for steroid glucuronides in these experiments. Mathur et al (19) have done an extensive study on excretion of metabolites of estradiol-17β by the chicken, and they found no evidence for the presence of glucuronides in the urine. The present work indicates that glucuronide conjugation of diethylstilbestrol is a significant excretory pathway. Thus it must be concluded that the synthetic estrogen diethylstilbestrol is cleared by the kidney in a different manner than is the naturally occurring phenolic steroid, estradiol-17β, and its derivatives.

The data presented here verify the preliminary observation of Hopwood et al (78) (79) who presented very tentative evidence that glucuronic acid conjugation of diethylstilbestrol occurs in the chicken.

The rabbit excretes estrogens mainly in the urine, and almost exclusively as a diglycoside, estradiol-3-glucuronide-17α-N-acetylglucosaminide (48). Very small amounts of estradiol-3-glucuronide-17β-D glucopyranoside have been isolated from rabbit urine (7). The estrogen conjugates in the bile are similar to those found in the urine, except that the bile appears to contain a small amount of monoconjugate. This

monoconjugate fraction consists of the 3-glucuronides of estrone, 17α-estradiol, and 17β-estradiol.

The guinea pig excretes estrone in the urine, largely in the free form. The estrogen metabolites identified were estrone glucuronide and 17β-estradiol-glucuronide. The extent of conjugation appears to depend on the route of administration of the estrogen. Successively less free estrogen is obtained in the urine when injection is intravenous, subcutaneous or intraperitoneal. This indicates that the guinea pig kidney has the ability to excrete free or conjugated estrogens and the amounts reaching the kidney depend on the extent of estrogen circulation through the liver. Thus intracardial injection of estrone leads to the excretion of 80% of the urinary estrone in the free form. Subcutaneous injection produces 37% and intraperitoneal 18% in the free form. In these cases the steroid is presumably absorbed more slowly, and undergoes more liver metabolism before being presented to the kidney. The guinea pig thus possesses a considerable ability to excrete free estrogens by renal clearance, in contrast to other animals which require the estrogens to be first conjugated with a polar group before elimination.

The cat excretes estrogens almost exclusively in the bile (see Table IV). The biliary products are estrone sulfate, estradiol-17β-sulfate and estradiol-3, 17β-disulfate. A small amount of glucuronide was observed in the cat bile by thin-layer chromatography and this result was confirmed by

β-glucuronidase hydrolysis. The cat urine contained approximately the same proportions of estrogen sulfate to glucuronide conjugates as did the bile. These results agree with those reported by Karim and Taylor (82).

Oehme and Davis (94) have found that cats are susceptible to phenol toxicity, which they attributed to lack of conjugate formation, caused principally by glucuronyl transferase deficiency, which would lead to slow excretion of the phenol. Our experiments provide no evidence that the cat had a slower rate of estrogen excretion than did the other animals. It was observed that the monosulfate fraction of the urine consisted mainly of estradiol-17β-sulfate. The preference for the alkyl hydroxyl group over the phenolic hydroxyl may well account for the difference in estrogen and phenol excretion.

The cat excretes approximately 98% of the recovered radioactivity in the bile and essentially none is reabsorbed. This confirms the results of Karim and Taylor (82). Sandberg et al have shown that the guinea pig excretes 60% of its estrogens in the bile, of which 87% is reabsorbed by the intestine and excreted in the urine (70). The above results are obtained from the difference in excretion pattern of intact and bile-fistula animals, and are a true picture only if the animals do not re-excrete in the bile material which has been re-absorbed from the intestine. With this assumption it can be calculated from the present results that the rabbit excretes 20-30% of injected

estrogen in the bile, and that 50-75% of this is reabsorbed and finally excreted in the urine. Further, since the conjugate in bile is mainly the same double conjugate which appears in the urine, it can be assumed that this material is reabsorbed by the intestine. Intestinal reabsorption and renal clearance appears to play a very minor role in the chicken. However, enterohepatic circulation may be present.

None of the mammalian species studied showed the same biliary excretion pattern, which agrees with the results of Sandberg et al (70), and may be due to different estrogen metabolites present in each of these species. Previous work has shown that free estrogen and glucuronic acid conjugates may be absorbed by the intestine more readily than sulfuric acid conjugates (95) (96) (97) (98), although this appears to vary with the intestinal site and steroid studied (70) (99) (100) (101).

V. SUMMARY

1. The pattern of excretion of some estrogens was examined in the rabbit, chicken, guinea pig and cat.

2. The rabbit eliminates a major portion of its estrogens in the urine. The estrogen metabolites in the bile of the rabbit are very similar to those observed in the urine, estradiol-3 glucuronoside-17α-N acetyl glucosaminide being the major metabolite.

3. The guinea pig, in contrast to the other animals, is able to eliminate a considerable amount of estrone or 17β-estradiol in the urine in the free form.

4. The chicken excretes estrogens both as glucuronide and sulfate conjugates in the bile. Diethylstilbestrol glucuronide and sulfate conjugates were found in the bile, urine and feces of the hen.

5. The cat excretes estrogens almost exclusively in the bile. These estrogen metabolites are to a large extent conjugates of sulfuric acid. Glucuronic acid conjugation is a very minor estrogen detoxification process in this species.

6. The species studied vary widely in their biliary and urinary excretion patterns.

C H A P T E R IV

I. INTRODUCTION - PERFUSION EXPERIMENTS

Steroids are carried in the blood circulation in the conjugated form (102). Very little is known of the nature of the estrogen metabolites carried in the blood of rabbits, chickens or guinea pigs. The form of conjugation is incompletely known and only a small number of conjugated estrogens have been isolated. This is due at least in part, to technical difficulties; the conjugated forms of the different estrogens generally occur in very small amounts. Despite the use of radioactive tracers, recovery of the estrogen in the blood is very low due to rapid excretion and total body dilution (103). In an effort to avoid these difficulties it was thought that an isolated liver perfusion unit utilizing a constant volume of blood would be ideally suited for determining the blood estrogen metabolites.

II. REVIEW OF SPECIES EXAMINED

The usefulness of liver perfusions for the study of steroid metabolism has been demonstrated (104) (105) (106) (107) (108). The three species - rabbit, chicken, and guinea pig, examined in this chapter have not been investigated using this technique. Thus, it was of interest to determine the estrogen metabolites present in the blood of these animals following isolated liver perfusion.

III. EXPERIMENTAL PROCEDURES

Two mature female animals of each species were used in these experiments. These animals were maintained on standard commercial diets and were not starved previous to the experiments.

Each animal was anesthetized with pentobarbital sodium and the liver exposed through a median incision. The bile duct(s) were located, ligated and transected. The inferior vena cava was ligated cranial to the right renal vein. Heparin (1000 units) was injected into the isolated portal vein, which was then clamped with a hemostat. The thorax was immediately opened and the liver, with the attached diaphragm, was excised from the animal and placed diaphragmatic surface downwards in a glass organ dish. The portal vein and gall bladder were immediately cathereterized with polyethylene tubing (PE 90 and PE 190 respectively, Becton, Dickinson Co., N.J., U.S.A.). The perfusion was commenced through the portal vein catheter approximately 5-10 minutes after excision. Care was taken to avoid the admission of air into the portal vein. The venous outflow was returned to the perfusate reservoir through the transected inferior vena cava. The perfusion pressure was adjusted to 18 cm. of water and maintained at this level throughout the experiment.

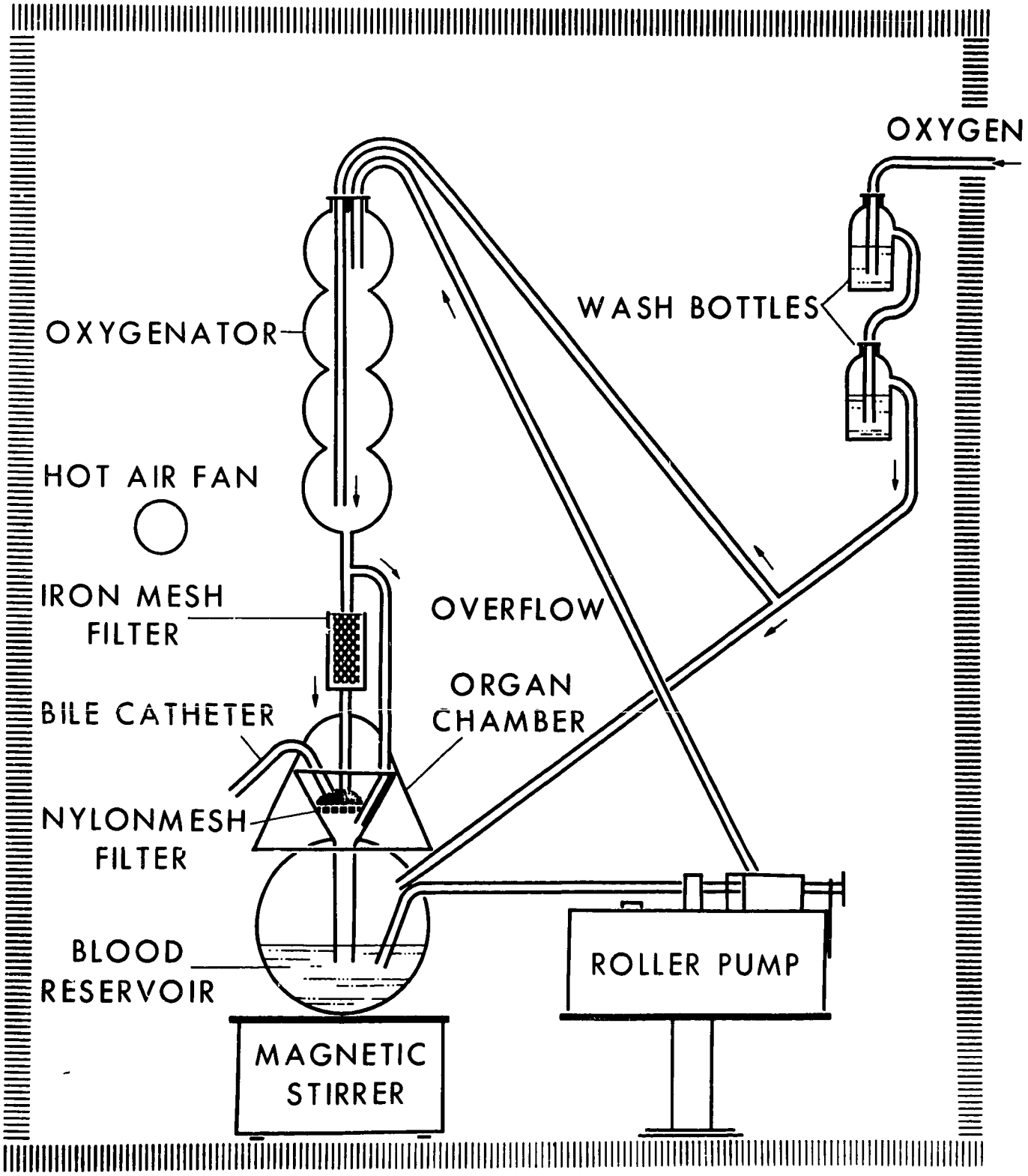
The perfusion apparatus was adapted from that described by Miller *et al* (109) with the following modifications (see

Figure VII). The whole blood perfusate was circulated with a roller pump (Buchler Co., Fort Lee, N.J., U.S.A.). The blood in the reservoir was gently agitated, with the use of a magnetic stirrer, to ensure complete and continuous mixing. Blood clots were removed by filtration through nylon mesh and an iron mesh filter taken from a disposable blood transfusion set (Abbott Laboratories, North Chicago, Ill., U.S.A.). The apparatus consisted entirely of glass and siliconized tygon tubing, and was located in an insulated cabinet maintained at a temperature of 35-40^{o} by hot air fans.

The perfusate consisted of 500 units of heparin, 10 mg of sulfobromophthalein (BSP) and 100 ml of whole blood. The blood was collected by cardiac puncture immediately prior to each experiment from animals of the species to be perfused. The perfusate was introduced into the perfusion apparatus 15 min before organ hook up to ensure adequate oxygenation. Oxygen-carbon dioxide exchange took place in a multibulbed tower and in the perfusate reservoir from a saline saturated mixture of 95% oxygen and 5% carbon dioxide. The flow rate was maintained at 8-12 ml per minute throughout the experiment.

The liver was perfused for 15 min to allow it to stabilize before 9 μci of estrone-6, 7-H^3 (specific activity 48.0 ci/mM) was injected into the effluent blood stream. The vehicle was 0.25 ml of ethanol and 5 ml of isotonic saline.

FIGURE VII APPARATUS FOR THE PERFUSION OF ISOLATED ORGANS. FOR DESCRIPTION SEE TEXT.



In the chicken liver perfusion experiments 3.6 μci of diethylstilbestrol monoethyl-1-^{14}C (specific activity 56.6 mci/mM) was also injected. At this time and at 30, 60, 120 minutes after the addition of the steroid, 5-10 ml blood was removed for standard blood chemistry determinations (see Table VII). All initial perfusate samples were within the normal ranges as given by the Canadian Council on Animal Care (110).

At the end of two hours, approximately 500 ml of isotonic saline was perfused through the apparatus to thoroughly wash the liver of any remaining blood. The blood and saline were immediately centrifuged and the supernatant was applied to an Amberlite column as described in Chapter III. The radioactivity remaining with the red blood cells was approximately 1-5% of the total injected. This was determined by carefully washing the hemolyzed cells with Biosolv, BBS-3. This fraction was discarded. The liver was weighed, homogenized in excess ethanol, and the ethanol fraction was evaporated to dryness, taken up in water and applied to the Amberlite column.

The determination of estrogen metabolites in the blood and liver extracts was carried out as described in Chapters II and III of this thesis.

IV. RESULTS AND DISCUSSION

The radioactive tracer recovery from all experiments

TABLE VII

Routine blood chemistry of liver perfusates at 0 and at 2 hours. Initial samples were all in the normal range.

Blood Chemistry	Rabbit			Guinea Pig			Chicken		
	Normal	Final #1	Final #2	Normal	Final #1	Final #2	Normal	Final #1	Final #2
Hematocrit ml/100 ml	36-46	-	-	37-45	-	-	30-42	-	-
Hemoglobin gm/100 ml	8-13	-	-	13-15	-	-	7-12	-	-
Serum Glutamic Oxalacetic Transaminase Units/100 ml	263	400	620	-	700	625	190-450	1000	500
Serum Glutamic Pyruvic Transaminase Units/100 ml	38	43	125	-	95	105	0	19	15
Total Plasma Protein gm/100 ml	4.4	4.3	4.4	5.2-6.0	2.4	3.5	2-5.5	2.6	2.4
Blood Glucose mg %	85-110	615	1050	60-110	930	630	125-200	540	520

TABLE VII (Continued)

Blood Chemistry	Rabbit Normal	Rabbit Final #1	Rabbit Final #2	Guinea Pig Normal	Guinea Pig Final #1	Guinea Pig Final #2	Chicken Normal	Chicken Final #1	Chicken Final #2
Blood Urea Nitrogen mg %	5-22	13	17	8-20	17	19	0.4-1	2	2
Plasma Sodium mequiv /l	141-156	149	142	120-155	136	138	-	150	151
Plasma Potassium mequiv /l	5.5-6.0	-	-	6.5-7.8	13.2	12.9	4.5-6.5	-	-
Plasma Chloride mequiv /l	90-110	108	99	95-115	116	108	116-140	134	132
% BSP Retention 30 min	-	33.7	32.9	-	77.4	70.6	-	74	74
% BSP Retention 60 min	-	11.9	3.3	-	65.8	52	-	20	69.2
% BSP Retention 120 min	-	-	13.9	-	60.8	52.8	-	18.7	37.4

All blood chemistry determinations were done by the Ottawa General Hospital Laboratories using routine clinical methods.

was approximately 50-70%; of which 7-10% was located in the liver and the remainder in the blood perfusate.

Table VIII shows the estrogen conjugates found in the blood perfusate. The results of the individual animals were similar enough to permit the use of the average in Table VIII. The major metabolite observed in rabbit blood perfusate was the diconjugate estradiol-3-glucuronide-17α-N-acetylglucosaminide. This is also the predominant excretion product identified in rabbit urine (76) and rabbit bile. Approximately 30% of the estrogen was found to be monoglucuronide. All of the estrogen conjugate observed in guinea pig blood was monoglucuronide. The chicken liver possesses the ability to conjugate both the synthetic estrogen, diethylstilbestrol, and the natural estrogens with either glucuronic or sulfuric acid. Approximately 50% of the natural estrogens were found in the perfusate as monoconjugates, the other 50% as diconjugates. The monoconjugates were predominately monosulfates of both the steroid estrogens and diethylstilbestrol. The diconjugate of diethylstilbestrol was formed to a lesser degree than the monoconjugate as compared to the case of the natural estrogens. All three possible diconjugates, namely disulfate, diglucuronide and sulfate-glucuronide were demonstrated in the diconjugate fraction.

Table IX shows the estrogen aglycones of the major monoconjugate fraction for each of the species. The estrogens

TABLE VIII

Metabolites of estrone and diethylstilbestrol found in blood following liver perfusion of three species.

All figures are expressed as percentages of the total radioactivity recovered.

Estrogen Metabolite	Rabbit	Guinea Pig	Chicken	
Injected Substrate	Estrone	Estrone	Estrone	Stilbestrol
Free Estrogen	4.6	1.6	9.8	10.1
Monoconjugate	29.8	98.4	48.2	69.1
Monoglucuronide	29.8	98.4	6.2	13.7
Monosulfate	-	-	42.0	56.2
Diconjugate	65.6	-	42.0	20.8
Estradiol-3-glucuronide -17α-N-acetylglucosaminide	65.6	-	-	-
Diglucuronide	-	-	3.1	0.9
Disulfate	-	-	36.6	6.9
Sulfate-Glucuronide	-	-	3.3	3.0

TABLE IX

Estrogen aglycones of the monoconjugate fraction in blood following liver perfusion of three species.

All figures expressed as percentage of total monoconjugate fraction.

Estrogen aglycone	Rabbit	Guinea Pig	Chicken
Estrone	29	82	14
Estradiol-17α	71	-	-
Estradiol-17β	-	18	86

observed in the rabbit perfusate were estrone and estradiol-17α. The aglycone of the diconjugate fraction was estradiol-17α. The guinea pig perfusate contained estrone and estradiol-17β in the proportion of 4:1. The opposite was revealed for the chicken in that estradiol-17β was the major aglycone observed. Estriol, in trace amounts, was also found as an aglycone of the monoconjugate fraction in the chicken. This is in accordance with previous work which shows that estriols are natural products of the chicken (111) (33).

The conjugated estrogens in the ethanol extract of the perfused livers were of approximately the same proportions as found in the perfusate. However, a large amount, 40-50%, of the estrogens were in the free form as compared to a maximum of 10% for the perfusate. The explanation for this observation may be enzyme hydrolysis of conjugated estrogens prior to ethanol extraction. This may, however, be an indication that the estrogens are in the free form intracellularly and in the combined form extracellularly.

The estrogen metabolites found in the blood perfusate following perfusion of the isolated liver are very similar to those observed in the excretory products of the intact animal. This data supports the view that perfusion of the intact liver with oxygenated blood permits the liver itself to absorb the estrogen, synthesize estrogen conjugates and to contribute these to the circulating plasma in a manner closely approximating the

physiological as reflected in the excretory products of intact normal animals. Although the liver degenerates rapidly (see Table VII) two factors make this organ suitable for this use, 1) the liver possesses a very large metabolic capacity, so that about 80% of the organ may be destroyed before seriously impairing synthesis and detoxification, 2) the liver retains its ability to metabolize steroids even in the presence of gross hepatic destruction. It has been demonstrated, with hepatotoxins, that the liver retains the property of steroid metabolism despite losing other properties such as the capacity to conjugate bilirubin with glucuronic acid (11a) (113) (114) (115) (116).

V. SUMMARY

1. The isolated livers of three species were perfused with whole blood to determine the metabolites of estrone formed by the liver and released into the blood.

2. The rabbit liver formed and released estradiol-3-glucuronide-17α N-acetylglucosaminide and estradiol-3-glucuronide.

3. The guinea pig liver conjugated estrone with glucuronic acid and released it in the perfusate.

4. The chicken is capable of forming sulfuric acid and glucuronic acid conjugates of both the natural estrogens and the synthetic estrogen, diethylstilbestrol.

5. The estrogen metabolites found in the blood following perfusion of the isolated liver are similar to those observed in the excretory products of the intact animal.

C H A P T E R V

I. GENERAL SUMMARY

1) Rabbit liver *in vitro* possess the ability to conjugate phenolic estrogens with glucuronic or sulfuric acid. Sulfuric acid conjugation appears to play a very minor role and sulfates are not found in the blood or excretory products. The liver releases estradiol-3-glucuronide-17α-N-acetylglucosaminide and estradiol-3 glucuronide into the bile and blood. The kidney removes only the diconjugate from the blood. This is important, since the rabbit excretes estrogens predominantly in the urine. The majority of the material excreted in the bile and subsequently reabsorbed and excreted in the kidney is estradiol-3-glucuronide-17α-N-acetyl glucosaminide.

2) Chicken hepatic enzymes are capable of transfering a sulfate or glucuronide to the synthetic estrogen, diethyl-stilbestrol or to the natural estrogens but sulfate conjugation is the preferred form for both substrates. These estrogens are absorbed from the blood by the liver, conjugated with sulfuric and glucuronic acid and released to the blood and bile. The biliary pathway is the more important excretion route. The intestine reabsorbs only a small amount of the excreted material assuming there is no major enterohepatic circulation. The intestinal environment appears to contain a large hydrolytic enzyme activity, resulting in a large amount

of free estrogen and no diconjugate in the feces. Blood estrogens are excreted into the urine as the sulfate conjugates of the natural estrogens and of diethylstilbestrol. In the case of diethylstilbestrol the glucuronide is also excreted.

3) The guinea pig was examined in this treatise because it was a common laboratory rodent and because this animal exhibited an odd pattern of estrogen excretion in preliminary experiments. The liver of the guinea pig in vitro is able to form estrogen conjugates with sulfuric and glucuronic acid with equal capability. However, only the monoglucuronides appear in the blood following isolated liver perfusion. The guinea pig is able to excrete free estrogens in the urine if these are presented to the kidney, while the other animals examined require the estrogens to be first conjugated before the kidney can excrete them.

4) The cat was investigated because of its history of being susceptible to toxicological conditions. This animal demonstrated a very low level of glucuronyl transferase in in vitro experiments. This was in accord with the in vivo results, in that the cat excreted the estrogens in the sulfate form. The liver extracts estrogens from the blood, conjugates them with sulfuric acid and excretes them almost entirely in the bile. If enterohepatic circulation is present the kidney does not appear capable of clearing estrogen sulfates.

II. CONCLUSIONS

The results obtained in this work show that the mechanisms by which the phenolic steroid estrogens are conjugated and excreted vary greatly between species. In the rabbit, while sulfates can be formed by liver tissue *in vitro* in the presence of appropriate co-factors, this formation is quantitatively minor as compared to glucuronide formation (Table II). The fact that glucuronides but not sulfates of administered estrogens are excreted in the rabbit is probably explained by this predominance of glucuronide forming capacity, although the inability of the rabbit hepatic cell to release estrogen sulfates or a lack of co-factors for sulfate conjugation *in vivo* may be responsible for their absence from urine, bile and blood.

In contrast to the rabbit, the chicken liver (Table II) is much more facile in the *in vitro* formation of estrogen sulfates than the glucuronides, and this accords with the excretion of these compounds as sulfates in the urine (19). However, in the case of the chicken, significant amounts of estrogen glucuronides are excreted in the bile. Thus, again in contrast to the rabbit, the estrogen conjugates in chicken bile and urine are not the same.

The guinea pig, unlike both the rabbit and the chicken, has a high *in vitro* capacity for the production of both sulfates and glucuronides of the estrogens, but only the

glucuronides are excreted in the urine, or formed when liver is perfused with estrone. It is possible that a lack of co-factors for sulfate conjugation in vivo or estrogen sulfate permeability may be responsible for this effect.

The cat differs from all the other species in that it excretes estrogens almost exclusively in the bile and almost exclusively as sulfates. The lack of glucuronide conjugates of the phenolic steroids appears to derive from the inability of the liver to form glucuronides in vitro and more specifically the lack of glucuronyl transferase (46) (47).

In the rabbit and guinea pig, the synthetic estrogen diethylstilbestrol was conjugated as glucuronide as well as sulfate by liver tissue in vitro, and, in contrast to the phenolic steroids, was excreted partially as a glucuronide in the urine. In the cat, some formation of stilbestrol glucuronide was demonstrated in vitro although phenolic steroid glucuronides were not found.

These results indicate that the in vitro ability of liver tissue from a given animal to form sulfates or glucuronides of the natural or synthetic estrogens is not a reliable guide to the mechanisms by which these compounds may be handled in an in vivo situation. Further, from the differences between the modes of excretion of stilbestrol and the phenolic steroids by the hen and the cat, it is obvious that molecular structure affects the type of conjugation employed. It is likely that

these species differences extend to many compounds other than the estrogens, and it is therefore most important that they be considered when using small animals as prototypes for man in toxicological studies.

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