Title

The biliary excretion and enterohepatic circulation of diethylstilbestrol and diethylstilbestrol monoglucuronide in the Rhesus monkey

Author

Mroszczak, Edward Joseph

Publication Date

1974

Peer reviewed|Thesis/dissertation

THE BILIARY EXCRETION AND ENTEROHEPATIC CIRCULATION OF DIETHYLSTILBESTROL AND DIETHYLSTILBESTROL MONOGLUCURONIDE IN THE RHESUS MONKEY

by

Edward Joseph Kroszczak
B.S., State University of New York at Buffalo, 1967
M.S., University of Wisconsin, 1969

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA

[redacted]

Degree Conferred: . . . . . .

THE BILIARY EXCRETION AND ENTEROHEPATIC CIRCULATION OF DIETHYLSTILBESTROL AND DIETHYLSTILBESTROL MONOGLUCURONIDE IN THE RHESUS MONKEY.

Edward J. MROSZCZAK, Ph.D.
University of California, San Francisco, 1974

Adult, male rhesus monkeys were prepared surgically with indwelling arterial-venous catheters as well as permanent catheters to the common bile duct (cystic duct ligated) and duodenum (opposite the sphincter of Oddi). After surgery, the monkey was placed in a restraining primate chair for chronic usage. A continuous intravascular infusion of heparinized saline was necessary to prevent clotting. The externalized hepatic and duodenal catheters could be connected to allow a normal enterohepatic circulation of bile, when the monkey was not being used experimentally.

Diethylstilbestrol-^{14}C was used in all in vivo studies and was assayed by liquid scintillation counting, after separation of unchanged compound from metabolites. Throughout the experiments, hepatic bile was totally collected and a duodenal or intravenous infusion of sodium taurocholate was maintained to assure a steady bile flow rate. Urine was collected after voluntary voiding into a tray which separated urine from feces. Arterial blood was sampled at appropriate times within an experiment.

After an intravenous injection of diethylstilbestrol-^{14}C, approximately 58% of the dose is excreted into the urine and 30% into the bile after 24 hours. Diethylstilbestrol and sodium diethylstilbestrol monoglucuronide, prepared biosynthetically using female New Zealand white rabbits, served as chromatographic standards in elucidating the metabolism of diethylstilbestrol in the rhesus monkey. In bile, only diethylstilbestrol monoglucuronide-^{14}C was found while multiple, unidentified metabolites

appeared in the urine. No unchanged compound is excreted into the urine or bile. Blood samples contained unchanged compound and the monoglucuronide.

Pharmacokinetic analysis of post-injection (800 μg containing 38.5 μCi) diethylstilbestrol-^{14}C blood levels indicated that the three compartment open model was most compatible with the observed data. A digital computer (IBM 360/50) generated the following equation describing the model: $C_b = 4{,}470 \cdot e^{-.1221 \cdot t} + 2{,}890 \cdot e^{-.0349 \cdot t} + 574 \cdot e^{-.0068 \cdot t}$ where C_b represents the blood concentration levels of diethylstilbestrol-^{14}C as a function of time (t). The pre-exponential terms represent intercepts on the ordinate at zero time (DPM/ml) and the exponential terms represent hybrid rate constants (min^{-1}).

In vitro solubility studies of sodium diethylstilbestrol monoglucuronide were carried out at ambient room temperature (23.5 to 24.5°C) in isotonic sodium phosphate buffer solutions (pH = 8.00, μ = 0.170). In plain buffer the solubility of the monoglucuronide is 1.37 mg/ml, however, this could be increased several times by the addition of taurocholate alone or a taurocholate-lecithin combination (molar ratio 5.7 to 1) in amounts which exceed the CMC. In contrast, the addition of sodium taurodehydrocholate, which does not form micelles in the concentration ranges studied, did not enhance the solubility of the monoglucuronide.

Blood samples were taken and total collections of bile and urine were made during a constant intravenous infusion of diethylstilbestrol-^{14}C (equivalent to 179 μg/min) which was maintained throughout the experiment. Bile flow rate was brought to an initial constant level (.095 ml/min) with a duodenal infusion of taurocholate (2.9 μmol/min) and was then increased to a higher steady state level (0.149 ml/min) in response to an increased

taurocholate infusion rate (13.6 μ mol/min). The results of this experiment indicated that a bile flow dependent transport maximum exists for the excretion of diethylstilbestrol monoglucuronide into the bile.

Separate experiments were undertaken in which solutions of diethylstilbestrol-^{14}C or diethylstilbestrol monoglucuronide-^{14}C were administered duodenally. The results of these experiments indicated that the extensive enterohepatic circulation of diethylstilbestrol is mediated by bacterial enzymes (specifically β-glucuronidase) located in the large intestine of the rhesus monkey. Therefore, in an intact animal (given diethylstilbestrol) diethylstilbestrol monoglucuronide would be excreted into the bile and deposited in the duodenum. Since the polar glucuronide is not well absorbed, it will pass down to the bacteria rich environment of the large intestine. Here, enzymatic hydrolysis liberates free diethylstilbestrol which can be absorbed, resulting in an enterohepatic circulation.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

GENERAL ASPECTS OF THE BILIARY SYSTEM

Anatomy

Any consideration of the elimination of foreign compounds and especially one involving biliary excretion necessitates a detailed discussion of the biliary system. The overall anatomy and physiology of the liver is very well described (1). One therefore can forego a discussion of the liver and concentrate on the anatomy of the biliary system which includes the bile canaliculi, ductules, intrahepatic ducts, the hepatic duct, the cystic duct, the gall bladder and the common bile duct (2). The following discription applies to man and primates in general which are very similar with respect to anatomy of the biliary tree.

The biliary system can be considered to begin with the bile canaliculi. These canaliculi consist of two grooves in the contact surfaces of liver cells and have no distinct walls of their own. Numerous microvilli project into the lumen of the canaliculi. Although some disagreement exists, it would seem that the only entrance into the canaliculi is through the hepatic parenchymal cell wall, but the possibility exists that there is a direct connection to the Space of Disse (3). The bile canaliculi are numerous and form a network between the liver cells, imparting strength to the entire liver muralium (4).

Bile canaliculi empty into the ductules or colangioles, an extensive, wide-meshed network of intralobular ductules which is continuous throughout the liver. These ductules have walls which are extremely thin and are well endowed with a venous and arterial blood supply. The small ductules discharge into slightly larger ducts within the liver which form plexuses surrounding the portal vein branches. In addition, arterial capillary branches

form a subepithelial plexus around the larger bile ducts (5). Finally, the larger bile ducts converge to form the extrahepatic-right and left hepatic ducts which anastomose forming the common bile duct. Further down, the common bile duct is joined by the cystic duct which originates from the gall bladder. The pancreatic ducts join just prior to termination in an elaborate musculature, collectively known as the sphincter of Oddi which opens into the duodenum (6).

It would seem that the highly perfused ductules and ducts would have a secretive and reabsorptive capacity and both have been demonstrated (7,8,9). In this respect the biliary system could be compared to the kidney with the exception that a high capacity filtration mechanism found in the glomerulus of the kidney does not exist in the biliary system. It has been shown in the rat that bile pressures normally exceed portal pressures by 50 per cent or more, establishing conclusively that the basic mechanism of fluid transfer (or bile formation) cannot be filtration. Therefore, the elaboration of bile is essentially a secretory process involving the active transfer of various elements in bile which determine directly or indirectly those fluid movements which are observed (10).

Mechanism of Bile Formation

The question then arises as to what is the exact nature of the driving force for bile formation. It has been demonstrated in dogs that acute interruption of the normal enterohepatic circulation of bile salts results in a progressive diminuation of bile flow (11). This was demonstrated to be due to the diminishing rate of bile salt excretion and could be overcome by the administration of sodium taurocholate (a typical bile salt) intravenously. It has been postulated that all bile salts are actively secreted at the can-

alicular level and this process is the prime driving force for bile production (12,13,14). These workers have demonstrated the linear dependence of bile flow and inorganic anion excretion (chloride and bicarbonate) on the rate of taurocholate secretion. Various mechanisms can account for the role of sodium taurocholate as the major choleretic in bile. First, one can consider that taurocholate is actively secreted at the bile canaliculi and this results in the entry of water and diffusible ions by osmosis and passive diffusion. Obviously, anion secretion requires concomitant transport of sodium and other cations for charge balance and the active transport of taurocholate along with sodium causes water to be transported into the canaliculi. Bile salts are known to exist in bile as part of complex micellar structures with only a portion of the molecules being ionized. Charge neutralization will still require sodium cations to balance the anionic charge of the micelle. Transport of sodium and water therefore ensues until the system becomes isosmotic with respect to blood. The net output of water and electrolytes is therefore subject to the quantity of bile salt in the lumen of the biliary tree. Whatever the mechanism, it is clear that bile salt transport is the major determinant of bile flow and electrolyte composition. It is believed that the hormone secretin acts further downstream from the canaliculi (the bile ductular system) and its choleretic effect is due to active transport of solute into the lumen with consequent passage of water and diffusable electrolytes. It should also be mentioned that certain organic anions and cations also possess a choleretic activity.

Under normal circumstances, of course, bile salts secreted by the liver pass into the intestine and are absorbed back into the hepatic portal circulation primarily in the ileum (15). Normal processes such as waking and sleeping, feeding and fasting and gastrointestinal hormone secretion can ef-

fect the enterohepatic circulation of bile salts. As is the case with all smooth muscle, the biliary tract is subject to a variety of humoral and neurogenic stimuli as well as direct vagal effects (16).

Micellar Structures of Bile

The bile salts are surface active compounds and appear to be present in all vertebrates. An excellent review of the clinical implications due to the unique properties of those compounds is presented by Hofmann (17). Although the bile salts can be likened to anionic detergents, their molecular structure is quite different from that of the typical detergent. Bile salts, the primary components of bile, are association colloids which form polymolecular aggregates known as micelles. These compounds contain both hydrophobic and hydrophilic regions and are thus known as amphiphilic compounds. They are important in lipid solubilization and transport in the bile and fat digestion and absorption in the intestine (17).

As mentioned, bile plays a major role in promoting fat digestion and fatty acid absorption. In the intestine, pancreatic lipase reacts at the surface of the dispersed fat droplets and fatty acids and monoglycerides are brought into a micellar solution with the bile salts. Without the bile salts the process of fat digestion and solubilization of these various substances could not occur.

In hepatic bile it is found that bile salt concentrations are as high as 50 mM while in the intestine the concentration may drop to as low as 5 mM, due to dilution by the various fluids. However, the critical micellar concentration (CMC) of the bile salts is about 3 to 4 mM and therefore at least a fraction of the bile molecules are associated into micelles, even in the intestinal contents.

There are basically three bile acids of importance in primates. They are the dihydroxy bile acids (deoxycholate and chenodeoxycholate) and the trihydroxy bile acid (cholate). These bile acids are conjugated with taurine or glycine and therefore a total of six bile salts are possible. In bile, virtually all bile salts are present as conjugates. This conjugation lowers the pKa of the bile acids and thus ensures that they will remain in solution while in bile and intestinal contents. No major differences have been found regarding the micelle forming properties of the dihydroxy and trihydroxy conjugates of either glycine or taurine.

Human bile appears to be a transparent solution, even though it contains a high concentration of cholesterol far above its water solubility. This cholesterol must be present in lipoprotein or micellar complex form, since it is virtually insoluble by itself in water. However, a third component, lecithin, is found to be necessary to solubilize cholesterol to an appreciable degree. Lecithin alone is insoluble in water but has a remarkably high solubility in bile salt solutions. As the bile salt micelle is swollen by lecithin, the micelle can dissolve more cholesterol. Hence a rather complex mixed micelle composed of bile salt, lecithin, and cholesterol exists in bile. The ratio of components is critical and perturbation can result in turbidity or even separation and precipitation of cholesterol as crystals.

The mixed micelle contains many negative surface charges and resembles a bead of ion exchange resin at the molecular level. Cations, primarily sodium, will be found in association with the negative charges of the bile salt anions. Since osmotic pressure is related to the total number of particles present and since the micelle with its bound counter ions behaves to some extent as a single particle, the osmotic pressure of bile salt solutions is far lower than one would calculate on a purely stochiometric basis. If it

were not for this micellular association a bile solution would be very hypertonic. It is in fact isosmotic with blood due to the formation of these micellar aggregates.

A compound closely resembling cholic acid (a trihydroxy bile acid) is dehydrocholic acid (Decholin). The only difference between them is that Decholin has three keto groups in place of the hydroxyl groups of cholate. Decholin is not normally found in bile and its absence of hydroxyl groups renders it incapable of micelle formation and therefore it cannot solubilize cholesterol or lecithin nor enhance fat absorption.

EXCRETION OF FOREIGN COMPOUNDS INTO BILE

Physical-Chemical Considerations

A large number of compounds and their metabolites are known to be excreted into the bile (18,19) some of which have been hypothesized to undergo an enterohepatic circulation which can significantly effect their disposition (20). Much of the work involving biliary excretion has been concentrated on organic anions. Most compounds are excreted in bile conjugated with glucuronic acid, glycine, taurine, sulfuric acid and glutathione and as such are present as anions. For an anion to be excreted to an appreciable degree in the bile it has been suggested (21) that it should possess three general characteristics; 1.) a molecular weight of at least 325 ± 50, 2.) a polar anionic group in its structure, and 3.) a certain molecular structure - as yet ill defined. This molecular structure feature undoubtedly includes a specific charge distribution as well as a minimum lipid solubility below which little biliary excretion occurs. Also, it should be considered that the molecular weight requirement refers to the compound excreted into the bile only. Hence a molecule may have a molecular weight of 250 and as such not fit the requirements. However, if it is conjugated with glucuronic acid, the metabolite will have a molecular weight of 444 which is well above the molecular weight range required.

Relatively little information is available on the types of organic bases eliminated in the bile. Of the compounds studied, they seem to have one characteristic in common: a polar quaternary amine group at one end of the molecule and one or more nonpolar ring structures at the opposite end. In comparing those organic cations excreted in the bile with the above described anions, it is interesting to note that in both cases the presence

of a polar ionizable group as well as a nonpolar portion (two or more aromatic or saturated rings) are features resulting in extensive biliary excretion. Also, as in the kidney, there appear to be two separate mechanisms in the liver for the secretion of organic anions and cations (22,23).

A number of organic compounds which are neither anions nor cations are excreted in the bile. The cardiac glycosides are important examples of such compounds. Here, the presence of a water-soluble sugar residue in the molecule is the one structural feature which stands out as being necessary for extensive biliary elimination. In a series of compounds, those containing the most sugar residues (the most polar) exhibit the highest biliary excretion. Again we can see a similarity between the organic anions and cations and those glycosides which are well excreted in the bile. They all have high molecular weight, a degree of polarity and contain at least two aromatic or saturated rings (nonpolar portion) in the molecule (21).

Species Variation in Biliary Excretion

With regard to species differences in biliary excretion, several factors must be considered. One must not only consider species differences but also the physical-chemical properties of the compounds under study since both are inter-related. To begin, one may roughly organize the various species studied into three groups (21). Group I contains the rat, dog and hen which are generally good biliary excretors. Group II contains the cat and sheep which are intermediate and Group III includes the rabbit, guinea pig, rhesus monkey and probably man - relatively poor biliary excretors.

If one now attempts to correlate the biliary excretion of organic anions in the various species the following picture ensues. Anions with a molecular weight below 300 are excreted poorly in all species. In the molecular weight

range of 300-500 species differences occur in accordance with the three groups listed. For example, Group I would excrete 20-30% of a dose of succinylsulfathiazole into the bile, Group II 5-15% and Group III 0-1%. For those compounds with a molecular weight range of 500-1200, biliary excretion is uniformly high in all species listed.

It should be noted both that man and the rhesus monkey are both in the same group (poor biliary excretors). Much of the previous research with diethylstilbestrol has been done on the rat and dog which are the best biliary excretors studied. In this respect the rhesus monkey is the model of choice if correlations to man are to be made.

Role of Bile Micelles in Biliary Excretion

The relation between biliary bile salt excretion and biliary cholesterol and phospholipid excretion has been studied in rats (24). Infusion of taurocholate, a micelle-forming bile salt, causes a concomitant increase in biliary bile salt excretion and biliary lipid excretion. However, with infusion of dehydrocholate, a nonmicelle-forming bile salt biliary lipid excretion increased negligibly. These studies prove that micelle formation is an important mechanism in biliary lipid excretion, but this alone could not account for the quantitative relation between biliary bile salt and lipid excretion.

In this study, both compounds investigated (cholesterol and lecithin) are normal components of bile and the bile salt mixed-micelle system. Hence, such a close correlation with bile salt excretion rate would not be unexpected. This may be related to the formation of a specific macromolecular complex in the liver and its subsequent excretion in the bile.

Another group (25) studied the drugs imipramine and desmethylimipramine

in the rat. They demonstrated that both compounds were taken up by the bile salt-phospholipid-micelles of bile in vitro. They were also able to calculate the molecular composition of the bile salt-lecithin-imipramine micelle to be 60:10:7 with a molecular weight of 36,000. It was further demonstrated (again using in vitro models) that while pure bile salt micelles could take up these drugs, the presence of phospholipid enhanced their solubilization. These workers also studied both drugs in rats and were able to show that the intact compounds and their metabolites appeared to be taken up into bile micelles and transported as such, somewhat analogous to a concentrative transfer process (25).

The anions bromsulphthalein (BSP) and bilirubin are known to be extensively excreted into bile. While no conclusive studies have been made to show that there is a micellar interaction with these anions in bile, this has been suggested for bilirubin (26). The biliary excretion of BSP is directly influenced by taurocholate excretion rate and here a carrier system of some sort was postulated but no studies of a possible interaction with bile micelles were undertaken (27).

It is obvious that the presence of micelles in bile can directly account for the biliary excretion of certain endogenous compounds. It has also been shown that certain foreign compounds can interact with these bile micelles and it was suggested that this interaction may be the controlling mechanism for excretion into the bile. These studies are important in that they show conclusively that the biliary micelles can function as a "carrier" of both endogenous and foreign compounds. Little more can be said since so little work in this area has been done. However, it is clear that the presence of these micellar structures in bile must be considered in the study of any compound which undergoes biliary excretion. While much more can be dis-

cussed in the area of biliary excretion it is hoped that the relatively small area covered above will adequately serve as an introduction to certain key areas which can be further pursued if desired.

THE RHESUS MONKEY AS AN EXPERIMENTAL MODEL FOR THE STUDY OF BILIARY EXCRETION

Before any research project is begun one must consider the goals and how they can best be attained. One of the most important considerations is the appropriate experimental model to be used. While it has not always been true in the study of biliary excretion and enterohepatic circulation, an animal model resembling man on an anatomic and physiologic level should be preferred. In addition, rather extensive surgery is required to gain access to the biliary pathways. It would be desirable to devise a model in which surgical trauma is minimized and anesthesia eliminated. Ideally, the animal should be allowed to recover completely from the surgical trauma and anesthesia and be maintained as a chronic preparation. It was found that the monkey was best suited to this end.

The type of monkey studied most commonly in this laboratory is the rhesus monkey (species-Macaca Mulatta). The use of the rhesus monkey is not new and has proved to be a valuable tool in many research projects. Basically, this monkey is very easy to handle and in our laboratory has proved to be a friendly and sociable animal. On the evolutionary scale the similarity of monkey to man is obvious. It has, in fact, been shown in various toxicologic studies, that once it is established that a given drug has a similar action in both monkey and man, the rhesus monkey is the experimental model of choice (28). The rhesus monkey is very adaptable to a chronic experimental situation and is virtually an ideal animal in which to study the biliary excretion and enterohepatic circulation of drugs.

It has already been made clear that man and monkey are very similar with respect to the anatomy of the biliary tree and in their ability to excrete foreign compounds into the bile. A review of some additional past

research will point out specifically, important areas of similarity and dissimilarity with respect to: various hemodynamic factors, bacterial environment of the intestine, and normal composition of bile. Finally, the suitability of the rhesus monkey as a chronic preparation will be discussed.

Studies have been completed which describe the normal distribution of cardiac output in the unanesthetized, restrained rhesus monkey (29). This was accomplished using radioactively labeled microspheres injected into the left ventricle of the heart. These workers have demonstrated that the per cent of cardiac output transitting specific organs in the monkey are in the same range as those reported in man. For example, the per cent of cardiac output in man versus monkey for various organs are: liver 28% vs 19%, kidney 23% vs 12%, heart 5% vs 5% and brain 14% vs 7%. However, in the monkey a much higher total cardiac output per 100 Gm body weight was observed. This high total of cardiac output is reflected in the much higher flow seen in most of the regional organs for the monkey compared to man. This was especially noticeable (comparing man vs monkey) in flow per 100 Gm of tissue (ml/min) in the liver 58 vs 148, kidney 420 vs 543, heart 84 vs 324 and brain 54 vs 80. It is also noteworthy that these monkeys had an unusually low hematocrit (28.2 to 32.2) compared to the normal value in human subjects (45.0).

Another important factor to consider is the bacterial environment of the intestinal tract. Since bile empties directly into the duodenum and from there can either be reabsorbed or passed into the feces, any factors which modify this are important. It is well known that man has almost no bacteria in the intestine from the pyloric sphincter to the ileo-caecal valve. This is also true of the rhesus monkey (30). On the other hand the rat and dog have bacteria throughout the small and large intestine. This is particularly true of the rat which is highly infested with bacteria throughout

the intestine.

This is significant since certain bacteria such as E coli are known to produce β-glucuronidase, an enzyme which can cleave glucuronides which are excreted into bile. Hence, if one assumes that the unconjugated form of a drug is more completely absorbed than the conjugate, one may get a false impression of the potential for enterohepatic circulation of a drug in man if the rat was used as the experimental model. For example, a drug administered to a rat if excreted into the bile as a glucuronide will be readily cleaved in the intestine, due to the presence of bacterial glucuronidase. The unconjugated drug thus produced could then be absorbed efficiently and an enterohepatic circulation of this drug would be established. However, in man or monkey this potential for enterohepatic circulation may not be observed at all or to a much smaller degree due to a relative lack of bacterial produced glucuronidase in the small intestine (31). These bacteria can also produce other alterations of drug molecules which can result in various toxic metabolites which may be observed in rat or dog but not necessarily in man or monkey (32).

Dowling and co-workers have used the rhesus monkey to study the enterohepatic circulation of bile (33). They were able to exteriorize the normal extrahepatic biliary pathway and by interposing an electronic stream-splitter in the circuit, the normal enterohepatic circulation of bile could be interrupted to an accurately controlled degree (34). Their results clearly demonstrated that the rhesus monkey could be successfully used, on a chronic basis, to study the enterohepatic circulation of bile and other drugs. It has also been shown that the composition of bile in this primate closely resembles the composition of bile in man (35,36).

The methods employed by Dowling are far superior to those of other

workers in the field of biliary excretion. Dowling demonstrated that the rhesus monkey can be surgically prepared far in advance of a given experiment and during the interim placed in a restraining chair. In this way one can avoid the use of any anesthesia during an experiment and also the severe trauma of surgery - both of which can alter the physiologic significance of the results. In addition, monkeys placed in restraining chairs, if well taken care of, can last for months - considerably reducing the costs involved in working with such expensive primates.

DIETHYLSTILBESTROL AS A MODEL COMPOUND FOR THE STUDY OF BILIARY EXCRETION

Diethylstilbestrol is a synthetic estrogen widely used both orally and parenterally in man and animals. It is a neutral compound, insoluble in water but soluble in alcohol, ether, chloroform and various oils. It can be recrystallized from benzene as small plates and has a melting point of 170°C and a molecular weight of 268.3. It is relatively non-toxic having an LD-50, orally, in mice of 3 grams per kilogram. In the physiologic system it will be non-ionized since it has a pKa of approximately 10.

Diethylstilbestrol is known to undergo cis-trans isomerization (37). This isomerization has been the subject of much controversy but of the two forms, the trans isomer is believed to be more biologically active because of its structural similarity with estradiol (38). The importance of the trans configuration in regard to biological activity was demonstrated by Walton and Brownlee (39) who showed the trans dipropionate diethylstilbestrol derivative to be 600 times more active than the cis derivative. The cis isomer is found mainly in solvents of low dielectric constant such as benzene where it may account for as much as 25% of the diethylstilbestrol present. In high dielectric constant solvents and in vivo one can expect the trans isomer to predominate. In fact it has been proposed that any activity seen with the cis isomer is due to isomerization to the trans isomer in vivo.

Diethylstilbestrol is capable of producing all the pharmacologic and therapeutic responses attributed to natural estrogens. It is indicated for the relief of symptoms of the menopause; in senile vaginitis; for the relief or prevention of painful engorgement of the breasts postpartum; for control of functional uterine bleeding; in carcinoma of the prostate; and

in mammary carcinoma of postmenopausal women. The dose can vary from 0.1 to 15 mg daily and has recently been recommended in much higher doses as a post-coital contraceptive.

Diethylstilbestrol is also capable of producing malignant tumors in experimental animals. Cancers of the breast, uterus, abdominal cavity, testicular Leydig-cell, and lymph may be incited by large doses of this drug. More recently it has been proposed that maternal ingestion of diethylstilbestrol during pregnancy increases the risk of vaginal adenocarcinoma developing years later in the offspring exposed (40,41).

The synthetic estrogens have been in use for some 25 years, the most important of these being diethylstilbestrol (42). It is well known that the naturally occuring estrogens are extensively excreted into the bile of man and may undergo an enterohepatic circulation (43). However, no information appears to be available regarding the disposition of diethylstilbestrol in primates or in any other animal.

Although many studies have been made in the past on the biliary excretion of diethylstilbestrol, few have been directed at defining the basic mechanisms involved and the overall in vivo effect excretion into the bile can produce. Fisher (44) and Hanahan (45) have shown that in the bile fistula rat, after the injection of diethylstilbestrol-^{14}C, 94% of the radioactivity appeared in the bile and 2% in the urine after 24 hours. In the bile 3% of the recovered radioactivity is diethylstilbestrol, 72% diethylstilbestrol monoglucuronide and 25% a combination of at least three other metabolites which were not identified. In other species it was found that in the dog, 60 to 90% appeared in the bile and 10 to 30% in the urine (45). The rabbit is exceptional in that about 70% of the administered dose is found in the urine (mainly as the glucuronide) 6 days following the oral

administration of diethylstilbestrol (47). Note, that all the bile recovery experiments were done with total collection of bile during or immediately after anesthesia and under acute non-physiologic conditions.

It has also been shown that when diethylstilbestrol is administered a steroid-induced cholestasis results (48). This effect has been correlated with a depression of bile flow rate the mechanism of which is still conjectural. This report indicates that bile flow rate may influence the biliary excretion of diethylstilbestrol or its metabolites and leads into the questions regarding the basic mechanisms involved.

As will be pointed out in subsequent sections it appeared that diethylstilbestrol would be a fairly simple compound to study. It will be shown that only the monoglucuronide is present in bile and co-exists with unchanged compound in the blood. Also the separation of unchanged compound from metabolite is a fast and efficient process. The urine apparently contains multiple metabolites. Hence diethylstilbestrol appears to be an ideal compound with which to investigate the various aspects of biliary excretion.

In summary, while it is known to be excreted in the bile of various species of animals, little has been done to explain how or why this occurs. In short, we have a pharmacologically active compound in widespread usage today with a significant toxic potential. However, beyond these facts there are many gaps in our understanding of the fate of this compound in vivo.

PROJECT GOALS

A compound which undergoes biliary excretion and entereohepatic circulation poses at least seven problems which merit study as follows: (i) what happens to the compound in the blood, (ii) how does the compound pass from the blood to the hepatic cell, (iii) what happens to the compound in the hepatic cell, (iv) how does this compound (or its metabolites) pass from the hepatic cell into the bile canaliculi, (v) what happens to the compound (or its metabolites) in the bile, (vi) what happens to the compound (or its metabolites) in the intestine, and (vii) how does the compound (or its metabolites) pass from the intestine into the blood? To answer all of these questions would require more than one research project and more than one experimental model. In my research I propose to consider mainly questions i, iii, iv, v, and vii with respect to the synthetic estrogen diethylstilbestrol and one of its metabolites diethylstilbestrol monoglucuronide.

As previously mentioned, no work is available concerning the biological fate of diethylstilbestrol in primates. Since both man and monkey can be regarded as poor biliary excretors, much of the previous work done in the rat and dog (good biliary excretors) is not necessarily applicable. Therefore, the initial goal will be to determine to what extent diethylstilbestrol is excreted into the bile of the rhesus monkey. Logically, the next goal will be to determine the form in which it is excreted into bile. If possible, one would desire to define diethylstilbestrol metabolism as completely as possible and elucidate those metabolic end products present in blood, bile, and possibly urine.

By studying the blood levels of diethylstilbestrol, one can describe

a pharmacokinetic model and from this obtain its biologic half-life, volume of distribution and clearance, all of which are important in rational drug therapy and avoidance of potential toxicities.

A detailed study of the excretory pattern in bile is proposed. It is imperative that bile flow rate be regulated throughout the experimental procedures. This is accomplished by the infusion of sodium taurocholate, a typical bile salt. Without this replacement, the volume of bile produced per unit time progressively decreases making sampling difficult and possibly also exerting an effect on the excretion of diethylstilbestrol (or its metabolites) in the bile. It will also be important to study the effect of bile flow rate on the excretion of diethylstilbestrol (and its metabolite). Past studies have shown that the choleresis induced by bile salts enhances the biliary excretion of cholesterol and lecithin (49) and also bromsulphthalein (27). However, choleresis can also depress the excretion of various compounds such as phenol red and bromcresol green (50). These reports indicate that bile flow rate influences the biliary excretion of many compounds and a detailed study of this effect is proposed. Another common occurence in the biliary excretion of various compounds is the presence of a maximal excretory rate which may be altered by varying the bile salt excretion rate. Both of these effects will be studied with respect to diethylstilbestrol and its metabolites. Closely related to these features is the ability of a compound itself to cause an increase or decrease in bile flow rate due to its excretion into the bile.

Another aspect to be studied is the potential for an enterohepatic circulation of diethylstilbestrol and its metabolites. Past studies done in this area (in the rat) may not be entirely valid due to the differences of intestinal bacteria populations (between rodents and primates) as

discussed earlier. This aspect of biliary excretion can also have an effect on the biologic half-life of a compound and the potential build up of metabolites (51).

Perhaps the most important question to consider is; what is the primary driving force for excretion into the bile? The possibility that incorporation into bile micelles is a significant factor will be considered, since it is well known that bile micelles primarily composed of bile salts and lecithin do exist. The ability of these bile micelles to incorporate various fat soluble compounds has been discussed (52). However, whether the incorporation into the core of biliary micelles, or some other type of association with bile salts provides the driving force for biliary excretion has never been clearly established. Various in vivo and in vitro experiments will be conducted in order to clarify some of these questions.

Diethylstilbestrol and its major metabolite diethylstilbestrol monoglucuronide provide the basic tools for the investigation into the various aspects and potential mechanisms of biliary excretion. The parent compound is available and the metabolite can be prepared and this allows one to carry out various studies not otherwise possible. These facts combined with the knowledge that diethylstilbestrol (as with the naturally occuring estrogens) has a good potential for biliary excretion and enterohepatic circulation make these project goals feasible.

REFERENCES

1. Rappaport, A.M., Schaffner, F. and Popper, H., in "Diseases of the Liver" Third Edition, Edited by Leon Schiff, J.B. Lippincott Co., Philadelphia, 1965, Ch. 1 and Ch. 2.

2. Elias, H., Embryology, Histology and Anatomy of the Biliary System, in "The Biliary System" Edited by W. Taylor, Blackwells Scientific Publications, Oxford, 1965, pp. 1-13.

3. Rouiller, C., Les Canalicules Biliaires, Acta Anat., 26, 94 (1956).

4. Elias, H., A Re-Examination of the Structure of the Mammalian Liver, Am. J. Anat., 85, 379 (1949).

5. Elias, H. and Petty, D., Terminal Distribution of the Hepatic Artery, Anat. Rec., 116, 9 (1953).

6. Giermann, H. and Holle, G., Stereoskopische und Mikroskopische Untersuchungen zur Pathologie des Schleimhautreliefs und Klappenapparates der Papilla Vateri, Acta Hep-Splen., 8, 189 (1961).

7. Despopoulos, A. and Sonnenberg, H., Congruence of Excretory Functions of Liver and Kidney: Sulfonamides, Am. J. of Physiol., 212, 1117 (1967).

8. Clark, A.G., Hirom, P.C., Millburn, P., Smith, R.L. and Williams, R. T., Reabsorption from the Biliary System as a Factor Influencing the Biliary Excretion of Organic Anions, Proc. of the Biochem. Soc., 115, 62P (1969).

9. Clark, A.G., Hirom, P.C., Millburn, P. and Smith, R.L., Absorption of some Organic Compounds from the Biliary System of the Rat, J. Pharm. Pharmac., 23, 150 (1971).

10. Brauer, R.W., Hepatic Blood Supply and the Secretion of Bile, in "The Biliary System", Edited by W. Taylor, Blackwells Scientific Publications, Oxford, 1965, pp. 41-67.

11. Wheeler, A.O. and Ramos, O.L., Bile Flow and Composition after Taurocholate Infusion, J. Clin. Invest., 39, 161 (1960).

12. Sperber, I., Secretion of Organic Anions in the Formation of Urine and Bile, Pharmacol. Rev., 11, 109 (1959).

13. Sperber, I., Biliary Excretion and Choleresis, Proc. First Internat. Pharmacol. Meeting, 4, 137 (1963).

14. Presig, R., Cooper, H.L., and Wheeler, H.O., The Relationship between Taurocholate Secretion Rate and Bile Production in the Dog During Cholinergic Blockade and Secretin Administration, J. Clin. Invest., 41, 1152 (1962).

15. Small, D.M., Dowling, R.H. and Redinger, R.N., The Enterohepatic Circulation of Bile Salts, Arch. Intern. Med., 130, 552 (1972).

16. Fritz, M.E. and Brooks, F.P., Control of Bile Flow in the Cholecystectomized Dog, Am. J. Physiol., 204, 825 (1963).

17. Hofmann, A.F., Clinical Implications of Physicochemical Studies on Bile Salts, Gastroenterology, 48, 484 (1965).

18. Smith, R.L., The Biliary Excretion and Enterohepatic Circulation of Drugs and Other Organic Compounds, Prog. in Drug Res., 9, 299 (1966).

19. Schanker, L.S., Secretion of Organic Compounds in Bile, in "Handbook of Physiology" Vol V, Edited by C.F. Code and W. Heidel, Waverly Press, Inc., Baltimore, Md., 1968, pp. 2433-2450.

20. Williams, R.T., Millburn, P. and Smith, R.L., The Influence of Enterohepatic Circulation on Toxicity of Drugs, N.Y. Acad. Sci., 123, 110 (1965).

21. Millburn, P., Factors in the Biliary Excretion of Organic Compounds, in "Metabolic Conjugation and Metabolic Hydrolysis" Vol II, Edited by Fishman, Academic Press, New York, 1970, pp. 1-74.

22. Schanker, L.S. and Solomon, H.M., Active Transport of Quaternary Ammonium Compounds into Bile, Am. J. Physiol., 204, 829 (1963).

23. Weiner, I.M. and Mudge, G.H., Renal Tubular Mechanisms for Excretion of Organic Acids and Bases, Am. J. of Med., 36, 743 (1964).

24. Hardison, W.G.M. and Apter, J.T., Micellar Theory of Biliary Cholesterol Excretion, Am. J. Physiol., 222, 61 (1972).

25. Bickel, M.H., Metabolism and Biliary Excretion of the Lipophilic Drug Molecules, Imipramine and Desmethylimipramine in the Rat - II (Uptake into Bile Micelles), Biochem. Pharmcol., 19, 2437 (1970).

26. Gartner, L.M., Lane, D.L. and Cornelius, C.E., Bilirubin Transport by Liver in Adult Macaca Mulatta, Am. J. Physiol., 220, 1528 (1971).

27. O'Maille, E.R.L., Richards, T.G., and Short, A.H., Factors Determining the Maximal Rate of Organic Anion Secretion by the Liver and Further Evidence on the Hepatic Site of Action of the Hormone Secretin, J. Physiol., 186, 424 (1966).

28. Udall, V., The Challenge of Selecting the Suitable Animal Species in Toxicology, in "The Problems of Species Difference and Statistics in Toxicology" Vol XI, Edited by Baker, Tripod, and Jacob, Excerpta Medica Foundation, Amsterdam, 1970, pp 70-72.

29. Forsyth, R.P., Nies, A.S., Wyler, F., Neutze, J. and Melmon, K.L., Normal Distribution of Cardiac Output in the Unanesthetized, Restrained Rhesus Monkey, J. Applied Physiol., 25, 736 (1968).

30. Scheline, R.R., Drug Metabolism by Intestinal Microorganisms, J. Pharm. Sci., 57, 2021 (1968).

31. Clark, A.G., Fischer, L.J., Millburn, P., Smith, R.L., and Williams, R.T., The Role of Gut Flora in the Enterohepatic Circulation of Stilboestrol in the Rat, Biochem. J., 112, P17 (1969).

32. Wapole, A.L., Williams, M.H.C., and Roberts, D.C., The Carcinogenic Action of 4-Aminodiphenyl and 3:2'-Dimethyl-4-Aminodiphenyl, Brit. J. Indust. Med., 9, 255 (1952).

33. Dowling, R.H., Mack, E., Picott, J., Berger, J. and Small, D.M., Experimental Model for the Study of the Enterohepatic Circulation of Bile in Rhesus Monkeys, J. Lab. Clin. Med., 72, 169 (1968).

34. Berger, J., Redinger, R. and Small, D.M., Instrument for Sampling and Measuring Bile Flow, Med. and Biol. Engng., 8, 19, (1970).

35. Wiggins, H. and Wooton, I., Studies in the Bile Acids, Biochem. J., 70, 349 (1958).

36. Haslewood, G.A.D., The Biological Significance of Chemical Differences in Bile Salts, Biol. Rev., 39, 537 (1964).

37. Winkler, V.W., Nyman, M.A., and Egan, R.S., Diethylstilbestrol *cis-trans* Isomerization and Estrogen Activity of Diethylstilbestrol Isomers, Steroids, 17, 197 (1971).

38. Grundy, J., Artificial Estrogens, Chem. Rev., 57, 281 (1957).

39. Walton, E., and Brownlee, G., Isomers of Stilboestrol and its Esters, Nature, 151, 305 (1943).

40. Herbst, A.L., Ulfelder, H., Poskanzer, D.C., Adenocarcinoma of the Vagina, New Eng. J. Med., 284, 878 (1971).

41. Greenwald, P., Barlow, J.J., Nasca, P.C., and Burnett, W.S., Vaginal Cancer After Maternal Treatment with Synthetic Estrogens, New Eng. J. Med., 285, 390 (1971).

42. Solmssen, U.V., Synthetic Estrogens and the Relationship Between their Structure and their Activity, Chem. Rev., 37, 481 (1945).

43. Adlercreutz, H., Estrogens in Human Bile, in "The Biliary System", Edited by W. Taylor, Blackwell Scientific Publications, Oxford, 1965, pp. 369-384.

44. Fisher, L.J., Millburn, P., Smith, R.L., and Williams, R.T., The Fate of ^{14}C Stilboestrol in the Rat, Biochem. J., 100, 69P (1966).

45. Hanahan, D.J., Doskalakis, E.G., Edwards, T., and Dauben, H.J., The Metabolic Pattern of ^{14}C-Diethylstilbestrol, Endocrin., 53, 163 (1953).

46. Twombly, G.H., and Schoenewaldt, E.F., Tissue Localization and Excretion Routes of Radioactive Diethylstilbestrol, Cancer, 4, 296 (1951).

47. Dodgson, K.S., Garton, G.A., Stubbs, A.L., and Williams, R.T., Studies in Detoxication - On the Glucuronides of Stilboestrol, Hexoestrol, and Dienoestrol, Biochem. J., 42, 357 (1948).

48. Despopoulos, A., Hepatic and Renal Excretory Metabolism of Bile Salts: A Background for Understanding Steroid Induced Cholestasis, J. Pharmacol. Exptl. Ther., 176, 273 (1971).

49. Schersten, T., Relationship Between the Biliary Excretion of Bile Acids and the Excretion of Water, Lecithin, and Cholesterol in Man, Eur. J. Clin. Invest., 1, 242 (1971).

50. Sperber, I., The Biliary Excretion and Choleretic Effect of Some Phenolsulphonephthaleins, Acta Physiol. Scand., 42: Suppl. 145: 129 (1957).

51. Keberle, H., Hoffmann, K., and Bernhard, K., The Metabolism of Glutethimide (Doriden), Experientia, 18, 105 (1962).

52. Hofmann, A.F., The Function of Bile Salts in Fat Absorption, Biochem. J., 89, 57 (1963).

SURGERY, MEDICAL CARE AND MAINTENANCE OF THE RHESUS MONKEY

Male rhesus monkeys, species - Macaca Mulatta, weighing from 4.1 to 5.5 kilograms were used. These monkeys are distributed through the New York Primate Laboratory which obtains them from India. Immediately after arrival, the monkeys are placed in quarantine for a period of not less than five weeks. This screening period is necessary since the use of animals nearer to man on the evolutionary scale brings with it the problems of contagious diseases which can be easily transmitted from monkey to man. During the quarantine the monkeys are checked for viral infections, parasites and tuberculosis. It is of particular importance to rule out the presence of Virus B, an endemic disease of rhesus monkeys which may cause encephalitis in man with a very high mortality rate.

Prior to surgery, the monkey can be immobilized with ketamine HCl (Vetalar-Parke Davis), 10 mg/Kg by intramuscular injection. This renders the monkey immobile but not unconscious and he can then be easily handled. After transferring the monkey out of the cage one can administer a hypnotic dose of sodium pentobarbital (Diabutal-Diamond Labs), 30 mg/Kg by intravenous injection.

The initial surgery to be performed involves insertion of permanent arterial-venous catheters. First the monkey is shaved over the lower abdominal region and prepared with tincture of iodine solution. A left flank incision is then made running obliquely from the iliac crest to the midinguinal region. The left external iliac vein and artery are then exposed and polyvinyl catheters (Resinite #20-Western Fibrous Glass Prod. Co.) introduced and passed cephalad, until the tips of the catheters reach the inferior vena cava and descending aorta distal to the renal arteries.

The catheters were then sutured firmly to fascia before being exteriorized through puncture holes in the skin near the umbilicus. Post surgically the monkey is allowed to recover in a prone position after he has been placed in a primate chair and kept on his back until completely conscious. The monkey is then set upright and both catheters connected to an infusion pump (Harvard Apparatus) containing 5 units of heparin per ml of normal saline (see plate I). This heparinized saline is pumped through the catheters at a rate of 1 ml per hour per syringe. In addition, each day the catheters are flushed with about 8 ml of heparinized saline to insure that no clots develop. Using this procedure the catheters may be kept functional for months. During a given experiment one can administer drug as a bolus or infusion via the venous catheter and obtain blood samples from the arterial catheter. If an infection develops post surgically a penicillin-streptomycin combination (Streptillin-Trico Pharm. Co.) can be administered intramuscularly - 1 ml per day for five days. Generally, the monkey is not used experimentally for at least 10 days after surgery.

Two weeks after implantation of the catheters, hepatic bile duct surgery was performed. The monkey was prepared as before but additionally, food was withheld for 24 hours prior to surgery. Water was given ad libitum.

An abdominal midline incision was made and the external hepatic ducts exposed (see plate II). The cystic duct was isolated and severed from the common bile duct. The proximal and distal ends of the severed cystic duct were ligated and the gall bladder left in place. Then the common bile duct was severed from the duodenum just above the sphincter of Oddi and the duodenal portion of the duct ligated. A latex T-tube (size 8 Fr. Bardex-C.R. Bard, Inc.) was inserted into the common bile duct via an incision distal to the point of entry of the ligated cystic duct. One arm of the T-tube was

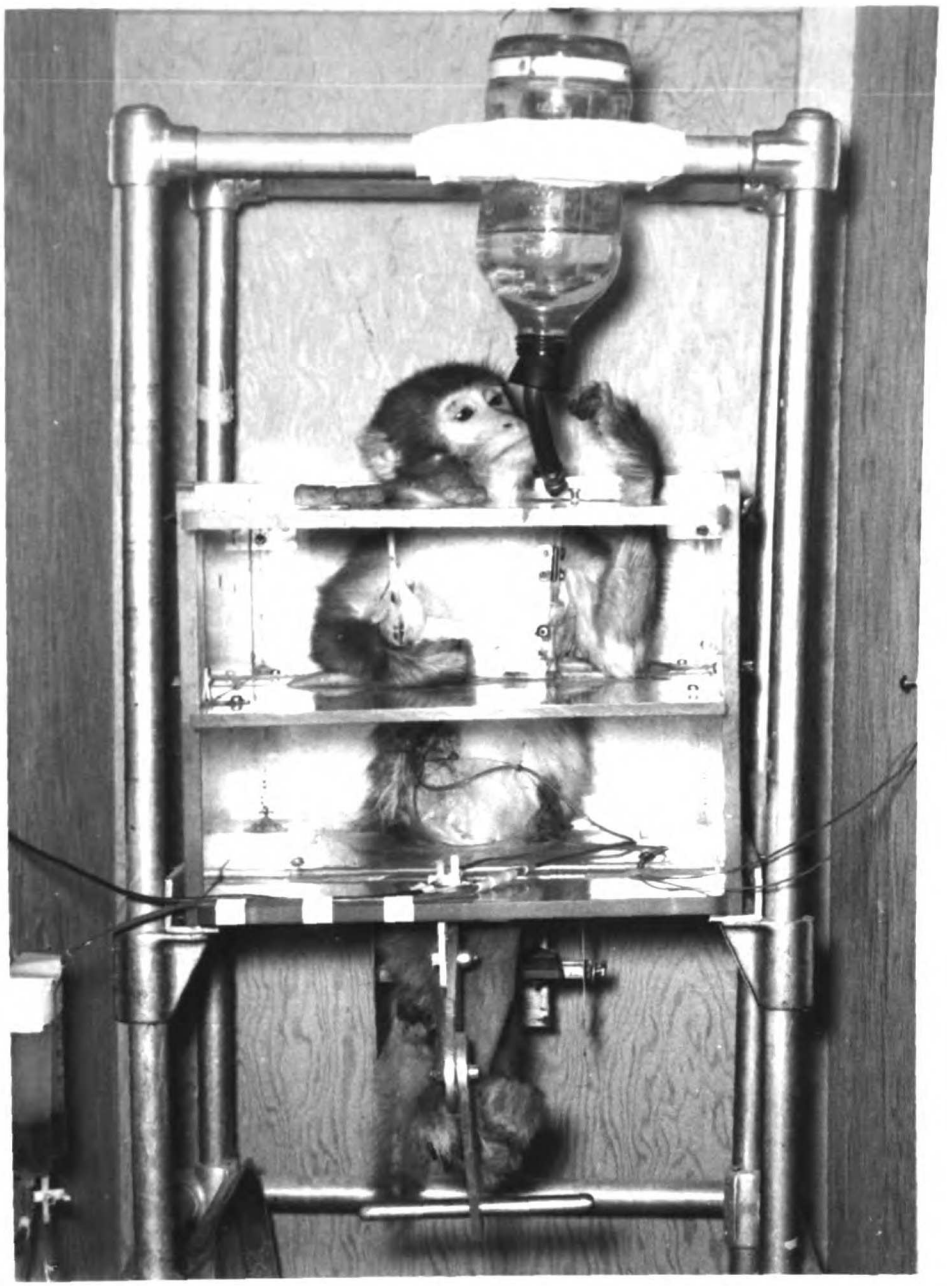


Plate I - Rhesus monkey in primate chair.

Plate II -

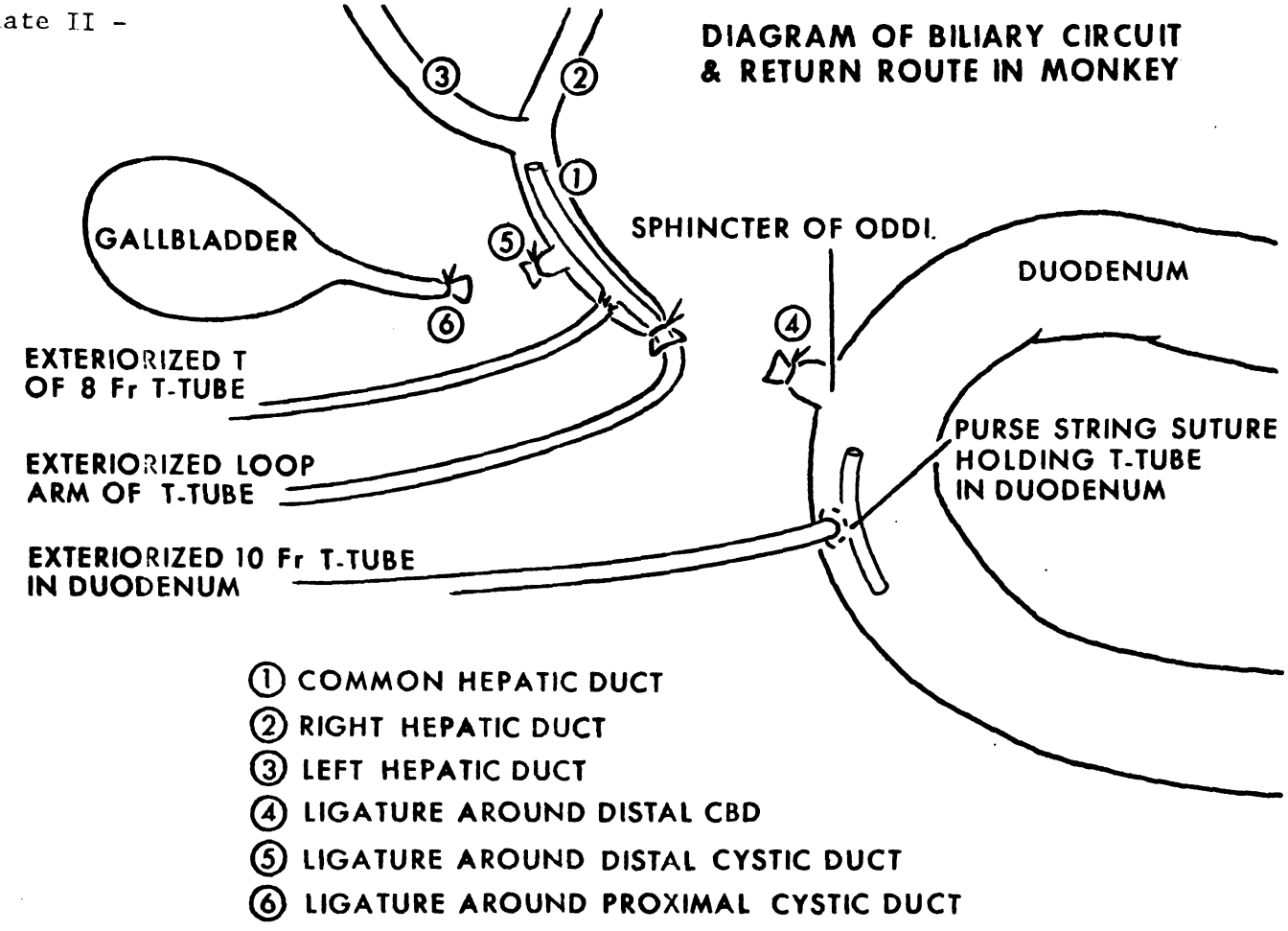


DIAGRAM OF BILIARY CIRCUIT
& RETURN ROUTE IN MONKEY
GALLBLADDER
SPHINCTER OF ODDI.
DUODENUM
EXTERIORIZED T
OF 8 Fr T-TUBE
EXTERIORIZED LOOP
ARM OF T-TUBE
EXTERIORIZED 10 Fr T-TUBE
IN DUODENUM
PURSE STRING SUTURE
HOLDING T-TUBE
IN DUODENUM
① COMMON HEPATIC DUCT
② RIGHT HEPATIC DUCT
③ LEFT HEPATIC DUCT
④ LIGATURE AROUND DISTAL CBD
⑤ LIGATURE AROUND DISTAL CYSTIC DUCT
⑥ LIGATURE AROUND PROXIMAL CYSTIC DUCT

previously cut so as to be positioned just below the juncture of the right and left hepatic bile ducts while the other arm was passed through the distal portion of the common bile duct previously severed from the duodenum. Both the straight length "T" portion and the distal arm of the T-tube were then exteriorized through punch holes in the skin. The T-tube was secured into the common bile duct by ties at both points of exit. This type of circuit has the advantage of anchoring the T-tube into place and also allows one to flush the line periodically as necessary. A second T-tube (size 10 Fr.) was inserted into the proximal third of the duodenum, approximately opposite to the sphincter of Oddi and the straight "T" portion of the tube was exteriorized through a punch hole in the skin. A purse string suture was made to hold the duodenal tube in place. The abdomen was closed in layers, and the monkey again was placed in the primate chair and allowed to recover from anesthesia in a prone position. Food was withheld for 2 to 3 days after surgery but water given ad libitum. Following the operation 1 ml of penicillin-streptomycin was given daily by intramuscular injection for 5 days. This circuit allows complete drainage from the hepatic ducts, bypassing the gall bladder, and returns bile to the duodenum at approximately the same site as would occur under physiologic conditions. It should be noted that in one monkey only the straight "T" length portion of the hepatic T-tube was exteriorized while both arms of the tube, suitably cut, were placed into the common bile duct, the distal portion of the duct was ligated.

After surgery the exteriorized hepatic and duodenal tubes (plate III) were connected via a peristaltic pump (Harvard) in order to maintain a normal enterohepatic circulation of bile when the monkey is not being used (plate IV). The interposing of a peristaltic pump was necessary since

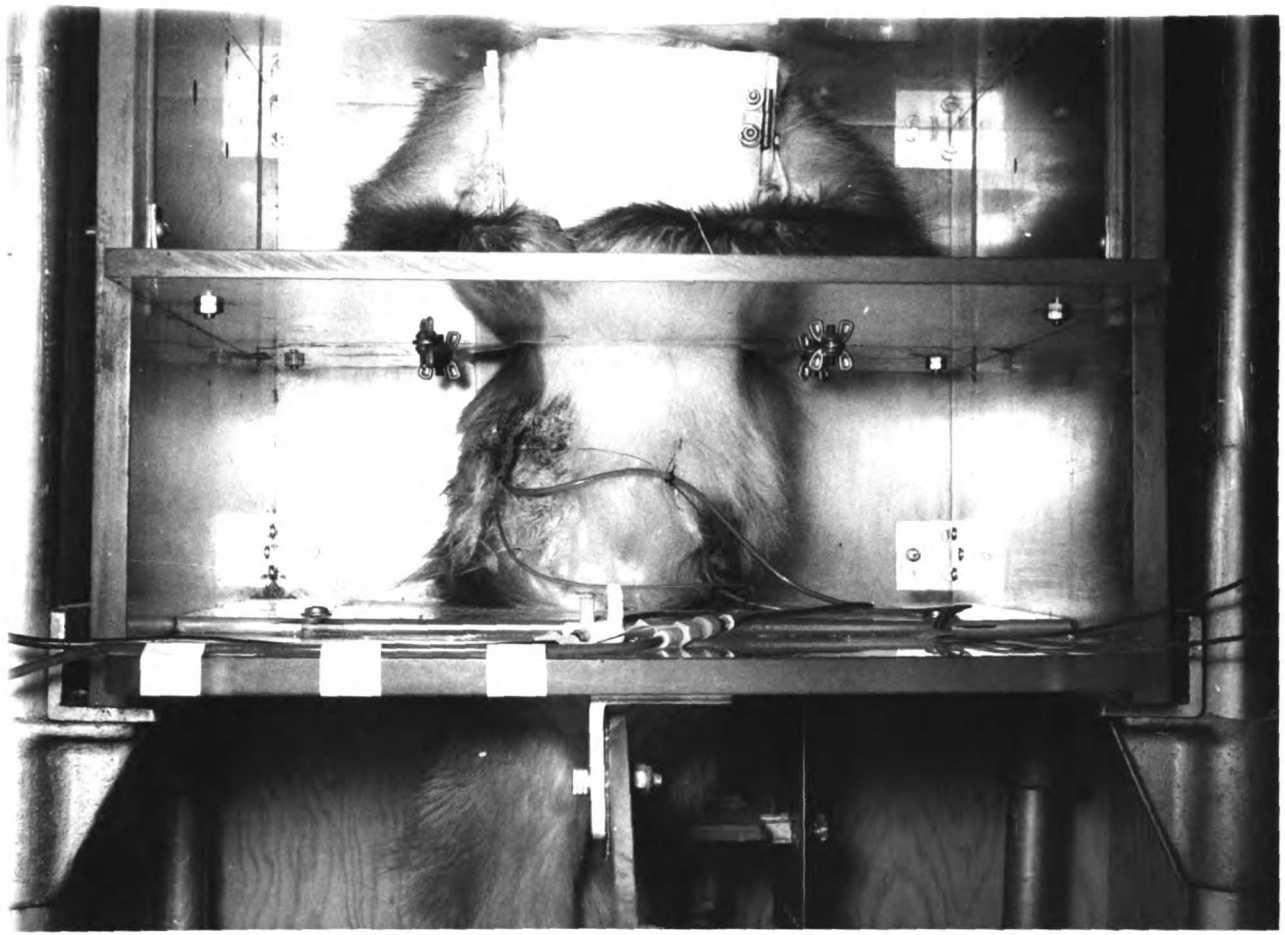


Plate III - Abdominally exteriorized hepatic (large diameter) and duodenal (small diameter) catheters. The arterial and venous catheters which were exteriorized from the lower left quadrant of the abdomen can also be seen.

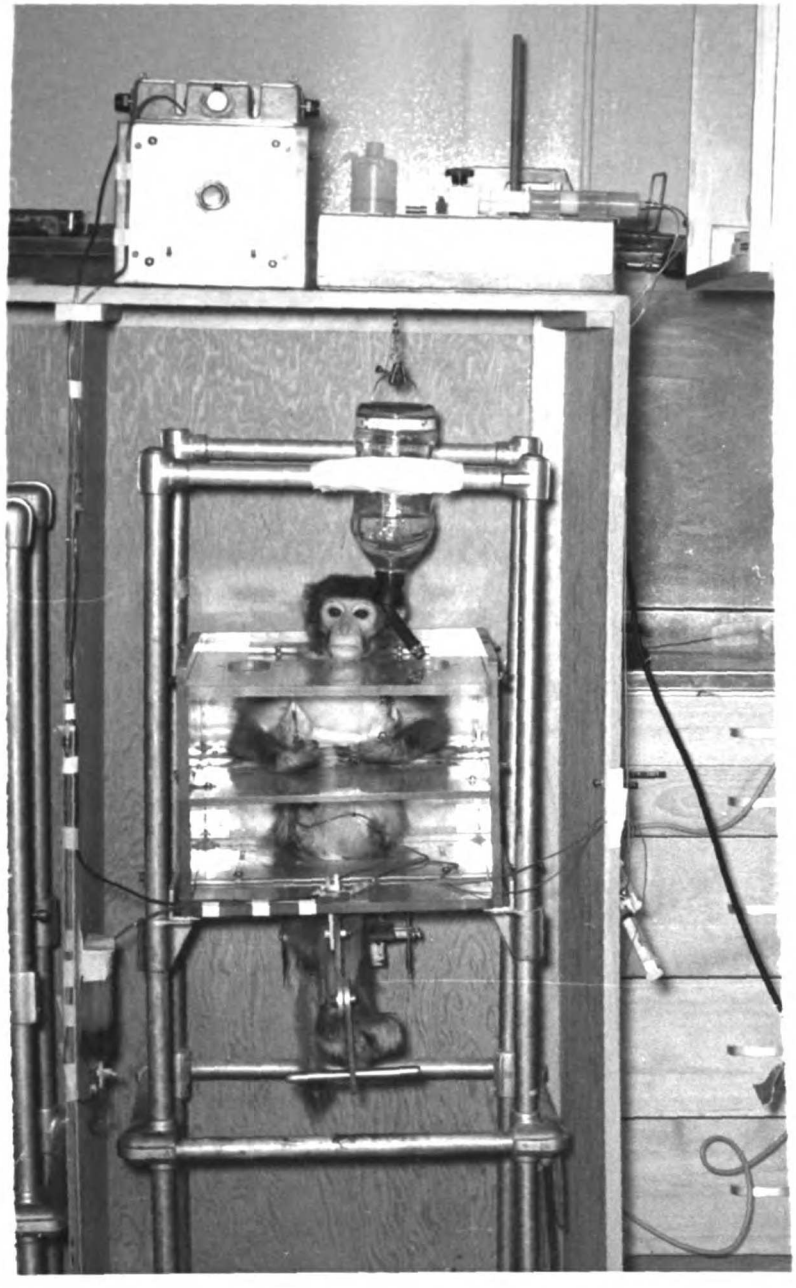


Plate IV - Full view of a surgically prepared rhesus monkey in a primate chair. Bile from the hepatic catheter is collected into reservoirs located to the right of the chair and then driven into the duodenal catheter by means of a peristaltic pump located above the chair. A constant intravenous and intra-arterial infusion of heparinized saline is maintained by means of an infusion pump also located above the chair.

precipitation of phospholipids and cholesterol, which occasionally occurs, caused clogging of the tubes and prevented normal hepatic bile pressures from maintaining proper drainage into the duodenum. This precipitation can cause considerable problems and one must flush the lines daily to prevent clogging.

In addition to the above precautions a regular routine of maintenance is required to keep the monkeys healthy. To prevent pressure sores, it is necessary to clean and dry the metal bars of the monkey seat each day and to inspect vulnerable areas where pressure necrosis may result. Various types of padding may be placed under the armpits and on the midplate where the monkey rests his elbows. The monkey can be immobilized with ketamine if it is necessary to remove him from the chair for care and cleaning.

The monkey is fed a regular diet of monkey chow (Purina) and water ad libitum. Also, it may be necessary to supplement his diet parenterally if blood tests warrent it with intramuscular Imferon (Lakeside), Pluribex (Pasadena Res. Lab.) and intravenous Ambex (Elanco Prod. Co. - Lilly). If well-cared for, a given monkey is well worth the cost and time of setting up. In our laboratories several monkeys have lasted a year and longer.

Hence, a monkey suitably prepared allows one to readily administer drugs parenterally and take blood samples without disturbing the monkey. The biliary circuit allows one to collect bile 100% or return it, if desired, via the duodenal tube. It is also possible to give an "oral" dose via the duodenal tube. Urine can be collected by means of a urine tray placed beneath the primate chair. All of this can be done without anesthesia or trauma to the monkey, approximating physiologic conditions.

BIOSYNTHESIS OF SODIUM DIETHYLSTILBESTROL MONOGLUCURONIDE USING NEW ZEALAND WHITE RABBITS

Diethylstilbestrol monoglucuronide (sodium salt) was prepared biosynthetically in rabbits after the method of Dodgson (1). Female, New Zealand white rabbits weighing from 4 to 6 kilograms were used. After several attempts with the former method which was not very successful several modifications were made.

Diethylstilbestrol (Matheson Coleman and Bell) was used as supplied without further purification. An intravenous solution was prepared by dissolving 200 mg of diethylstilbestrol in 2.0 ml of absolute ethanol which was then transferred to a 200 ml volumetric flask. This was well mixed with 50 ml of PEG-400 and then made up to 100 ml with sterile distilled water containing 500 units of heparin. This solution was administered via an ear vein at the rate of 0.25 ml per minute. The urinary bladder was cannulated and urine was collected over dry ice for two days following the infusion. Urine was thawed (just prior to extraction), adjusted to pH 3.0 and continuously extracted with a threefold volume of ether for 9 to 18 hours. The ether extracts were combined and evaporated to a volume of 100 ml and then 30 ml of a saturated solution of sodium bicarbonate was added. The sodium salt of diethylstilbestrol monoglucuronide precipitates at the interface. The crude product is collected, dried and dissolved in a minimum volume of methanol and recrystallized by addition of ethyl acetate. Subsequent recrystallizations are made from a methanol-ethyl acetate solution. A typical experiment produced 111 mg of metabolite, representing a 32% yield.

A portion of the sample was further dried over P_2O_5 at 78°C in a 5μ vacuum for 12 hours and subjected to elemental analysis. The analysis

found C-59.20% and H-6.03% which would correspond to the monohydrate of sodium diethylstilbestrol monoglucuronide which calculates to C-59.50% and H-6.05% both of which are $\pm$ 0.2 to 0.4%. To rule out the possibility of aromatic hydroxylation an IR spectrum of both diethylstilbestrol and its glucuronide was run (plate V) in KBr pressed discs. The spectrum shows characteristic evidence of 1-4 disubstitution of the aromatic ring between 800 to 900 cm-1 for both diethylstilbestrol and metabolite. One can also observe evidence for an ether linkage and alicyclic secondary alcohols (glucuronide) present between 1000 and 1100 cm^{-1} for metabolite but not parent compound. This evidence along with that of Dodgsen et al provides further support that the product isolated is in fact sodium diethylstilbestrol monoglucuronide.

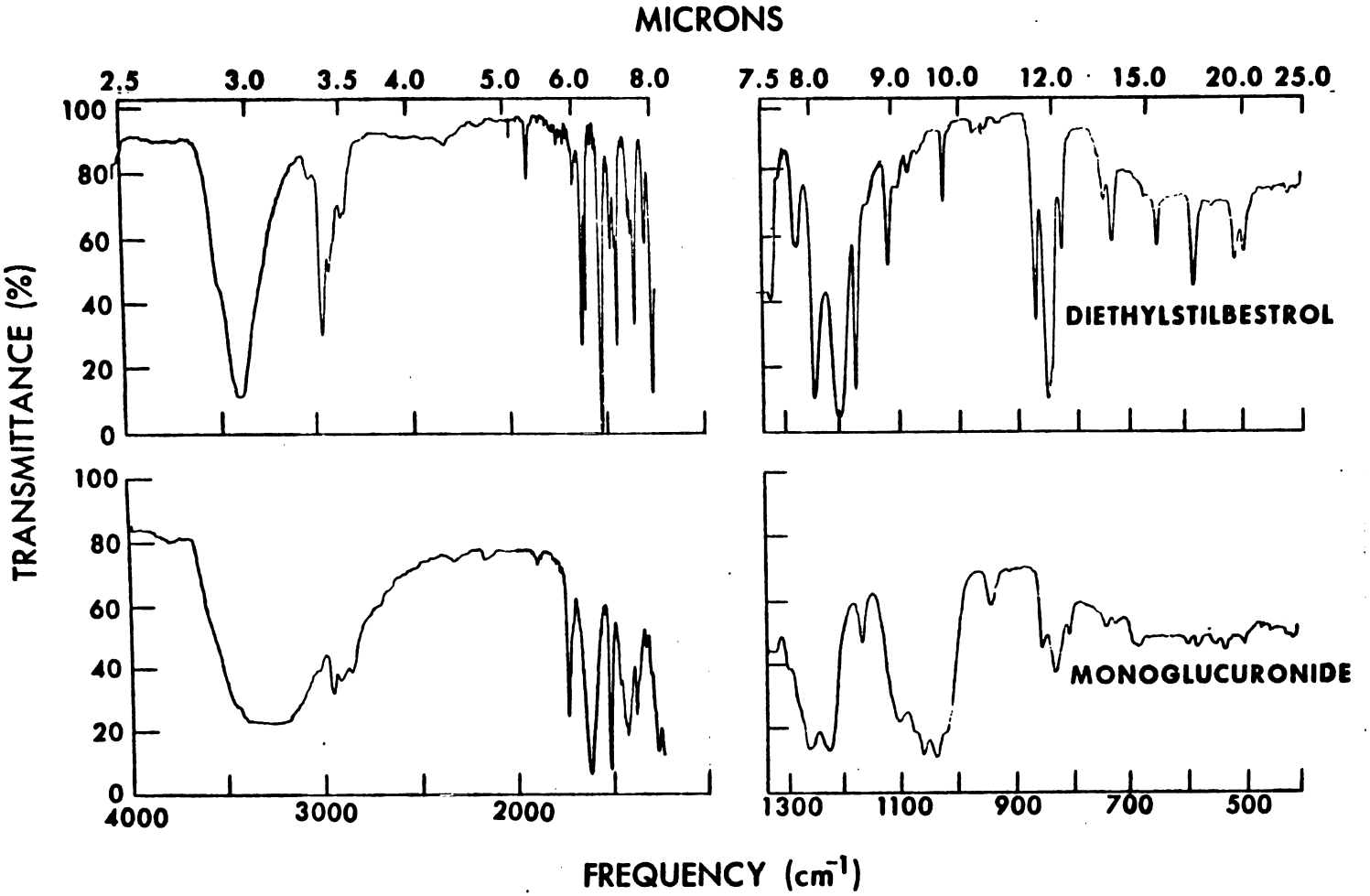


Plate V - Comparative infrared spectrograms of diethylstilbestrol and diethylstilbestrol monoglucuronide (monohydrate).

ANALYTICAL METHODS

In Vitro Assay and Separation Techniques for Diethylstilbestrol and Sodium Diethylstilbestrol Monoglucuronide

Non-radioactive diethylstilbestrol and its monoglucuronide were studied using various TLC and solvent extraction methods to determine an efficient method of separation. Unlabeled compound could be readily analyzed using direct ultraviolet spectrophotometric absorption techniques. Various other assays for non-radioactive diethylstilbestrol are also available (2,3,4). Diethylstilbestrol and metabolite were dissolved in 95% ethanol for all absorption measurements which were performed on the Cary 15 spectrophotometer. Diethylstilbestrol and diethylstilbestrol monoglucuronide both absorb maximally at 235 nm in 95% ethanol and exhibit linearity (Fig. 1) in the concentration ranges studied.

Thin layer chromatography (TLC) studies of diethylstilbestrol and metabolite were done using 5 x 20 cm glass silica gel plates, of 0.25 mm layer thickness, (EM Reagents Div. - Westbury, N.Y.). The compounds were dissolved in methanol (Spectroquality-MCB) as a 10 mg % solution and 0.05 to 0.10 ml were streaked onto the plate which was then developed in a solvent system of reagent grade n-propanol and concentrated aqueous ammonia (7:3). A second development system was prepared with reagent grade n-butanol and ammonium carbonate buffer (4:2). Development times ranged from six to eight hours. Immediately after air drying, the plates were placed on a TLC scraping device (Analabs-North Haven, Conn) and 5 mm segments were scraped into wide mouth vials. Diethylstilbestrol and metabolite were eluted from the silica gel with 95% ethanol and absorbence was measured after centrifugation. Additionally, some TLC plates were simply

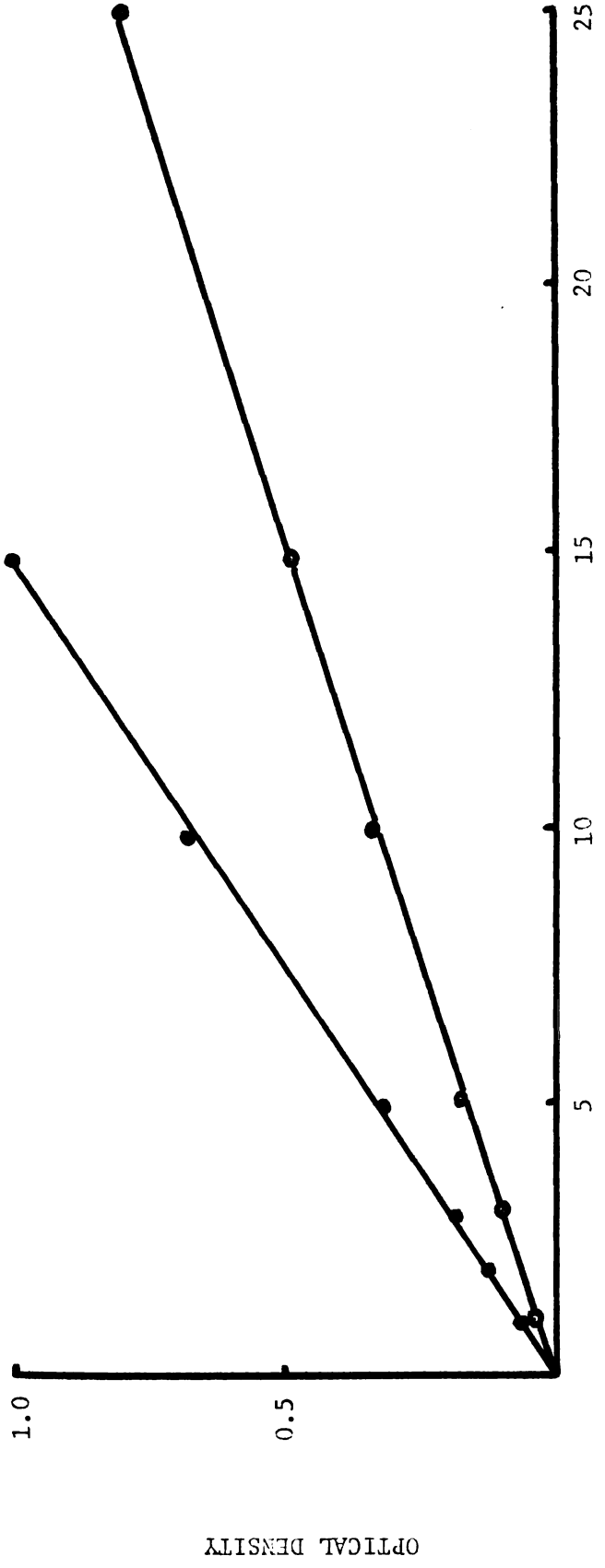


Figure 1 - Standard curves for diethylstilbestrol (●) and diethylstilbestrol monoglucuronide (●) in 95% ethanol.

visualized under a UV light source after development to determine R_f values. Chromatography studies were carried out in the two systems described for diethylstilbestrol and metabolite. A plot of optical density versus distance along the TLC plate indicates good separation using both systems (Figs:2,3). In the n-propanol plus aqueous ammonia system the average R_f with standard deviation in parenthesis for diethylstilbestrol was 0.874 (.044) and diethylstilbestrol monoglucuronide 0.372 (.042). In the n-butanol plus ammonium carbonate buffer system one obtains an R_f of 0.874 (.044) for diethylstilbestrol and 0.095 (.009) for diethylstilbestrol monoglucuronide. A similar pattern of chromatographic behavior was observed for these compounds by Fischer and Millburn (5).

Both compounds could also be separated using the Folch extraction procedure (6). Basically, this procedure employs a chloroform-methanol-water system which when combined in the proper proportions will cause a separation into two phases. The chloroform phase will contain non polar substances such as diethylstilbestrol while the methanol-water phase will contain the polar glucuronide. This extraction procedure will be further detailed in the bile salt assay section where it was also used with an additional precipitation step. However, in these studies the precipitation step was not included because the glucuronide is precipitated. Instead, methanol or ethanol solutions were prepared and aliquots transferred to test tubes which were then evaporated. One then can add 0.1 ml of distilled water and the chloroform-methanol solution and proceed as described in the bile salt assay section. After separation of the two phases, they are evaporated, the residue dissolved in 95% ethanol, and measured spectrophotometrically. In a typical experiment in which diethylstilbestrol and metabolite were studied separately in ethanol solutions, it was found

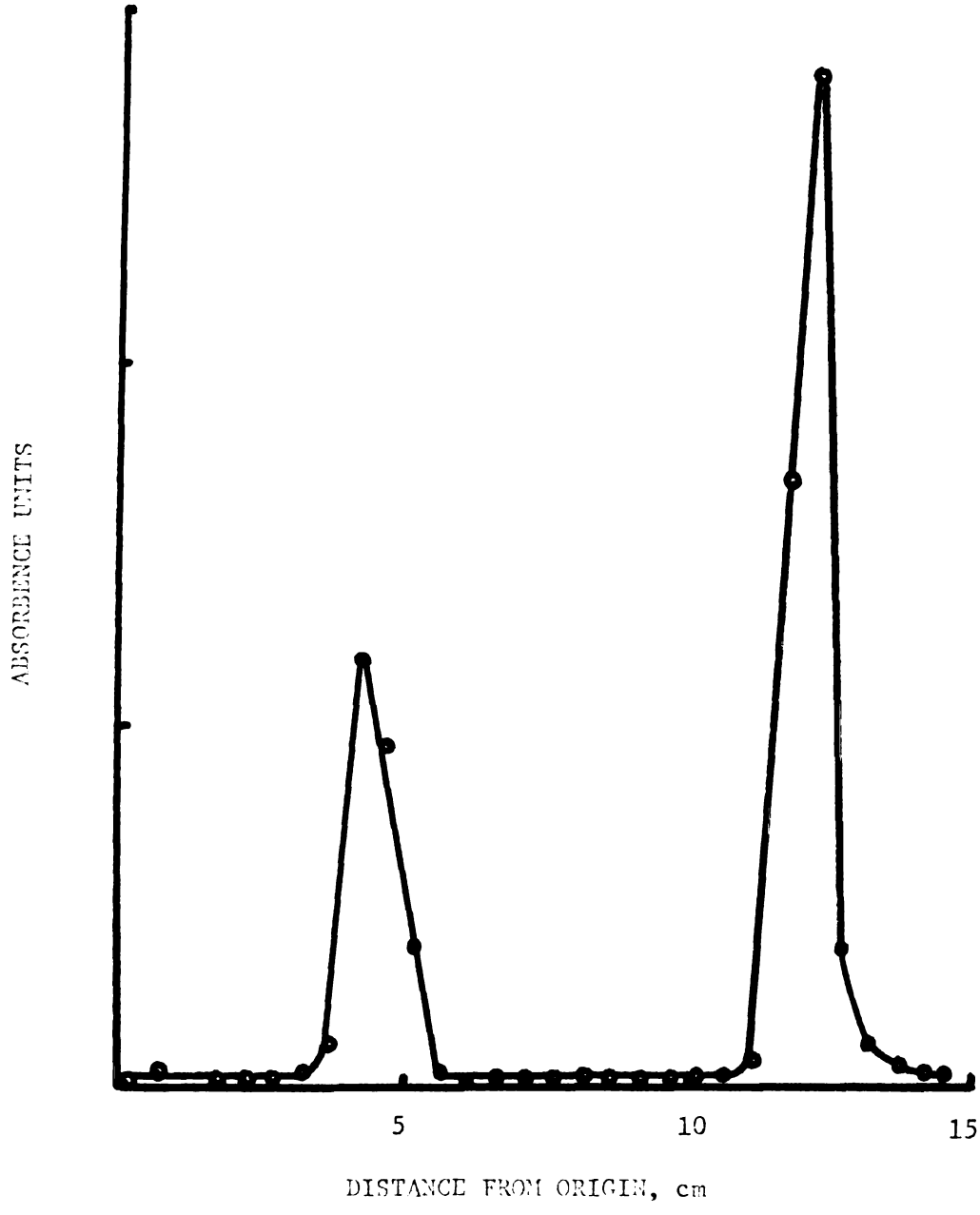


Figure 2 - Thin layer chromatogram of diethylstilbestrol (R_f=.86) and diethylstilbestrol monoglucuronide (R_f=.32) standards developed in the n-propanol plus aqueous ammonia (7:3) system.

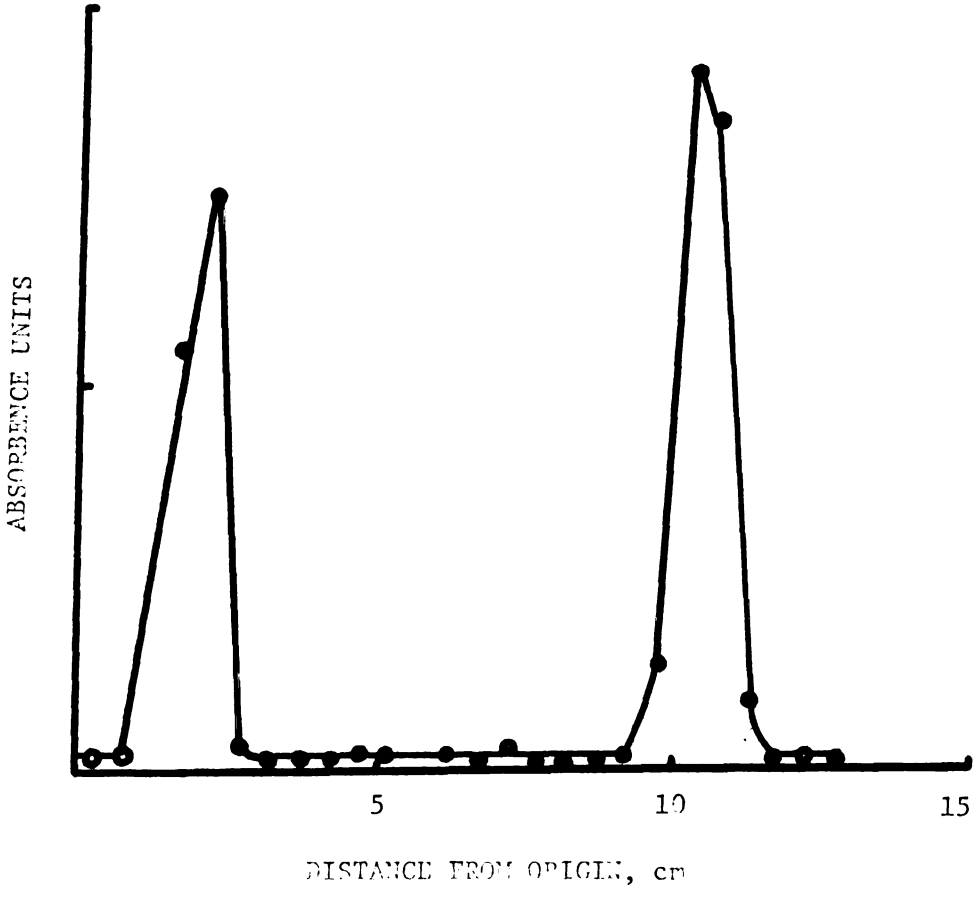


Figure 3 - Thin layer chromatogram of diethylstilbestrol (R_f=.81) and diethylstilbestrol monoglucuronide (R_f=.092) standards developed in the n-butanol plus ammonium carbonate buffer (4:2) system.

that diethylstilbestrol partitions into the methanol-water phase to the extent of 2.8% while 97.5% resides in the non-polar chloroform phase. Diethylstilbestrol monoglucuronide, however, partitions mainly into the polar methanol-water phase (94.0%) while almost none (0.1%) can be found in the chloroform phase. These experiments were carried out using a total amount of 100 μ gm of diethylstilbestrol and metabolite, an amount well above what one would encounter in biologic samples. Another series of experiments were done at lower levels and with diethylstilbestrol and metabolite present together in the initial solutions. This was accomplished by using radioactive diethylstilbestrol-^{14}C of known specific activity (the assay of which will be described in the next section) and cold metabolite - allowing one to assay for each independently, while both are present in the same system. Table 1 clearly demonstrates that efficient and reproducible separation is possible with varying combinations of diethylstilbestrol and metabolite present in the same initial ethanolic solution. The average per cent recovery (standard deviation in parenthesis)for diethylstilbestrol in the chloroform phase is 95.3 (1.60) and in the methanol-water phase 4.48 (0.05) with a total recovery of 99.7 (1.60). However, for diethylstilbestrol monoglucuronide one finds that 96.4% (4.13) partitions into the polar methanol-water phase while none could be detected in chloroform giving a total recovery of 96.4% (4.13).

Characterization of Diethylstilbestrol Monoethyl-1-C14

Diethylstilbestrol monoethyl-1-C14 of specific activity 39 m Ci/mmol was obtained from Amersham/Searle Corporation. Radiochemical purity at the time of purchase, determined by chromatography, was 98 to 99%. It is stored at room temperature as a dilute benzene solution sealed under nitrogen.

Table 1 - Recovery of Diethylstilbestrol (DES) and Sodium Diethylstilbestrol Monoglucuronide (NaDESGA) Separately and When Combined in Various Combinations and Carried Through the Folch Extraction Procedure.

Amount present in initial solution (μgram)		Per cent recovery[a] Methanol-water phase	Chloroform phase	Combined total
DES	10.20	4.5	96.9	101.4
NaDESGA	-	-	-	-
DES	7.65	4.5	93.1	97.6
NaDESGA	2.00	97.5	0.0	97.5
DES	5.10	4.5	95.8	100.3
NaDESGA	4.00	101.0	0.0	101.3
DES	2.55	4.4	95.2	99.6
NaDESGA	6.00	91.4	0.0	91.4
DES	-	-	-	-
NaDESGA	8.00	95.4	0.0	95.4

[a] Each figure represents an average of two experiments.

Prior to usage 1 μL (293,000 DPM) was tested chromatographically in the n-propanol plus aqueous ammonia system. After development, the TLC plate was scanned using the Autochron Radio Scanner (Varian Aerograph, Berthold). Figure 4 indicates that there are no radiochemical impurities detectable using this particular system and that the R_f value is the same one obtained using cold diethylstilbestrol.

Various Aspects of Liquid Scintillation Counting

Once the disintegration of a radionuclide begins, the probability of this disintegration is characteristic of the half-life of the isotope and because of the large number of events involved, follows a statistical law such as the normal or Gaussian distribution. Therefore, the accuracy of an observed radioactivity measurement is generally expressed in terms of deviation from the distribution law. In radioactive counting measurement the normal distribution law can be substituted by the Poisson distribution law allowing greater ease in the manipulation of counting data. When the number of observed events (counts) is relatively large, the standard deviation as calculated from both the normal and the Poisson distribution laws are almost identical in value.

The standard deviation (σ) of a given number of gross counts (n) is:

$$\sigma = \sqrt{n}$$

regardless of the length of time required to accumulate the counts. When the standard deviation is expressed as percentage error, the following expression is used:

$$\sigma\ \% = 100/\sqrt{n}$$

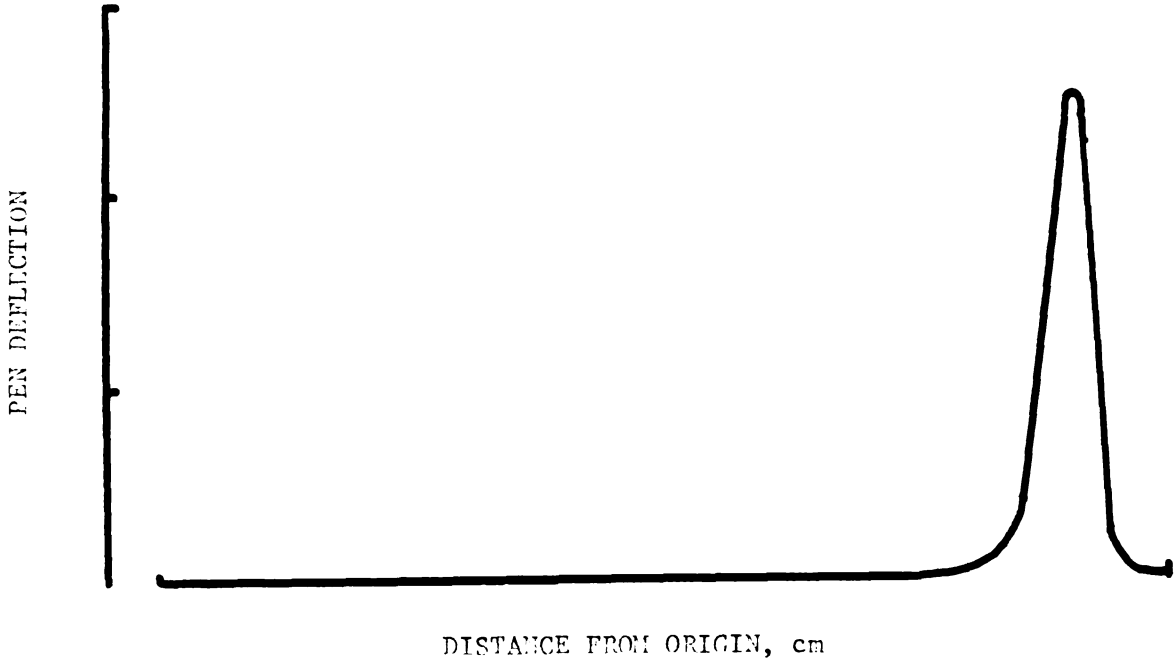


Figure 4 - Radioscan of diethylstilbestrol-^{14}C (R_f=.90) developed in the n-propanol plus aqueous ammonia (7:3) system. The following conditions were employed in obtaining the scan: measure range = 300, time constant = 3, and scan speed = 100 inches per hour.

Therefore to obtain a percent standard deviation of 1, one must accumulate 10,000 gross counts per minute. Similarly, if one obtains at least 5,000 gross counts per minute (cpm), σ % = 1.41. In all experiments a gross count of at least 5000 cpm was strived for. In addition to gross cpm one must also consider net cpm, that is the counts due to the activity of the sample less background. Some of the blood samples approach background and require a large number of counts (long counting time) to achieve an acceptable level of significance. With care in the handling and cleaning of equipment, such low level counting can be done. When samples which showed less than twice background counts are included in the data they will be pointed out since the degree of confidence here is greatly diminished. It should be noted that only a few of the blood samples approach a level of twice background or less, while the majority of samples particularly bile and urine greatly exceed background.

All liquid scintillation counting was done on the Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375. Prior to counting, radioactive compound was incorporated into 1.8 ml of methanol (spectroquality) or 100% ethanol (Rossville Gold Shield) which was placed into high quality glass or plastic counting vials (Scientific Products). Then 10 ml of a toluene (Scintillation Grade-Eastman) cocktail,prepared by adding 4 grams of Omnifluor (New England Nuclear) to one liter of toluene,was added. The following procedure was used in those assays involving TLC and subsequent liquid scintillation counting. After scraping into counting vials, 1.8 ml of methanol was added to the vial which was shaken for 15 minutes to elute all radioactivity from the silica gel. Then 10 ml of toluene cocktail was added, mixed well and allowed to settle for 24 hours in a refrigerator prior to counting. In those assays involving the Folch extrac-

tion procedure, both phases were completely evaporated after separation, the residue redissolved in 2.0 ml of methanol and a 1.8 ml aliquot was then transferred to a counting vial to which 10 ml of toluene was added.

The Packard Model 3375 has an Automatic External Standardization (AES) capacity which was used to correct for quenching. Since the most serious quenching to be encountered would be due to the bile samples which impart a yellow color to the cocktail, picric acid in methanol (10 mg/ml) was used to construct a quench curve (Fig. 5). As one adds methanol alone (1.8 ml) to toluene (10 ml) and then methanol containing picric acid to toluene the AES ratio will decline due to the increased quenching of the system. A plot of this ratio versus the per cent efficiency of counting results in a smooth curve on cartesian-coordinate graph paper. A known amount of toluene-^{14}C standard is added to each quenched sample allowing one to calculate the efficiency of counting of the sample.

Diethylstilbestrol-^{14}C

Recovery from Blood, Bile, and Urine

The recovery of diethylstilbestrol-^{14}C was measured from blood, bile and urine obtained fresh from a rhesus monkey. Diethylstilbestrol-^{14}C was initially dissolved in methanol and an aliquot of this was transferred to a screw cap test tube (29,300 DPM, equivalent to 0.0913 μgm). The methanol was evaporated and 0.1 ml of fresh whole blood, bile or urine was added to the test tube and mixed well. The test tubes were then placed in a water bath at 37°C for 60 to 90 minutes. Following this, 1.9 ml of absolute ethanol was added, mixed well and the samples were centrifuged at 2400 rpm for 10 minutes. A 1.8 ml aliquot of the supernatant was transferred to a counting vial and 10 ml of toluene cocktail added and the sample was

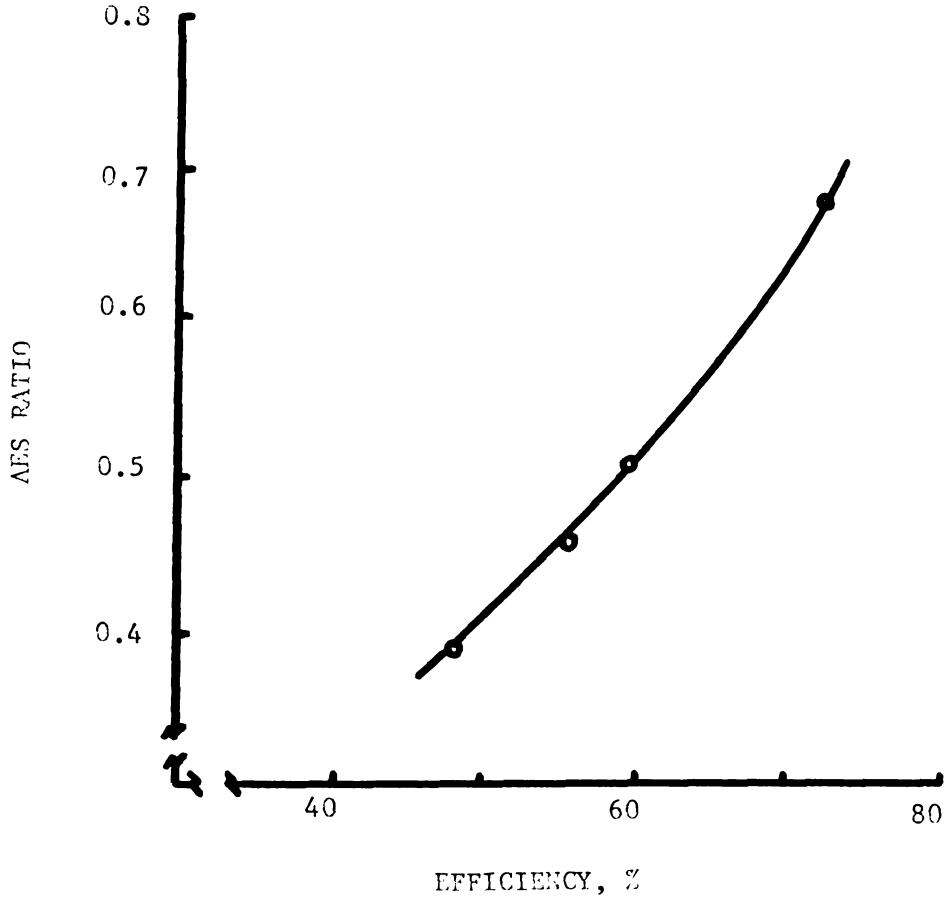


Figure 5 - Automatic External Standardization (AES) ratio versus counting efficiency in variably quenched samples containing toluene-^{14}C.

counted. In some experiments, the 1.8 ml ethanolic supernatant was transferred to another test tube, evaporated, and the residue redissolved in 0.1 ml of methanol. This volume was streaked onto TLC plates which were developed in the n-propanol plus aqueous ammonia system. This latter procedure was carried out to exclude the possibility of chemical alteration of parent compound in the various body fluids to be studied.

Blood recovery (standard deviation in parenthesis) averaged 101.2 (1.77)%, bile 98.9 (1.11)% and urine 103.8 (1.76)%. All three systems showed only one peak after TLC with an average R_f (standard deviation in parenthesis) in blood of 0.890 (.008), bile 0.908 (.017) and urine 0.875 (.024). Therefore, one can conclude that recovery from all the biologic fluids studied is essentially 100% (following ethanol precipitation) and that no metabolic alteration of parent compound occurs in these fluids (that can be detected in the TLC system used).

Rapid Spectrofluorometric Assay For Total Bile Salts in Bile

There are a number of assays available for the determination of bile salts[1] in various biological fluids. By far the most widely used method is gas-liquid chromatography (GLC), which is highly sensitive and specific for determination of the individual bile salts (7). However, if one is interested in total bile salts only, GLC can prove to be too time-consuming and costly. An enzymatic assay is also available, applicable to serum and other biological fluids (8,9). This is a reliable assay for total bile salts and can be used for the individual bile salts if the appropriate separation

[1]The term "bile salts" as used here refers to the sodium salts of the bile acids conjugated with taurine or glycine.

procedures are carried out before the assay. However, significant problems are encountered: preparation, purification, and standardization of the enzyme; preparation of the multiple component solutions; adherence to standard conditions; and finally, instability of the enzyme and cofactor used.

A number of spectrophotometric assays for bile salts have been developed. One of these (10) involves hydrolysis of the bile salts, a series of extractions, treatment in 65% sulfuric acid, and ultraviolet absorption analysis. This assay requires the simultaneous measurement of cholic and deoxycholic acids. A similar assay (11) involves many tedious extractions in the separation of glycine and taurine conjugates, and color development with furfural solution. It also requires simultaneous measurement of cholic and deoxycholic acids in bile. However, it does not require hydrolysis of the conjugates, which is known to cause structural alterations of the bile acid molecule that result in errors in the subsequent results (12). Each of the above spectrophotometric assays fails to recognize the presence of other bile acids, namely chenodeoxycholate and lithocholate, the former being present in high concentrations in human bile.

The method presented below is based on the well-known fact that the bile acids fluoresce after treatment with sulfuric acid (13). A number of workers have made use of this characteristic in the determination of bile acids in blood and bile. Levin et al. (14) used fluorescence to measure total bile salts in bile. However, before sulfuric acid treatment the bile acid conjugates were hydrolyzed, followed by a series of extractions. Also, the sample volume required for this method is much larger than in the method reported here. However, the assay does take advantage

of the fact that after specific treatment with sulfuric acid, the bile acids have fluorescence spectra that are sufficiently similar to permit the determination of total bile acids in unresolved mixtures of these acids. Panveliwalla et al. (15) used fluorometry after thin-layer chromatographic separation to measure individual bile salts in various biological fluids. This group did not hydrolyze the bile salts before treatment with sulfuric acid and were able to assay for the bile salts with good sensitivity.

The method presented here (16) is applicable to those procedures in which a rapid yet reliable assay of total bile salts in bile is desired. It involves a single precipitation and extraction step and then treatment with concentrated sulfuric acid under such conditions that the fluorescence spectrum resulting is such as to allow determination of all bile salts (except the conjugates of lithocholate) at one emission wavelength setting. In the assay a small sample volume is required and there is no need for prior hydrolysis of the conjugates, a time-consuming and destructive process. Finally, the method requires no unusual equipment or procedures; all reagents and the resultant fluorescent products are stable. All the bile acids except lithocholate are accounted for. However, since this particular bile acid is less than 3% of the total bile acids or completely absent in most mammals studied, the error caused by its omission will be small, although it does fluoresce significantly after sulfuric acid treatment and so it is likely that under conditions of the assay much of it is detected.

Materials and Apparatus. Absolute ethanol (Rossville-Gold Shield Alcohol, U.S.P.), chloroform (analytical grade), methanol (spectrograde), and sulfuric acid (analytical grade, 95.5 to 96.5%, relative density 1.84)

are used without further treatment. Barium hydroxide and zinc sulfate (analytical grade) solutions are prepared in distilled water as described by Levin et al. (11). The bile salts (Maybridge Chemical Co., Ltd., Tintagel, N. Cornwall, U.K.) are used without further purification. The sodium chloride is analytical grade and preparation of its solution, "pure solvents upper phase - - containing 0.29% sodium chloride," is described by Folch et al. (6).

Evaporation of samples is by nitrogen jet with the "N-Evap" (Model 106) apparatus. Mixing of samples is most efficiently accomplished with a vortex mixer such as the "Vari-Whirl" (Scientific Supplies Co.). A Perkin-Elmer MPF-2A Fluorescence Spectrophotometer is used to measure the fluorescent values however, any fluorometer can be used as long as the proper excitation and emission wavelengths and slit widths are set. In our laboratory the International Centrifuge (Model UV) and the "Porta Temp" water bath (Precision Scientific Co.) were available.

As with any fluorometric assay, all glassware used must be rigorously cleaned. In particular, the test tubes are routinely acid washed to remove any fluorescent contaminants. Teflon-lined screwcap test tubes (Kimax, 16 x 100 mm) are appropriate in all steps of the procedure.

Procedure. A 1.75 ml quantity of absolute ethanol is added to 0.1 ml of bile. Then, 0.1 ml of barium hydroxide solution, and 0.05 ml of zinc sulfate solution are added to precipitate protein and bilirubin (the latter causes high fluorescent readings owing to nonspecific color interference). After mixing, the samples are centrifuged at 2400 rpm for 10 min. One-tenth volume (0.2 ml) of the supernatant fluid is transferred to another test tube and evaporated. In order to separate the polar and nonpolar material the method of Folch et al. (6) was used. In this procedure the residue is taken up in 0.1 ml of distilled water and 39 volumes (3.9 ml) of chloro-

form-methanol solution (2:1 by vol). This is then mixed well with 0.22 volumes (0.88 ml) of sodium chloride "wash solution" (7 g/liter). The phases separate; the bile salts are partitioned into the more polar (methanol-water) upper phase, which is drawn off with a Pasteur pipet. Phospholipids, fatty acids, and cholesterol go into the less polar (chloroform) lower phase (17). To ensure quantitative removal of bile salts, the lower phase is rinsed three times with about 1 ml of "pure solvents upper phase - containing 0.29% sodium chloride" (6). These are combined and made up to a volume of 5.0 ml with methanol. A 0.25-ml aliquot is transferred to another test tube and evaporated. To the residue one adds 5.0 ml of concentrated sulfuric acid, mixes thoroughly, then heats in a water bath at 60 $\pm$ 1°C for 60 min. After this period the samples are immediately placed in an ice bath for 5 min. After removal and equilibration to room temperature, fluorescence is measured at an excitation wavelength of 470 nm and emission of 505 nm. The emission and excitation wavelength slit widths are each set at 10 nm bandpass.

Standard solutions of sodium taurocholate are prepared in distilled water ranging from 9.3 to 37.2 μmol/ml, the concentration range one would expect in most bile samples (18,19). A blank consisting of distilled water is run concurrently with the standards. The entire procedure results in a 10,000-fold dilution, so that a sample originally containing 9.3 μmol of sodium taurocholate per milliliter is diluted to a final concentration of 0.93 nmol/ml of sulfuric acid. This concentration is easily measured at the least sensitive setting on the instrument, and one can easily detect a taurocholate concentration of 0.372 nmol/ml (0.2 μg/ml) in sulfuric acid. A correction is made for the dilution factor and the average percent recovery for all the bile salts, and the concentration of

the bile sample is expressed as μmol of total bile salt per milliliter.

Discussion. Precipitation with ethanol along with barium hydroxide and zinc sulfate solution effectively removes the small amount of protein present as well as the potentially interfering bilirubin. In earlier procedures attempted in this laboratory, distilled water rather than ethanol was used and variable precipitation of the bile salts occurred, rendering the assay unreliable. The extraction step is sufficient to remove cholesterol, phospholipids, and fatty acids which partition into the lower phase and are discarded. Any steroids present will be in low concentration with respect to the bile salts and the dilution factor alone will make their interference negligible. Also, it would be expected that most steroids would enter the lower phase. Hence the assay appears to be specific for total bile salts. There is a possibility, however, that conjugates of drugs under study might interfere.

It should be noted that the instrument settings in this study may vary with different fluorometers. Thus it will be necessary for each laboratory to define the appropriate wavelength settings of their instrument.

Treatment of the bile salts with sulfuric acid as described produces overlapping emission spectra with an isosbestic point at 505 nm. This is seen in Figure 6, in which the emission spectra of the conjugates of cholate, deoxycholate, chenodeoxycholate, and lithocholate are shown (excitation 470 nm, 10-nm slit). These spectra are independent of whether a given bile acid is conjugated with taurine or glycine. It is probable that the conjugates are completely hydrolyzed during the heating process in sulfuric acid solution. Obviously, they also undergo dehydration and other reactions resulting in fluorescent moieties. As observed, the trihydroxy

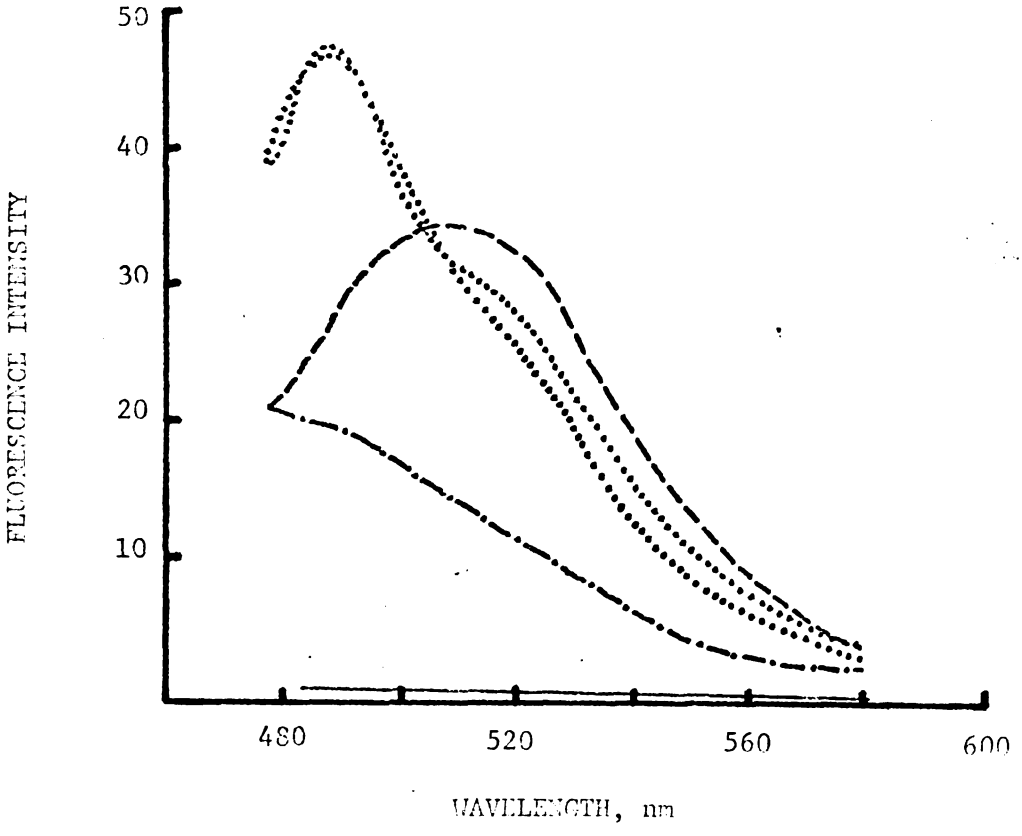


Figure 6 - Fluorescence emission spectra of the bile salts in concentrated sulfuric acid. The excitation wavelength is 470 nm, with both excitation and emission slit widths set at 10 nm. Final concentration of each bile salt is 1.86 nmol/ml in sulfuric acid. The legends trace the curve for the taurine or glycine conjugates of cholate (-----), deoxycholate and chenodeoxycholate (....), lithocholate (-.-.), and blank sulfuric acid (_____).

bile salts, sodium taurocholate-sodium glycocholate, produce a broad flattened emission spectrum. The dihydroxy bile salts, sodium taurodeoxycholate-sodium glycodeoxycholate and sodium taurochenodeoxycholate-sodium glycochenodeoxycholate, produce almost identical emission spectra and have a sharper peak occurring at a shorter wavelength than the trihydroxy bile salts. The overlapping emission spectra allow one to simultaneously assay any combination of these bile salts (except lithocholate conjugates). Under the conditions specified, equimolar solutions of the six bile salts, separately or in combination, produce essentially the same fluorescence. This is seen in Table 2, which lists the relative fluorescence intensity of each bile salt and a combination based on sodium taurocholate as the standard, which is assigned a value of 1.00.

The procedure as described involves a precipitation and extraction step. The extraction is a modification of that described by Folch et al. To test the extraction characteristics of this procedure, a combination of equimolar quantities of the six bile salts was prepared in distilled water at a concentration of 18.6 μmol/ml. This solution was carried through the extraction procedure as described and the resultant upper and lower phases were assayed for bile salts with both the GLC and fluorometric method. Essentially 100% of the bile salts enter the upper phase and none are found in the lower phase. In addition, a sample of rat bile was assayed for bile salts after extraction and again no bile salts were found in the lower phase. Hence, partitioning of bile salts into the more polar phase is complete.

Recovery studies of each of the bile salts were made after their addition to pooled rat bile samples, which were then carried through the entire procedure. The bile salts were added to give an initial concentra-

Table 2 - Relative Fluorescence Intensity of the Bile Salts Based on Sodium Taurocholate as the Standard.

Bile salts[a]	Relative fluorescence intensity[b]
Na taurocholate	1.00
Na taurodeoxycholate	1.03 (.07)
Na taurochenodeoxycholate	1.04 (.10)
Na glycocholate	1.06 (.01)
Na glycodeoxycholate	0.92 (.09)
Na glycochenodeoxycholate	1.00 (.12)
Combination[c]	0.99 (.09)

(a) Final concentration of each bile salt is 1.86 nmol/ml of sulfuric acid.

(b) An average of three experiments, each based on sodium taurocholate assigned a fluorescence intensity of 1.00 (Standard deviation in parenthesis). The average relative fluorescence intensity of the six bile salts is 1.01 with standard deviation of 0.05.

(c) An equal parts combination of the six bile salts to give the same final concentration.

tion of 9.3 μmol/ml over and above the concentration of bile salts already present in the bile sample. Table 3 lists the average percent recovery of each bile salt. Although there is some variability here - for example, sodium glycodeoxycholate is quite low (80.7% recovery, SD 9.1) - all the others are almost completely recovered. The average recovery of total bile salts is 95.7% (standard deviation, 8.5). This average was used when correcting for total bile salts in subsequent calculations.

Figure 7 indicates that linear fluorescence response is obtained when the aqueous standards of sodium taurocholate (9.3 to 37.2 μmol/ml) are carried through the procedure. This range of concentration should include most bile samples; however, if a very concentrated sample is encountered, it may be diluted with concentrated sulfuric acid after the heat treatment. Fluorescent readings taken soon after cooling and a week later were essentially the same for a series of samples (with the exception of the most dilute sample) in the same range of concentration as the aqueous standards (Table 4). For very dilute samples (0.372 nmol/ml of sulfuric acid) a significant change in fluorescence intensity may occur with time.

Reproducibility is observed both within a single run and from day to day. To test variation within a run, five 0.1-ml portions of a single bile sample were carried through the procedure. The average concentration determined was 5.56 μmol/ml of bile (SD, 0.311). On another bile sample nine determinations were made on different days to evaluate day-to-day variation. The average concentration calculated was 18.59 μmol/ml of bile (SD, 1.09). Note that bile samples are collected on ice and frozen soon thereafter. Samples may be thawed and refrozen for subsequent analysis without noticeable effect.

In order to check the validity of the assay, various bile samples

Table 3 - Recovery of Each Bile Salt From Pooled Rat Bile.

Bile salts[a]	Percent recovery[b]
Na taurocholate	103.0 (14.3)
Na taurodeoxycholate	97.3 (10.9)
Na taurochenodeoxycholate	104.0 (24.0)
Na glycocholate	93.0 (21.9)
Na glycodeoxycholate	80.7 (9.1)
Na glycochenodeoxycholate	96.0 (19.7)

(a) Each bile salt was added to pooled rat bile to give an initial concentration of 9.3 mol/ml above that already present in the bile sample.

(b) An average of three experiments with the standard deviation in parenthesis. The average percent recovery of the six bile salts is 95.7% with a standard deviation of 8.5%.

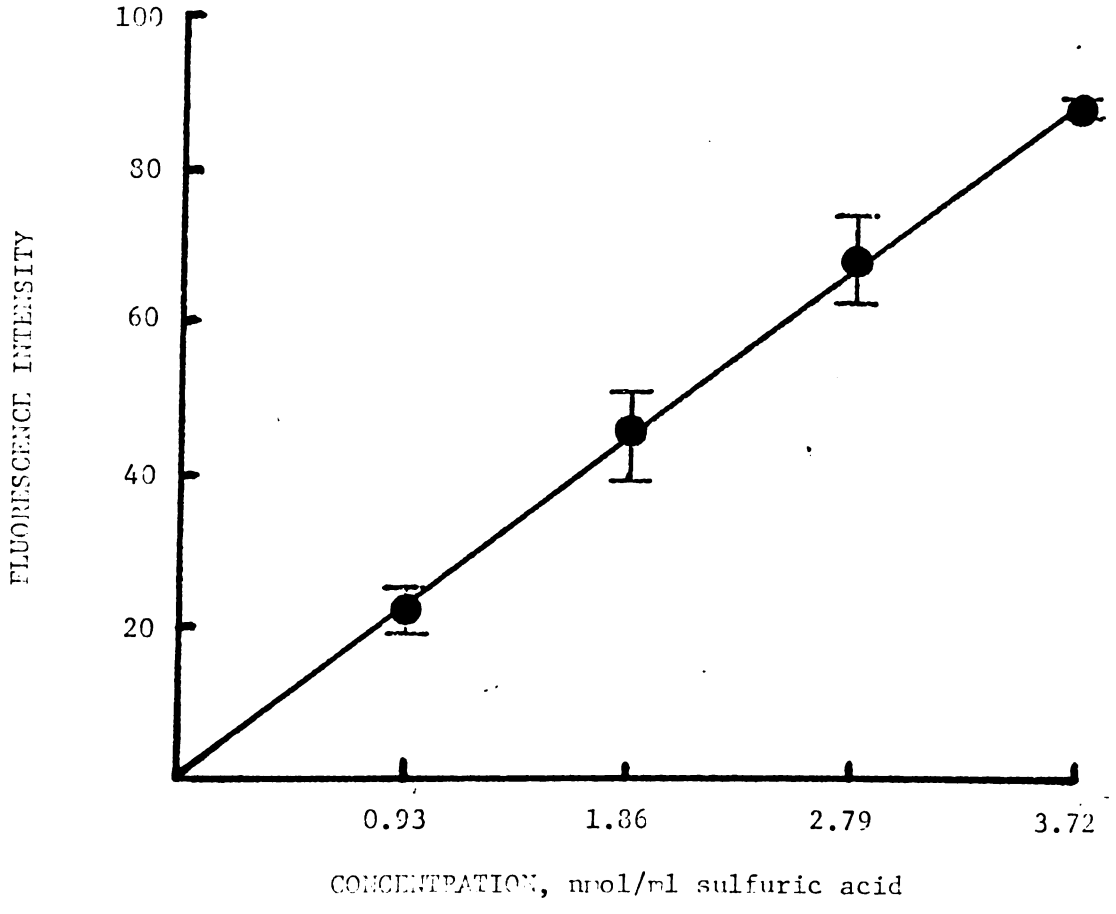


Figure 7 - A plot of fluorescence intensity vs final concentration of sodium taurocholate in sulfuric acid. Solid circles represent the average of three runs while the verticle bars include the average plus or minus the standard deviation. The calculated correlation coefficient is 1.000, the slope 23.97, and Y-intercept 0.650.

Table 4 - Stability of Sodium Taurocholate Solutions in Concentrated Sulfuric Acid

Concentration[a] (nmol/ml)	Initial fluorescence[b] intensity	Final fluorescence[b] intensity	Variation (% of initial)
0.372	24.0	27.0	+12.5%
0.744	41.0	38.5	-6.1%
1.116	63.2	63.0	-0.3%
1.860	100.0	100.0	0.0%

(a) Final concentration of sodium taurocholate in concentrated sulfuric acid.

(b) Initial fluorescence intensity is that read immediately after procedure while final fluorescence intensity is that read on the same samples, stored at room temperature, one week later.

were subjected to fluorometric, GLC, and enzymatic assay. On a sample of rat bile both the fluorometric and GLC assay were run and the results are shown in Table 5. As observed, both assays give essentially the same result; also, slightly less variability is obtained with the fluorometric assay. On a series of five different human bile samples, the fluorometric assay was performed in our laboratory while another group ran the enzymatic assay. The results are compared in Table 6. The data (20) were statistically evaluated, applying the t test to the differences of the paired observations to test the hypothesis that the mean difference = 0. One can then calculate $t = 0.211$, and consequently must accept the hypothesis that there is no difference between the calculated concentrations of the fluorometric and enzymatic assay.

Table 5 - Comparison of Total Bile Salt Concentration Determined by the Fluorometric and GLC Method.

Sample	Concentration (mol/ml of bile)	
	Fluorometric	GLC
Rat bile	24.2(2.4)[a]	22.6(2.9)[a]

[a] The average of four determinations with standard deviation in parentheses.

Table 6 - Comparison of Total Bile Salt Concentration Determined by the Fluorometric and Enzymatic Method.

Sample[a]	Concentration (μmol/ml bile)[b]	
	Fluorometric	Enzymatic
1.	1.4	1.8
2.	2.5	2.6
3.	7.2	6.4
4.	6.2	7.0
5.	8.5	8.4

(a) Different samples of human intestinal bile (1 & 2) and human t-tube bile (3-5).

(b) The fluorometric assay was done in our laboratory and the enzymatic assay in another laboratory, resulting in paired observations on each bile sample. The differences of the paired observations were calculated and statistically evaluated using the t test. The calculated t statistic is, $t = -0.211$.

REFERENCES

1. Dodgson, K.S., Garton, G.A., Stubbs, A.L., and Williams, R.T., Studies in Detoxication - On the Glucuronides of Stilboestrol, Hexestrol, and Dienoestrol, Biochem. J., 42, 357 (1948).

2. Dingemanse, E., The Colorimetric Assay of the Synthetic Oestrogenic Substance Diethylstilbestrol, Acta Brev. Neerl. Physiol., 10, 118 (1940).

3. Goodyear, J.M., Hatfield, L.S. and Marsh, M.M., Ultraviolet Irradiation and Absorptiometric Methods for the Determination of Diethylstilbestrol in Tablets, J. Am. Pharm. Assoc., Sci. Ed., 43, 605 (1954).

4. Goodyear, J.M. and Jenkinson, N.R., Irradiation Fluorometric Method for Estimation of Diethylstilbestrol in Beef Liver Tissue, Anal. Chem., 33, 853 (1961).

5. Fischer, L.J. and Millburn, P., Stilboestrol Transport and Glucuronide Formation in Everted Sacs of Rat Intestine, J. Pharmcol. Exptl. Ther., 175, 267 (1970).

6. Folch, J., Lees, J., and Stanley, G.H.S., A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem., 226, 497 (1957).

7. Nair, P.P., and Garcia, C., A Modified Gas-Liquid Chromatographic Procedure for the Rapid Determination of Bile Acids in Biological Fluids. Anal. Biochem., 29, 164 (1969).

8. Iwata, T., and Yamasaki, K., Enzymatic Determination and Thin-Layer Chromatography of Bile Acids in Blood. J. Biochem., 56, 424 (1964).

9. Palmer, R.H., The Enzymatic Assay of Bile Acids and Related 3 - Hydroxysteroids; Its Application to Serum and Other Biological Fluids. In Methods in Enzymology, XV; S.P. Colowick, Ed. Academic Press, New York, N.Y., 1969, pp 280-288.

10. Mosbach, E.H., Kalinsky, H.J., Halpern, E., and Kendall, F.E., Determination of Deoxycholic and Cholic Acids in Bile. Arch. Biochem., 51, 402 (1954).

11. Levin, S.J., Johnston, C.G., and Boyle, A.J., Spectrophotometric Determination of Several Bile Acids as Conjugates. Anal. Chem., 33, 1407 (1961).

12. Ahrens, E.H., Jr., and Craig, L.C., The Extraction and Separation of Bile Acids. J. Biol. Chem., 195, 763 (1952).

13. Benard, H., and Broer, Y., Fluorescence de Certaino Acides Biliares Apres Chauffage en Milieu Sulfurique. Bull. Soc. Chim. Biol., 42, 99 (1960).

14. Levin, S.J., Irvin, J.L., and Johnston, C.G., Spectrofluorometric Determination of Total Bile Acids in Bile. Anal. Chem., 33, 856 (1961).

15. Panveliwalla, D., Lewis, B., Wootton, I.D.P., and Tabaqchali, S., Determination of Individual Bile Acids in Biological Fluids by Thin-Layer Chromatography and Fluorimetry. J. Clin. Pathol., 23, 309 (1970).

16. Mroszczak, E. J. and Riegelman, S., Rapid Spectrofluorometric Assay for Total Bile Salts in Bile, Clin. Chem., 18, 987 (1972).

17. Bell, C.C., Jr., Vlachevic, Z.R., and Swell, L., Alterations in the Lipids of Human Hepatic Bile After the Oral Administration of Bile Salts. Surg. Gynecol. Obstet., 132, 36 (1971).

18. Portman, O.W., Importance of Diet, Species and Intestinal Flora in Bile and Metabolism. Fed. Proc., 21, 896 (1962).

19. Thureborn, E., Human Hepatic Bile: Composition Changes Due to Altered Enterohepatic Circulation. Acta. Chir. Scand., Suppl., 303, 1 (1962).

20. Dixon, W.J., and Massey, F.J., Jr., Inference: Two Populations, in "Introduction to Statistical Analysis", McGraw-Hill, New York, N.Y., 1969, pp 119-123.

IN VITRO SOLUBILITY STUDIES OF SODIUM DIETHYLSTILBESTROL MONOGLUCURONIDE

All trihydroxy and dihydroxy bile salts are capable of forming micelles, the size and number of which are influenced by bile salt concentration, counterion concentration, pH, and temperature. Very small or primary micelles are probably bonded hydrophobically while larger micelles are formed by the aggregation of small micelles through hydrogen bonding of hydroxyl groups. Sodium dehydrocholate, a triketo bile salt and a very potent choleretic does not form micelles in vitro (1). Phospholipids, in the presence of bile salts and water form molecular associations. Lecithin, an insoluble amphiphile with two hydrocarbon chains, is solubilized by bile salts, either free or conjugated, forming mixed bile salt-lecithin micelles (2).

It is known that certain compounds are excreted into the bile sequestered in the mixed micelles which are present (3,4). It has in fact been suggested that bile salts regulate the excretion of biliary lipids due to the formation of a specific macromolecular complex in the liver and its subsequent excretion in the bile (5). The role of biliary micelles in the excretion of anions is less clear, yet bile flow dependent excretion has been observed for bilirubin (6) and sulfobromophthalein sodium (7). In the former reference it was suggested that bilirubin may be sequestered within the bile acid micelle.

In light of these previous investigations, a study of the potential interaction of the anion sodium diethylstilbestrol monoglucuronide with bile salt micelles was begun. The ability of sodium taurocholate to form micelles with or without lecithin under various experimental conditions has been well defined in vitro (8). Therefore, it was decided that in

vitro systems would provide the most straight-forward approach in defining those interactions involved.

Materials and Apparatus. Sodium taurocholate (Maybridge, Ltd., Tintagel, N. Cornwall, U.K.), lecithin (L-α-lecithin dipalmitoyl, synthetic - General Biochemicals, Chagrin Falls, Ohio) and sodium taurodehydrocholate (Calbiochem, Los Angeles, Calif.) were used without further purification. Sodium diethylstilbestrol monoglucuronide was obtained biosynthetically from rabbits as previously described. Since monkey bile was found to have a pH ranging from 7.8 to 8.2 an isotonic sodium phosphate buffer of pH 8.00 was prepared as described by Sorensen (9). The buffer solution was prepared such that the sodium ion concentration was 0.154 molar and the ionic strength, $\mu = 0.170$.

Solubility studies were carried out in the following systems: phosphate buffer solution alone, buffer solution containing sodium taurocholate (concentration range 0.32 to 32.0 mM), buffer solution containing sodium taurocholate plus lecithin (molar ratio 5.7 to 1) in the concentration range for taurocholate of 0.077 to 7.7 mM, and buffer solution containing sodium taurodehydrocholate (concentration range 0.1 to 30 mM). In certain experiments actual monkey bile was used as such and in diluted form, all dilutions were done with the phosphate buffer solution. In the various aqueous in vitro model systems studied, phosphate buffer was the vehicle in all cases to insure that pH, ionic strength and sodium ion concentration would remain within very narrow limits and not of themselves influence the solubility studies. The sodium salts of taurocholate, taurodehydrocholate, and diethylstilbestrol monoglucuronide will contribute to the ionic strength and total sodium concentration in the final solutions. However, since the final system results in a very complex equilibrium of free ions and those

associated within the micelles no correction was made for these added moities and it was assumed that their contribution would not be a major factor (8).

Absorbance measurements were made on the Cary 15 spectrophotometer and pH measurements on the Corning pH meter (Model 10, Scientific Products). Continuous mixing of the aqueous systems was provided by an inversion mixing device (Linson Instruments, AB LARS Ljungberg & Co., Stockholm). Two different centrifuges (Spinco 152 Microfuge-Beckman and the Model HN-S centrifuge-International Equipment Co. Mass.) were used at different stages of the procedure.

Procedure. A 0.5 ml portion of each of the above described aqueous systems was placed in a test tube and sodium diethylstilbestrol monoglucuronide was added with vigorous mixing. When the glucuronide appeared to be in excess the test tubes were placed on the continuous shaking device and mixed for 12 and 24 hour periods. During this time the samples were frequently checked to make sure that an excess of glucuronide was always present. These studies were done at ambient room temperature which ranged from 23.5 to 24.5°C. After shaking was terminated, the mixture was centrifuged for four minutes on the Spinco 152 Microfuge and 0.1 ml of the clear supernatant was transferred to another test tube and made up to 10 ml volume with 95% ethanol (which precipitates the various buffer salts). This was mixed well and centrifuged again for 20 minutes at 2000 rpm on the model HN-S centrifuge and absorption measurements were made on the clear solution. In all cases a blank consisting of the exact system under study (without glucuronide) was also run. The corrected absorbance measurements were converted to concentration units (mg/ml) and these values represented the solubility of glucuronide at saturation.

Apparent saturation solubility was obtained at 12 hours. The data are presented as a log-log plot of total glucuronide dissolved in mg/ml versus millimolar concentration of the solubilizing bile salt system.

Results and Discussion. In Figure 8 one observes that taurocholate, alone and with lecithin, has the ability to increase the solubility of sodium diethylstilbestrol monoglucuronide. However, taurodehydrocholate has no solubilizing effect above that of buffer alone. At the highest concentrations (of solubilizer) studied the taurocholate system is observed to increase glucuronide solubility by 7.4 fold and the taurocholate plus lecithin system by 4.4 fold. In contrast, the average glucuronide concentration in the taurodehydrocholate system is the same as that in buffer. The trend of the solubility curves indicate that as taurocholate concentration is increased glucuronide solubility will increase further. Due to the lack of sufficient quantities of glucuronide additional points were not obtained. If one extrapolates the linear portions of the curves until they intersect it can be seen that the "break" in solubility for the taurocholate system occurs at approximately 3.7 mM. It has been reported that the critical micellar concentration (CMC) of taurocholate under similar conditions is between 3.2 and 4.0 mM (8). Similarly, in the taurocholate plus lecithin system the "break" in solubility occurs at approximately 0.82 mM taurocholate. It has been reported that the CMC for taurocholate-lecithin (molar ratio 5.7 to 1) is approximately 0.77 mM taurocholate (8). Sodium taurodehydrocholate does not form micelles in the concentration ranges studied (1) and no break in the solubility curve is observed.

In these aqueous systems the pH varied between 7.90 and 8.05 with no particular trend in any direction, therefore pH changes cannot account for

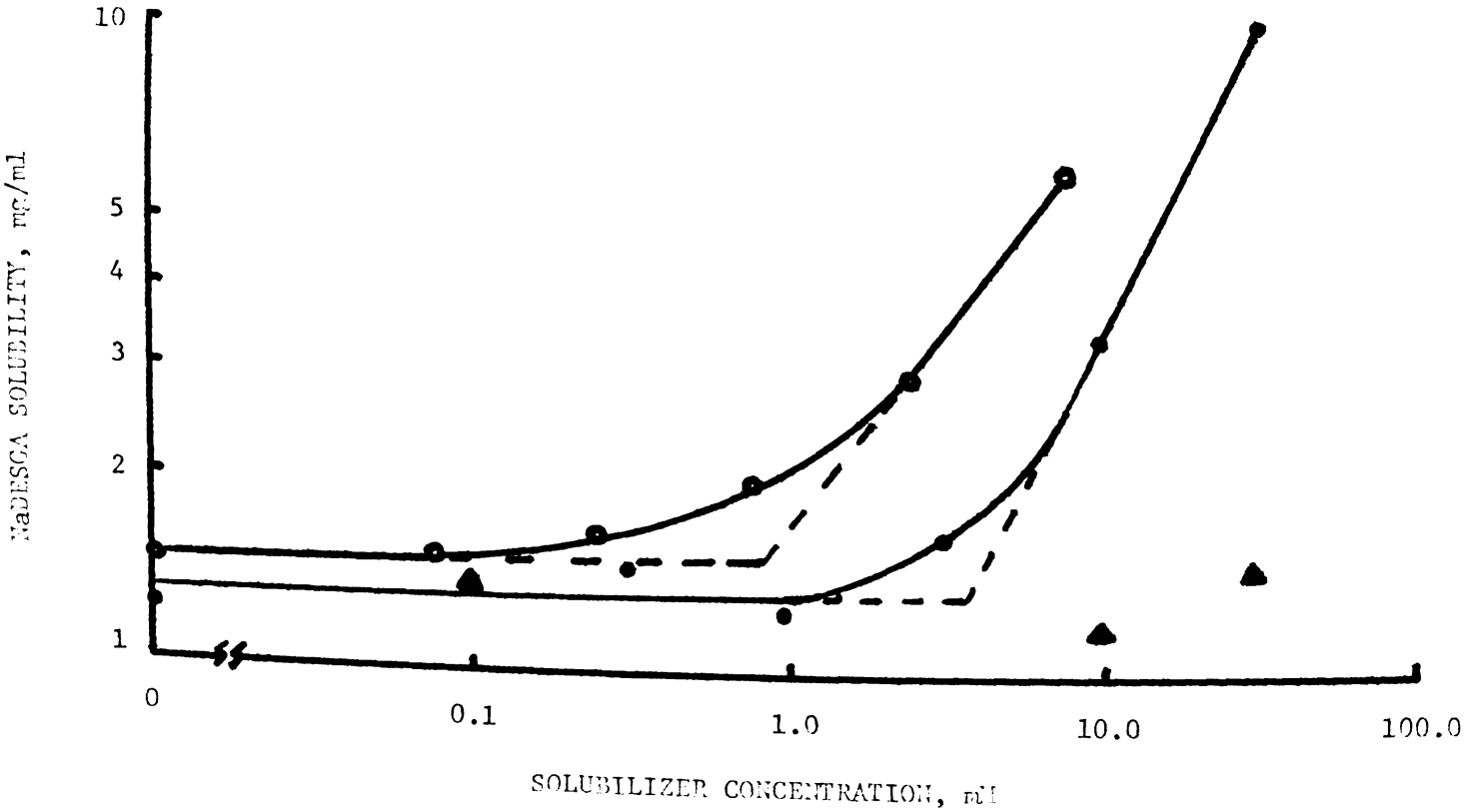


Figure 8 - A plot of the equilibrium solubility of sodium diethylstilbestrol monoglucuronide (NaDESGA) versus taurocholate concentration in phosphate buffer alone (●) and when combined with lecithin (◐). NaDESGA solubility versus taurodehydrocholate concentration in phosphate buffer is also indicated (▲). The extended broken lines cross at a point which approximates the taurocholate concentration at which NaDESGA solubility begins to increase.

the solubility differences. Additionally, in certain samples the possibility of hydrolysis of the glucuronide to free diethylstilbestrol was monitered. It was found that hydrolysis was negligible, amounting to 3% or less, again with no particular trend. Therefore, the observed concentrations of glucuronide cannot be due to hydrolyzed, solubilized diethylstilbestrol. A single trial run of taurodehydrocholate with lecithin was attempted and it was found that lecithin could not be solubilized in this system. As expected, the solubility of glucuronide with taurodehydrocholate alone and in the taurodehydrocholate plus lecithin system was the same. This rules out any solubilizing activity of lecithin alone since without the presence of the micelle forming taurocholate, lecithin itself is insoluble. It would appear then that some molecular association of sodium diethylstilbestrol monoglucuronide within the micelles that are present in the taurocholate systems is the only mechanism which could explain the data.

A further study was carried out in monkey bile. On the assumption that micellar solubilization of the glucuronide is an important factor, glucuronide solubility was determined in whole bile and bile which was diluted such that no micelles would be present. It was found that whole bile could solubilize sodium diethylstilbestrol monoglucuronide to the extent of 10.4 mg/ml (7.6 times the solubility in plain buffer) while bile diluted below the CMC for bile salts could not enhance glucuronide solubility (1.24 mg/ml) above that of buffer alone,1.37 (0.17 S.D.) mg/ml.

It should be pointed out that this latter study was of only 2 hours duration since the possibility of hydrolysis of the glucuronide by bile glucuronidase (10) can be a problem over longer periods of time. Another problem associated with the use of whole bile is the fact that it is an

unbuffered system and in this experiment the pH of whole bile changed from 8.04 initially to 9.00 at completion of the study. The bile which was diluted with buffer maintained a pH of 8.04 throughout the experiment. To exclude the possibility that the increase in pH was a factor, a solubility study was conducted in pH 10.0 buffer. It was found that glucuronide solubility at pH 10.0 was 1.68 mg/ml which is 1.2 times the solubility in pH 8.00 buffer. Hence, the pH increase in whole bile was not a significant factor in this study.

These studies clearly demonstrate that sodium diethylstilbestrol monoglucuronide forms an association with micellar structures both in synthetic aqueous solutions and in bile. The ability of taurocholate to solubilize the glucuronide is enhanced by the presence of lecithin. This is probably due to the fact that the bile salt-lecithin mixed micelle has a lower CMC for a given taurocholate concentration and more and larger micelles are present, hence a greater association and solubilization effect is possible.

Solubilization can be thought of as a mode of bringing into solution substances that are otherwise insoluble in a given medium. Solubilization involves the previous presence of a colloidal solution whose particles take up and incorporate within or upon themselves the otherwise insoluble material. In this instance it is the bile salts which are themselves freely soluble and colloidal and which take other substances into solution. However, in contrast to the general situation, sodium diethylstilbestrol monoglucuronide is not insoluble and it is charged negatively at a pH of 8.00 and therefore quite polar. Yet it can be further solubilized. This is in contrast to the general picture of a water insoluble, non-polar compound which resides inside the lipid like interior of a

micelle.

If one examines sodium diethylstilbestrol monoglucuronide it can be seen that this molecule is,in fact an amphiphilic compound. That is it has both a polar (hydrophilic) and non polar (lipophilic) portion within the molecule. One could theorize that such a molecule interacts with bile micelles in the following manner. The glucuronide can be envisioned as interdigitating with the associated molecules of the micelle in such a fashion that the relatively non-polar (phenolic) portion is alligned within the hydrocarbon interior of the micelle with its polar (glucuronide) portion extending out into the aqueous environment. In effect such a molecule will cause the micelle to conform into another association complex including sodium diethylstilbestrol monoglucuronide within its system. Considering the relatively "loose" configuration of bile salt mixed micelles (1) such an interaction is theoretically possible (11).

The micelle systems investigated above are saturated with respect to sodium diethylstilbestrol monoglucuronide which means both the micelles and aqueous environment are at saturation. If these systems were diluted with a blank micellar solution (no glucuronide present) the solute would re-equilibrate between the micellar and aqueous environment. At all times a significant amount of glucuronide would be dissolved in water. This may be contrasted to most solubilized compounds (water insoluble) where it would be observed that the solute partitions into the micelle to a very large extent with a considerably reduced number of freely dissolved molecules in water.

It is interesting to consider sodium diethylstilbestrol monoglucuronide in light of those requirements specified as being necessary for a compound to be well excreted into the bile (12). It does in fact fit all

the requirements in that it has the necessary molecular weight, is an anion, and has the required structural features that many other well excreted molecules also possess. That is, it contains a polar and non-polar portion and as the free acid (pH<3), exhibits a significant degree of lipid solubility. Perhaps this latter requirement is the most important.

If the interaction with bile micelles is an important requirement for good biliary excretion one can consider several mechanisms to explain why this may be so. One could consider this interaction to occur within the liver where a macromolecular complex (bile salt micelle) is formed and subsequently excreted into the canaliculi (5). Any compound with a high affinity for the bile micelle could therefore be carried along with it into the canaliculi. Alternatively one could postulate that all compounds are excreted into the bile but only those which strongly interact with the bile micelle (reducing the escaping tendency) remain, and are excreted in the bile. All other compounds are efficiently reabsorbed back into the blood and subsequently excreted into the urine.

Micellar solubilization has been theorized as being important for the biliary excretion of bilirubin (6) and shown definitively for the drugs imipramine and desmethylimipramine (3). In those studies involving bilirubin it was observed that bile osmolalities did not increase concurrently with marked increases in bile bilirubin concentrations suggesting that bilirubin conjugates are excreted as a sequestered molecule within a micelle, possibly in conjunction with the bile acids as previously postulated by Javitt (13). A discussion of the biliary excretion mechanisms of imipramine, desmethylimipramine and their conjugates has been presented earlier (Role of Bile Micelles in Biliary Excretion). Thus there are now at least three independent studies indicating that micelle solubilization

may play an important role in the biliary excretion of drugs. This provided a stimulating beginning for various in vivo investigations with diethylstilbestrol in an attempt to clarify some of the ambiguities concerning the biliary excretion of foreign compounds.

METABOLISM OF DIETHYLSTILBESTROL-^{14}C IN THE RHESUS MONKEY: ASSAY PROCEDURES IN BLOOD, BILE, AND URINE

The initial goal in the study of diethylstilbestrol-^{14}C in the rhesus monkey was to determine its metabolic end products in blood, bile, and urine and to develop suitable assay and separation techniques in these fluids. A study of the metabolism of diethylstilbestrol-^{14}C was undertaken by administering the drug as an intravenous bolus. Thin layer chromatography (TLC) techniques and the Folch extraction procedure were used to determine the presence of unchanged drug and metabolites in the various fluids.

Preparation of Solutions. Radioactive diethylstilbestrol-^{14}C was diluted with cold diethylstilbestrol as follows. First, an aliquot of a benzene solution of radioactive compound was accurately measured, transferred to a test tube and the benzene evaporated under a gentle nitrogen jet. Then cold diethylstilbestrol, previously dissolved in absolute ethanol, was added to the radioactive residue and completely dissolved. Polyethylene glycol 400 (PEG-400) was added, mixed well and then the system was diluted with distilled water. The resultant solution contained PEG-400 (50%), ethanol (2%) and distilled water (48%). A total dose of 38.5 μCi and 0.80 mg of diethylstilbestrol-^{14}C was contained in a 4.5 ml total volume of the injection solution. It was necessary to use PEG-400 as a component of the solvent due to the very low solubility of diethylstilbestrol in water. The general use and low toxicity of the high molecular weight polyethylene glycols has been well documented (14,15). Just prior to injection, this solution was sterilized by filtration through a Millipore filter (HAWG 013, HA 0.45μ, 13 mm) using a Swinny adapter. Sodium taurocholate (Gen. Biochem.), for duodenal infusion, was

prepared by dissolving 9.38 Gm of sodium taurocholate in 250 ml of distilled water (69.74 mol/ml). Adequate urine output was assured by an intravenous infusion of heparinized normal saline prepared by adding 5,000 units of a sterile solution of heparin to 1000 ml of sterile normal saline (5 units/ml).

Experimental Protocol. Food was withheld from about 5 hours prior to administration of drug and throughout the experiment but water was given ad libitum. Since bile was to be collected without return, it was necessary to replace bile salts. This was accomplished by infusing the sodium taurocholate solution via the duodenal catheter at the rate of 0.139 ml/min corresponding to a taurocholate input rate of 9.76 mol/min. This duodenal infusion was begun 4 hours prior to the beginning of the experiment and continued throughout in order to maintain a constant bile flow rate. The heparinized normal saline solution was infused at the rate of 30 ml/hour, from 2 hours prior to and throughout the experimental period. Diethylstilbestrol-^{14}C was administered as an intravenous bolus via the iliac vein and blood samples were collected via the iliac artery. Blood samples were immediately placed in heparinized, screw cap test tubes. Bile was collected using a fraction collector (Micro Fractionator-Gilson) which has the capacity to collect either exact volumes or for a preset length of time. Urine was collected immediately after voluntary urination by means of a screened tray, placed beneath the primate chair, which separated feces from urine. During a typical experiment approximately 25 ml of blood was withdrawn which represents less than ten per cent of total blood volume (367 ml) in a 4.1 Kg monkey.

Analytical Procedures. In those samples which were subjected to TLC the following procedure was followed. As soon as possible after sampling,

an aliquot of blood (0.1 to 0.4 ml), bile, or urine (0.1 ml) was transferred to a screw cap test tube and diluted with 20 volumes of absolute ethanol. The original samples were placed in a freezer immediately thereafter. These precautions were undertaken since the addition of ethanol to precipitate proteins or freezing of the original sample was necessary to prevent extensive hydrolysis of the conjugates present in urine and bile. The ethanolic solutions were centrifuged and a portion of the supernatant was transferred to another test tube and evaporated. The residue was taken up in 0.1 ml of methanol, streaked onto a TLC plate and the plates developed in the n-propanol-aqueous ammonia (7:3) system for 6 to 7 hours. After removal, the plates were air dried and 5 mm segments were scrapped into liquid scintillation counting vials. The silica gel was mixed with 1.8 ml of methanol for 15 minutes on a shaking apparatus to elute radioactive solutes. Finally, 10 ml of toluene cocktail was added, mixed well and the samples were allowed to settle for 24 hours in a freezer. These samples were then counted as discussed earlier (see Various Aspects of Liquid Scintillation Counting).

The Folch extraction procedure was employed in the analysis of blood samples as follows. After ethanol precipitation and evaporation, as described above, the residue is taken up in 0.1 ml of distilled water. Then a chloroform-methanol solution (2:1 v/v) is added and one proceeds as described in the analytical methods section (see In Vitro Assay and Separation Techniques for Diethylstilbestrol and Sodium Diethylstilbestrol Monoglucuronide). The bile and urine samples were assayed for total activity as follows. First the ethanol precipitation procedure was carried out as above. Next a portion of the ethanolic supernatant was transferred directly to counting vials and counted as previously discussed.

It has been previously shown that the ethanol precipitation did not result in loss of counts. Therefore 100% recovery was assumed after this procedure in blood, bile, and urine. However, in blood a recovery correction had to be made for losses incurred during the Folch extraction procedure. Suitable corrections were also necessary for quenching which averaged 71.0% in blood, 64.5% in bile, and 73.2% in urine.

Results and Discussion. In carrying out a study of this nature one must be certain that the compounds being assayed are stable in the sampled biological fluids during the periods of collection, storage, and separation. Preliminary studies indicated the presence of relatively large amounts of unchanged diethylstilbestrol as well as conjugates in the bile and urine. These large amounts of unconjugated diethylstilbestrol were possibly due to hydrolysis of metabolites and therefore studies were undertaken to collect and process the samples in a manner which would minimize this hydrolysis. Hence, any unchanged drug detected would only be that existing in the fluids prior to collection. It was found that extensive dilution with absolute ethanol or immediate freezing of the sample virtually eliminated hydrolysis of metabolites which occured if the samples were allowed to stand at room temperature for a period of time.

Even with these precautions, unchanged diethylstilbestrol was found in the ethanol processed samples to the extent of 6% of the total activity in bile and 4% in urine. It is our contention that this may be due to hydrolysis of metabolites either in the urinary bladder, in transit through the hepatic collecting ducts and catheter tubing and/or on standing in the collection vessels at room temperature during the short intervals prior to dilution with ethanol. Therefore, in this and future experiments it will be assumed that diethylstilbestrol is excreted into bile and urine only

in conjugated form. Such an assumption will not significantly effect conclusions drawn in later sections. This hydrolysis problem has plagued workers in the field of steroids (16) which are chemically similar to diethylstilbestrol.

In contrast to bile and urine, it was found that blood has little or no ability to hydrolyze diethylstilbestrol metabolites, since untreated blood samples allowed to stand for a few days gave the same TLC results as those which were immediately frozen or diluted with ethanol. Hence, we contend that the observed amounts of free and conjugated diethylstilbestrol in blood are real and not an artifact.

The results of the TLC separation experiments are shown in Figures 9-11. The presence of unchanged diethylstilbestrol and its monoglucuronide were verified by comparison with standards (see Analytical Methods) in which the R_f value for diethylstilbestrol had been found to fall within the 95% confidence limits of 0.85 to 0.90. In contrast, the 95% confidence interval for the monoglucuronide was found to be 0.32 to 0.42. The TLC scans for blood samples showed peaks of R_f value 0.87 and 0.34 respectively indicating the presence of both compounds. The bile samples showed one peak of R_f value 0.39 indicating that only diethylstilbestrol monoglucuronide could be detected by this method. However, the urine TLC samples were observed to have multiple peaks of R_f value 0.24 and 0.35 indicating that while the monoglucuronide may be present (R_f=0.35) at least one other metabolite is also in evidence. This low R_f compound represents a more polar metabolite, possibly a double conjugate (diglucuronide) or a mixed conjugate (glucuronide-sulfate) both of which are known to occur for estradiol, a compound very similar to diethylstilbestrol (17). Scans of the TLC plates of bile and urine also show peaks with a high R_f which would

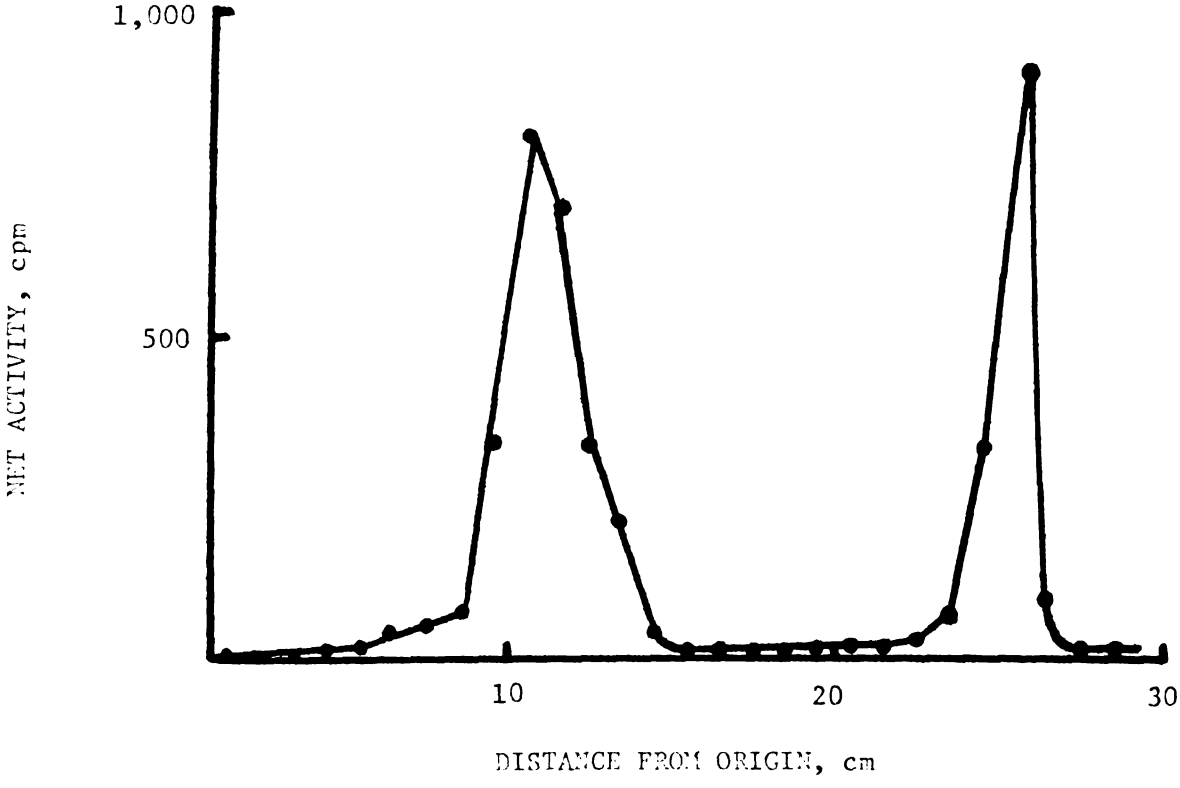


Figure 9 - Thin layer chromatogram of a blood sample obtained after an intravenous bolus of diethylstilbestrol-^{14}C. The development system consisted of n-propanol plus aqueous ammonia (7:3). Two peaks of R_f value 0.34 and 0.87 are obtained.

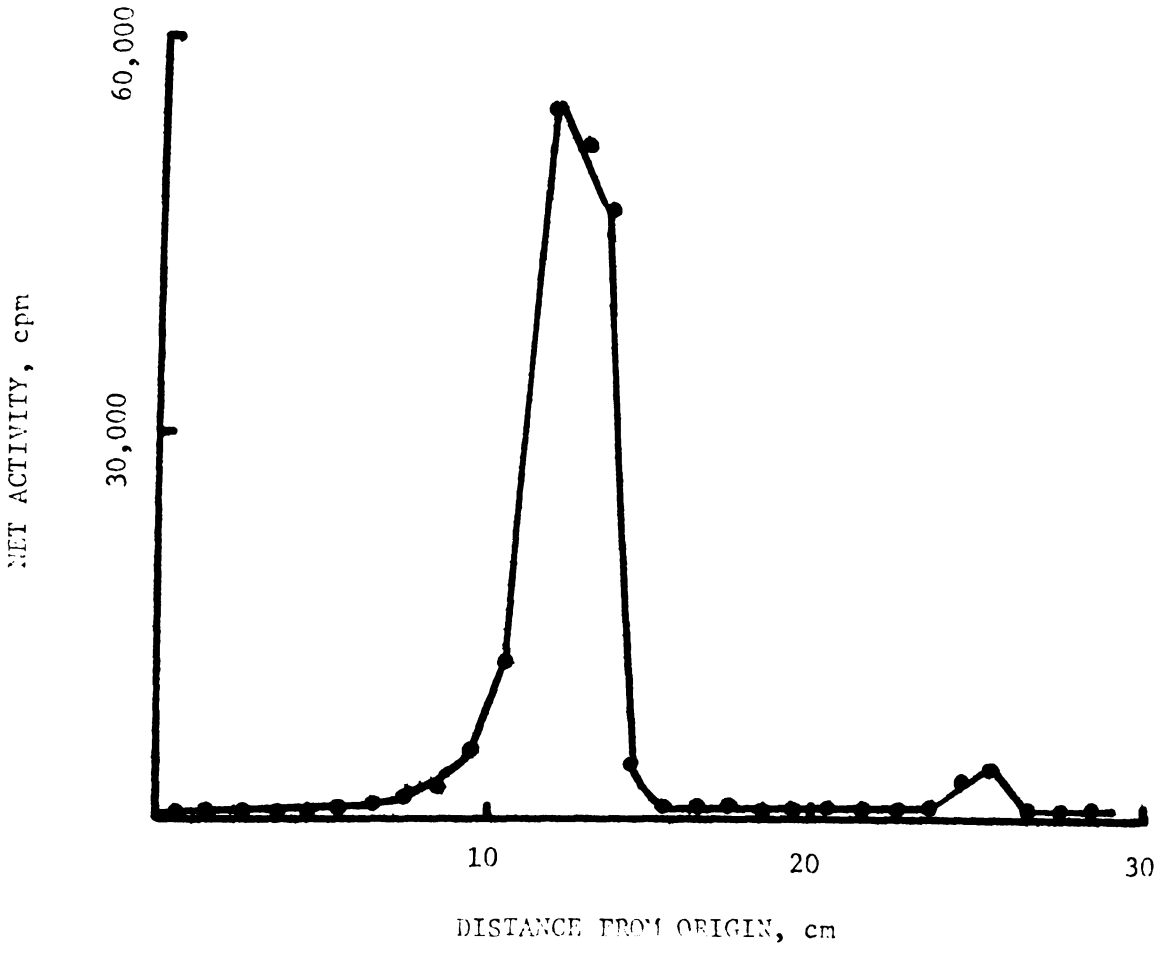


Figure 10 - Thin layer chromatogram of a bile sample obtained after an intravenous bolus of diethylstilbestrol-^{14}C. The development system consisted of n-propanol plus aqueous ammonia (7:3). A single major peak of R_f value 0.39 is obtained.

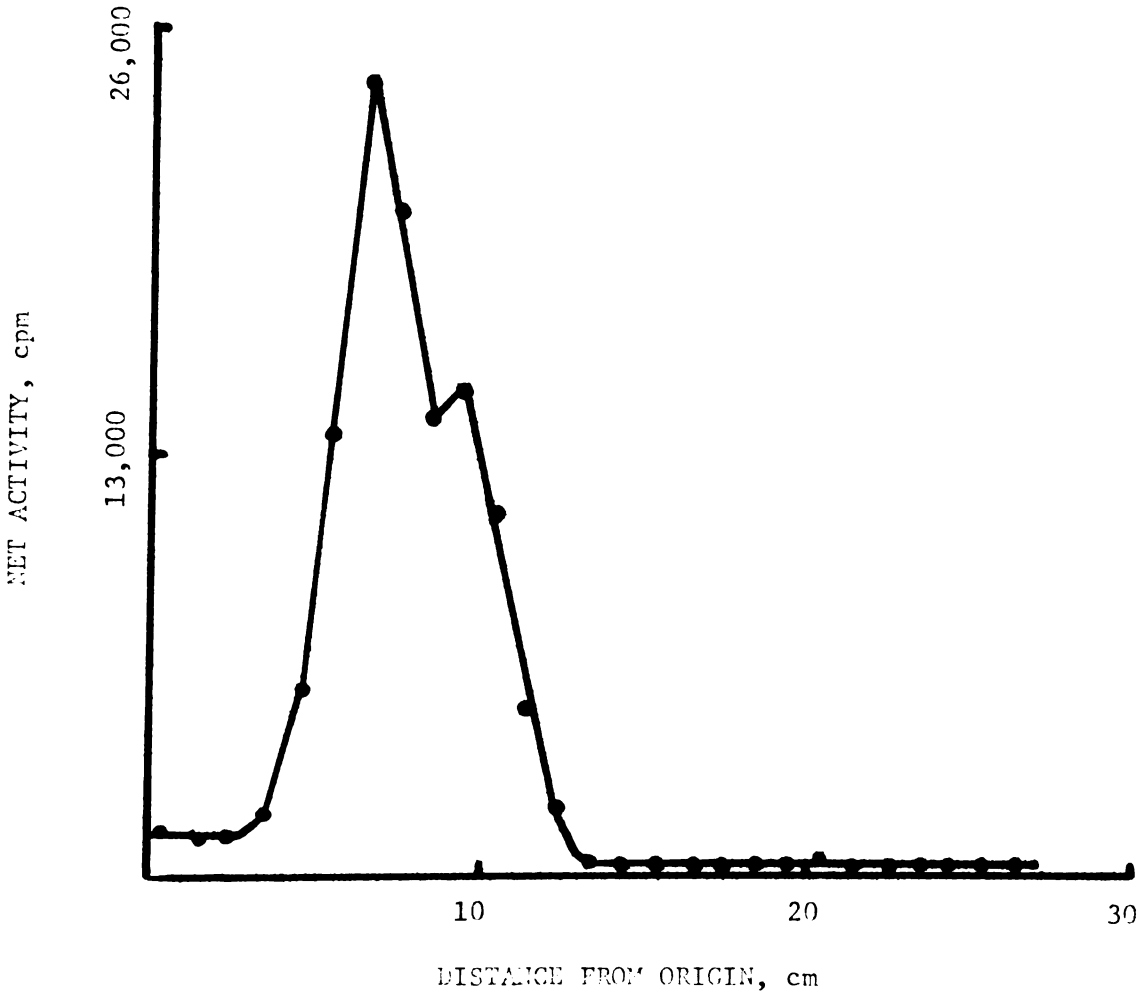


Figure 11 - Thin layer chromatogram of a urine sample obtained after an intravenous bolus of diethylstilbestrol-^{14}C. The development system consisted of n-propanol plus aqueous ammonia (7:3). Low R_f (0.24 and 0.35) multiple peaks are obtained.

correspond to unchanged diethylstilbestrol. However, as previously noted, the very small amounts of diethylstilbestrol indicated are very likely the result of hydrolysis of the conjugates which occured before the samples were processed.

This TLC data strongly supports the contention that in the rhesus monkey diethylstilbestrol monoglucuronide is the major metabolite, as previously reported for the rat and rabbit. While it would be worthwhile to further investigate the additional metabolite(s) in urine it was decided to focus on the biliary excretion and potential for enterohepatic circulation of diethylstilbestrol monoglucuronide. Hence urine data will be used primarily to account for the fraction of total dose recovered since further information cannot be obtained without separation and identification of all metabolites present. Even if it was decided to separate and identify those metabolites present in urine, it should be noted that this would add greatly to the complexity of the project. For example, if one were to assume that the second metabolite were the diglucuronide, the question of whether the diglucuronide came directly from diethylstilbestrol or, secondarily, from the monoglucuronide would have to be considered. This would entail additional experiments where both diethylstilbestrol and the monoglucuronide would have to be administered separately, or together with different labels. Both time and the amount of monoglucuronide (suitably labeled) which could be synthesized prohibited such additional investigations. Finally, the identification of metabolites requires the synthesis of such compounds (as was done with the monoglucuronide) and then TLC comparisons and various other tests with the synthesized compound and metabolites found in urine. Such a project is clearly a task requiring separate investigation in which the primary goal is the

elucidation of metabolites formed from diethylstilbestrol.

The separation characteristics of TLC versus the Folch extraction procedure are compared in Table 7 for blood, bile, and urine. Excellent agreement between the two procedures is obtained with the blood samples and,therefore,this assay method was used in place of TLC in all the remaining experiments as discussed earlier. In bile and urine, the same magnitude of separation is obtained but the Folch procedure apparently overestimates the amount of unchanged diethylstilbestrol present in these samples. Since it was decided to measure total activity in bile and urine (the unchanged diethylstilbestrol being proposed as an artifact) this discrepancy was not investigated further. The TLC and Folch procedure data obtained here provided the basis for the assay procedures in blood, bile, and urine and have been described in detail earlier in this section.

Table 7 - Comparative Efficiency of TLC and the Folch Extraction Procedure in Separating Diethylstilbestrol from Diethylstilbestrol Monoglucuronide Present in Blood, Bile and Urine Samples.

	Per cent of total activity in sample			
	Diethylstilbestrol		Diethylstilbestrol monoglucuronide	
Sample	TLC	Folch extraction	TLC	Folch extraction
Blood	35.5	35.1	64.5	64.9
Bile	3.4	5.7	96.6	94.3
Urine	0.2	3.6	99.8	96.4

INTRAVENOUS INJECTIONS OF DIETHYLSTILBESTROL-^{14}C

Having established the metabolic pattern of diethylstilbestrol, a study of the disposition characteristics of the drug in blood, bile, and urine was accomplished by administering an intravenous dose of diethylstilbestrol and carefully following the concentration-time course of unchanged drug and metabolite in these three fluids.

Methodology. An intravenous dose of diethylstilbestrol-^{14}C was administered to the same male rhesus monkey (wgt 4.1 Kg) on two separate occasions. Preparation of the dosage form, sampling of blood, bile, and urine and other procedures and conditions of the experiment are described in the previous section. In the first experiment a total dose of 48.3 μCi of diethylstilbestrol-^{14}C was administered. This had been diluted with cold diethylstilbestrol to 1,000 μg, resulting in a specific activity of 0.0483 μCi per μg. In the second study a dose of 38.5 μCi of diethylstilbestrol-^{14}C, diluted to 800 μg, resulting in a specific activity of 0.0481 μCi per μg was administered.

The dose in the semi-aqueous vehicle (4.5 ml) was injected into the iliac vein slowly, over a period of from 5 to 6 minutes. This procedure was followed since high concentrations of PEG-400 in the blood which could result following a rapid intravenous bolus have been shown to cause hemolysis (15). Also, the very rapid intravenous administration of the vehicle without drug caused noticeable excitability and stress in the monkey.

This infusion period is corrected for in the plots of diethylstilbestrol and metabolite blood levels, by subtracting the length of time of the infusion period (T) from real time (t), where the start of the infusion is

taken as zero time. Therefore, in these plots, a corrected time axis ($t' = t - \tau$) is used and "zero time" is actually the time at which the infusion ended. Pharmacokinetic analysis is done on the post-infusion blood level vs time curve.

Finally, a correction was also made for a "lag time" in the plots of biliary excretion rate data for metabolite. This correction can be made if one knows the volume of "dead space" in the externalized catheter tubing and the average bile flow rate during an experiment. The lag time so calculated was subtracted from real time resulting in a corrected midpoint time which more closely approximates what is occuring at the level of the external hepatic collection ducts. No corrections were made in the analysis of urinary data since urine was collected immediately after voluntary voiding.

Results and Discussion. In the first experiment blood, bile and urine were sampled for 24 hours and a plot of the cumulative per cent of dose excreted in urine and bile is seen in Figure 12. In this particular experiment 84.8% of the dose administered could be accounted for with 61.5% appearing in the urine and 23.3% in the bile. It can be seen that 95% of the total amount collected in both bile and urine is excreted within 5 hours after administration of the dose. However, even after 22 hours it appears that the asymptote has not yet been reached. If the sample collections had been carried out longer, it is possible that a greater portion of the dose could be accounted for. For practical purposes, it was decided to sample at discrete time intervals for 8 hours following the intravenous injections. Bile and urine samples, however, were collected out to 24 hours and pooled for calculation of the total amount recovered. At least two weeks were allowed between experiments.

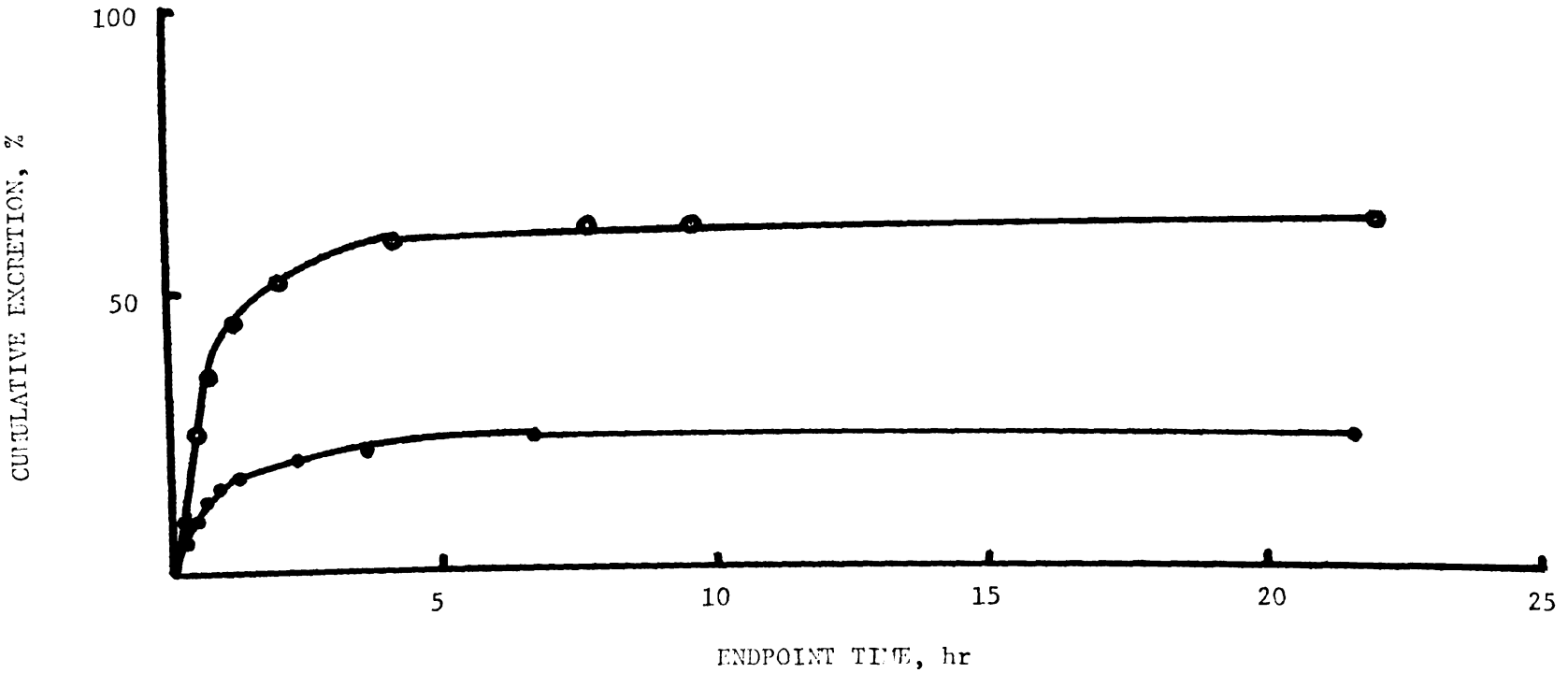


Figure 12 - A plot of the cumulative per cent of the dose excreted in urine (◎) and bile (●) after an intravenous bolus of diethylstilbestrol-^{14}C. After 24 hours, 61.5% of the dose was accounted for in the urine and 23.3% in the bile.

At that time activity levels in both bile and urine were down to background.

In the second experiment, 91% of the dose was recovered with 54.0% appearing in the urine and 37.0% in the bile. Since diethylstilbestrol is a fat soluble compound and residues can be found in cattle and poultry tissues after slaughter it can be theorized that accumulation also occurs in the monkey. That is, some diethylstilbestrol distributes into fatty depots after an intravenous injection and can remain there for long periods of time slowly leeching back out to be excreted in urine and bile long after the dose. Hence, in a 24 hour period one may not be able to account for 100% of the dose administered.

Pharmacokinetic Modeling Considerations. Drugs can distribute into tissues in a highly complex manner. In a real sense, the tissues probably contain different concentrations of the drug down to the subcellular level. Conceiving of the body as a multiple compartmental system allows one to reduce this almost infinite degree of complexity to a workable level. One may conceive of a model which represents drug distribution and elimination to be made up of a central compartment with inter-exchanging connections to one or more peripheral compartments. Such a model has been termed a mammillary model (18). For practical purposes many of the tissue compartments are small enough and exhibit sufficiently similar properties to be pooled into large groups according to their perfusion and partition characteristics (19) such as follows:

i) A highly perfused lean tissue group, consisting of the heart, lung, hepatoportal system, kidneys and endocrine glands.

ii) A poorly perfused lean tissue group consisting of the large mass of muscle and skin tissue.

iii) A fat group consisting of the adipose tissue, including bone marrow.

After an intravenous injection the resultant blood concentration data frequently appears to fit a bi- or tri-exponential function. This is considered to represent body distribution of the drug from a central into one or two peripheral compartments, with metabolism and excretion taking place from the central compartment. Generally one attempts to minimize the number of exponential terms which appear to be required to fit the actual experimental data.

Blood concentration data for diethylstilbestrol-^{14}C following intravenous administration at one dose level, employing a very intensive blood sampling schedule have been obtained in the second experiment. A pharmacokinetic analysis of the data allows one to propose a model for the disposition of diethylstilbestrol in the rhesus monkey. A semi-log plot of the blood concentration levels of diethylstilbestrol-^{14}C versus time is shown in Figure 13. After several attempts at fitting the data, it was concluded that the three body-compartmental model provided the best correlation to experimental data.

It should be noted that the last three points of Figure 13 were less than twice background, however they were included in the analysis. This was done since use of all the data (which is best fit by the 3 compartment model) provided much more compatable comparisons of the various model parameters (especially clearance values) with those observed in other studies.

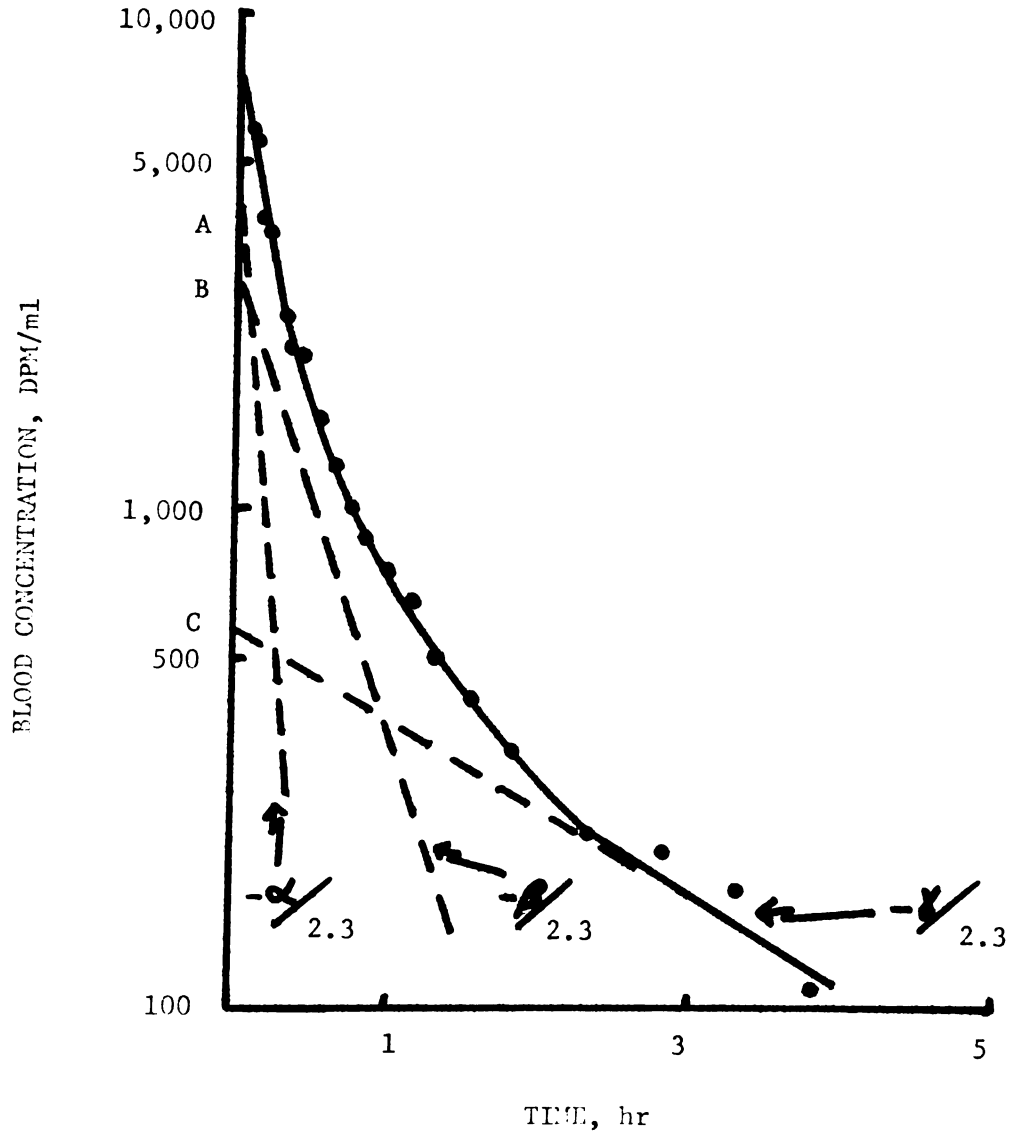
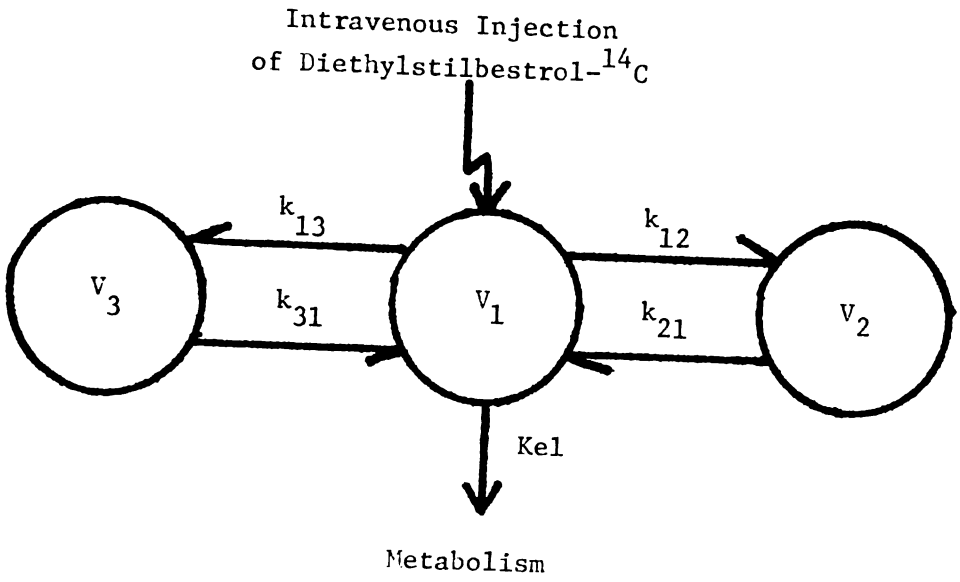


Figure 13 - A semilogarithmic plot of post infusion diethylstilbestrol-^{14}C blood levels versus time after an intravenous infusion of diethylstilbestrol-^{14}C. The solid circles are actual data points while the solid line is the computer generated fit to the data based on a three-compartmental open model. The curve may be resolved into its components yielding three linear segments (broken lines) with slopes of $-\alpha/2.3$, $-\beta/2.3$, $-\gamma/2.3$ and zero-time intercepts of A, B, and C respectively.

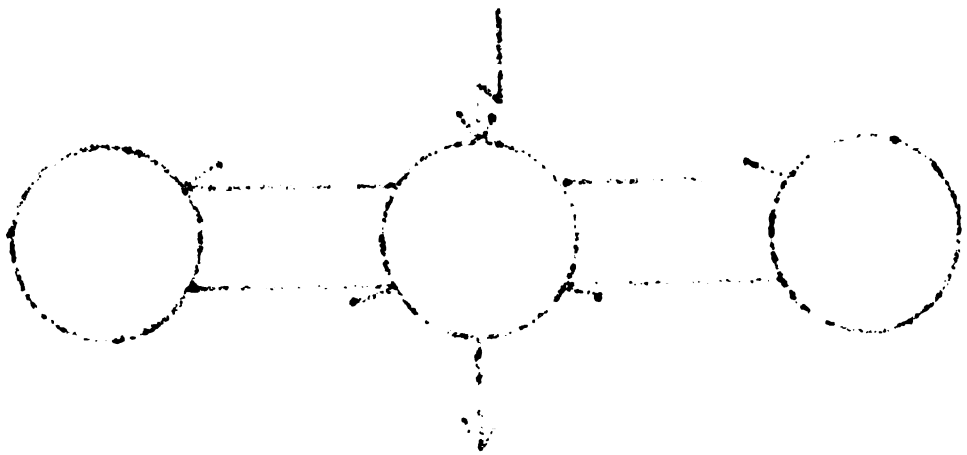


In the above schematic, the central (V_1) and peripheral compartments (V_2 and V_3) represent the various tissue groups just discussed. The rate constants between the compartments and out of compartment 1 are apparent first - order rate constants for distribution and metabolism, and it is assumed that metabolism takes place only in the central compartment. This model results in a tri-exponential equation (20) of the form:

$$C_B = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-\gamma t}$$

where C_B equals blood concentration levels of diethylstilbestrol (in the central compartment), A, B, and C represent intercepts on the ordinate at zero time, and α, β, and γ are hybrid rate constants, each influenced by all of the rate constants of the system. When plotted on semilogarithmic paper $-\alpha/2.3$, $-\beta/2.3$, and $-\gamma/2.3$ are the slopes of the respective exponential segments. By definition, $\alpha > \beta > \gamma$.

In actuality the following equation was the function defined and analyzed on the computer:



$$C_B = \frac{DOSE}{V_1} \cdot \left[\frac{(k_{21}-\alpha)\cdot(k_{31}-\alpha)}{(\beta-\alpha)\cdot(\gamma-\alpha)} \cdot e^{-\alpha t} + \frac{(k_{21}-\beta)\cdot(k_{31}-\beta)}{(\alpha-\beta)\cdot(\gamma-\beta)} \cdot e^{-\beta t} + \frac{(k_{21}-\gamma)\ (k_{31}-\gamma)}{(\alpha-\gamma)\cdot(\beta-\gamma)} \cdot e^{-\gamma t} \right]$$

The BMDx85 nonlinear least squares curve fitting program was used to fit the data on the IBM 360/50 digital computer.[1] This program results in a weighted (reciprocal blood concentration) least squares fit of a user specified function to data values by means of stepwise Gauss-Newton iterations on the specified parameters. Initial estimates of the parameters were obtained graphically and by use of the analog computer in the usual manner.

The computer generated parameters along with various other constants derived (21) from these parameters are presented in Table 8. The computer generated standard deviations, expressed as a per cent of the parameter value, are also included in the table. These standard deviations are quite large and in some cases approach the value of the estimated parameter. A detailed discussion of these parameter estimates and their standard deviations will not be dealt with here. However, the reader is referred to an excellent article by Boxenbaum et al. (21a) which does deal specifically with the interpretation of statistical estimations in pharmacokinetics. For the present, it will suffice to point out that when the standard deviation is a large value relative to the estimated parameter, this indicates that any parameter estimate in a relatively

[1]The program was prepared by the staff of Health Sciences Computing Facility, UCLA and further information can be obtained from the Biomed manual.

large region of the parameter space gives about equally good fit to the data. It should be strongly emphasized that this situation does not necessarily result from poor data; indeed, good data may result in an excellent computer fit, but with large standard deviations.

The derived constants (see Table 8) do not include standard deviations, which could be obtained using more exact equations which include the standard deviations of the computer generated parameters. For a detailed discussion of these equations the reader is again referred to Boxenbaum et al. (21a). The constant (Kel) can be calculated by use of the following equation:

$$Kel = \frac{(A+B+C)\cdot(\alpha\cdot\beta\cdot\gamma)}{A\cdot\beta\cdot\gamma + B\cdot\alpha\cdot\gamma + C\cdot\alpha\cdot\beta}$$

where Kel equals the metabolic rate constant(s) since no free compound is excreted in urine or bile. In order to calculate the rate constants k_{12} and k_{13} the following definitions are useful:

Let

$$E\ 1 = k_{12} + k_{13} + Kel$$

$$E\ 2 = k_{21}$$

$$E\ 3 = k_{31}$$

$$G\ 1 = \alpha + \beta + \gamma$$

$$G\ 2 = \alpha\cdot\beta + \beta\cdot\gamma + \alpha\cdot\gamma$$

$$G\ 3 = \alpha\cdot\beta\cdot\gamma$$

then,

$$E\ 1 = G\ 1 - E\ 2 - E\ 3$$

After calculating the values for E 1, E 2, E 3, G 1, G 2, and G 3, the individual rate constants k_{12} and k_{13} can be determined from:

$$k_{13} = \frac{G\ 3 - E\ 3 \cdot (G\ 2 - E\ 1 \cdot E\ 3 - E\ 2 \cdot E\ 3)}{E\ 3 \cdot (E\ 3 - E\ 2)}$$

and,

$$k_{12} = E\ 1 - k_{13} - Kel$$

As previously mentioned, pharmacokinetic analysis was done on the post-infusion blood level curve of diethylstilbestrol-^{14}C. Pharmacokinetic parameters are usually derived from the exponential equations which describe the blood level curve after a single rapid intravenous injection. However, it has been demonstrated (21b) that all the usual pharmacokinetic parameters obtained after a rapid intravenous bolus can also be obtained from postinfusion blood curves. These workers pointed out that the hybrid rate constants (α, β, and γ) are the same whether they are obtained from a post-infusion curve or a single intravenous bolus curve. However, the intercepts of the exponential terms (A, B, and C) are different and the differences increase as the infusion time lengthens. Since these intercepts are often used to calculate kinetic constants intrinsic to multicompartmental models, appropriate corrections must be made.

The intercepts which would be obtained from an intravenous bolus curve can be calculated from the following equation:

$$A_i = \frac{k_i \cdot \tau}{1 - e^{-k_i \cdot \tau}} \cdot A_i'$$

where Ai is the real intercept and Ai' is the intercept obtained (after extrapolation to t' = 0) from the post-infusion curve (where t' = t -). Of course, is the infusion time and ki represents the hybrid rate constant of the exponential term. The above equation was used to calculate those intercepts which would be obtained from blood level data after an intravenous bolus. These corrected intercepts were then used to calculate Kel by the equation previously listed and the volume of distribution for the central compartment, V_1, where:

$$V_1 = \frac{DOSE}{A+B+C}$$

$$V_1 = \frac{85,410,198 \text{ DPM}}{10,293.1 \text{ DPM/ml}}$$

$$V_1 = 8,298 \text{ ml}$$

As expected, this value differs from that calculated by the digital computer (Table 8) since digital computation was performed on post-infusion data.

This model presents some interesting considerations in terms of the obtained parameters. For example, in a 4.1 Kg monkey the circulating blood volume would be approximately 367 ml which is far less than V_1 (8,298 ml). In fact this volume is far in excess of any volume compartment which could be conceived of in an animal whose total body water would be approximately 2,460 ml. This volume term (V_1) represents the volume of the central compartment and at steady state the drug would be distributed into V_1 and the two peripheral compartments (V_2 and V_3) as well. The following relationships allow one to calculate the volumes of these peripheral compartments:

Table 8 - Pharmacokinetic Parameters Obtained by Resolving Post-Infusion Diethylstilbestrol Blood Concentration Data Into a Three Compartment Model.

Parameter	Value[(a)]	Units	Comment
V_1	10,766(8.9)	ml	Computer generated parameters
k_{21}	0.07141(82.3)	min^{-1}	
k_{31}	0.01045(99.9)	min^{-1}	
α	0.12213(43.8)	min^{-1}	
β	0.03494(66.9)	min^{-1}	
γ	0.00682(81.9)	min^{-1}	
Kel	0.04446	min^{-1}	Constants calculated from computer generated parameters
k_{12}	0.02234	min^{-1}	
k_{13}	0.01523	min^{-1}	

(a) The computer generated asymptotic standard deviation, expressed as a per cent of the value, is enclosed in parentheses.

$$k_{12} \cdot V_1 = k_{21} \cdot V_2$$

$$k_{13} \cdot V_1 = k_{31} \cdot V_3$$

also,

$$Vdss = V_1 + V_2 + V_3$$

The volumes of V_2 and V_3 are 2,596 ml and 12,093 ml respectively. If one combines all of these volumes, the total volume of distribution at steady state (Vdss) is 22,987 ml. Such large values for volume constants can cause great confusion (21c). In the case of V_1 one can only emphasize that it is not equal to the total body volume in which the drug would be distributed at steady state conditions, nor does it equal the volume of the blood or plasma. It is most logical to conceive of this so called volume constant as simply a parameter of the equation required to convert the mass of the drug we conceive to be in this imaginary central compartment into concentration units. It is not unusual to find extremely large volumes of distribution for various non polar, lipid soluble drugs (22). In contrast, very polar-water soluble compounds, huge macromolecules, or highly protein bould substances will have a volume of distribution which approximates blood volume (23).

The microscopic rate constant k_{31} represents the rate of transfer of drug out of the third compartment back into the central compartment. It may be noted that this constant is very small and approximates the value of γ. This correlates well with the theory that diethylstilbestrol is taken up into a poorly perfused tissue compartment (V_3) and then leeches out very slowly over a prolonged period of time. Hence, residual levels

of drug can be found in the body long after administration of the dose. One should recall however that this experiment was done with 100% collection of bile which does not allow for entero hepatic recycling (EHC) of drug. If EHC is established (in an intact animal) this could also contribute to the persistance of drug in the body.

Another important parameter to comment upon is mean clearance ($\dot{V}cl$). Clearance may be defined as the volume of blood completely cleared of drug per unit time, by processes of metabolism and excretion. It is a measure of the functional ability of an organ (or organs) to remove a drug from the body. The estimated clearance of a drug can vary anywhere from zero up to but not exceeding blood flow to the eliminating organ (except in the case where blood itself can metabolize drug). The mean blood clearance of diethylstilbestrol-^{14}C was estimated using the trapezoid rule to calculate the area under the blood level vs time curve (AUC) and knowing the dose administered:

$$\dot{V}cl = \frac{DOSE}{AUC}$$

$$\dot{V}cl = \frac{85,410,198 \text{ DPM}}{249,175 \frac{\text{DPM}\cdot\text{min}}{\text{ml}}}$$

This value (342.8 ml/min) is very large and in fact exceeds the reported value for liver blood flow (218 ml/min, S.D. 78) in the rhesus monkey. This indicates that there may be other organs responsible for the metabolic clearance of diethylstilbestrol. One may postulate that the kidneys (blood flow - 138 ml/min, S.D. 52) and a portion of the gastro-intestinal tract (blood flow - 126 ml/min, S.D. 57) are additional clearing organs. In the previous section evidence for metabolism occuring in the kidney was

presented. If a portion of the additional conjugates seen in the urine are formed directly from unchanged diethylstilbestrol entering the kidney from the blood, this organ would then be serving as a clearing organ. The estimated mean clearance could therefore exceed liver blood flow. Also, information which will be presented later clearly implicates the intestinal tract as a potential metabolizing organ. It has been previously demonstrated that the blood itself does not metabolize diethylstilbestrol. Methods are available which allow one to determine the exact contribution of additional clearing organs (24) but this was beyond the scope of the present investigation.

While the model proposed and conclusions drawn may have validity, the limitations of the model should be pointed out. It must be noted that only one dose level was studied and in only one monkey. Hence the model may hold here but the possibility of dose dependent kinetics which would be observed at higher dose levels could change the model. The fact that bile was collected but not returned, preventing EHC of drug, can also modify the model as already pointed out.

Diethylstilbestrol-^{14}C and diethylstilbestrol monoglucuronide-^{14}C activities found in blood and bile samples are plotted in Figure 14. The blood data is expressed in DPM per ml and the bile data in terms of DPM per minute, however one can easily convert to amounts knowing the specific activity of diethylstilbestrol-^{14}C (106,838 DPM/μg) and metabolite (61,436 DPM/μg). It can be observed that blood levels for metabolite exceed those of unchanged diethylstilbestrol at all time points. One can calculate (using the trapezoid rule) that the area under the blood concentration-time curve for metabolite exceeds parent compound by approximately 3 fold. A very rapid appearance of metabolite with a peak blood level

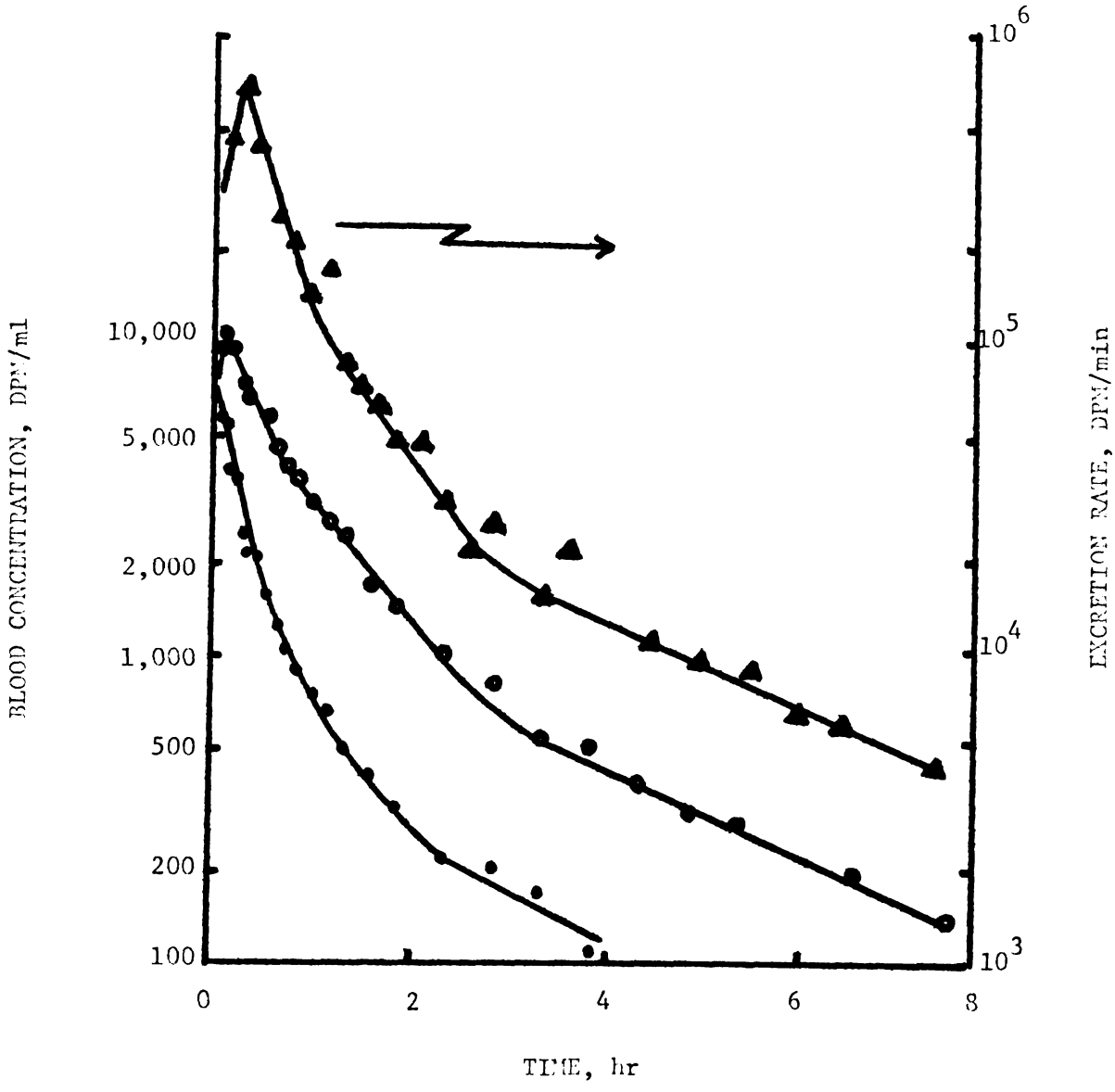


Figure 14 - A semilogarithmic plot of the blood levels of diethylstilbestrol-^{14}C (●) and diethylstilbestrol monoglucuronide-^{14}C (◎) vs time along with the biliary excretion rate of diethylstilbestrol monoglucuronide-^{14}C (▲) vs midpoint time-following the intravenous injection of diethylstilbestrol-^{14}C.

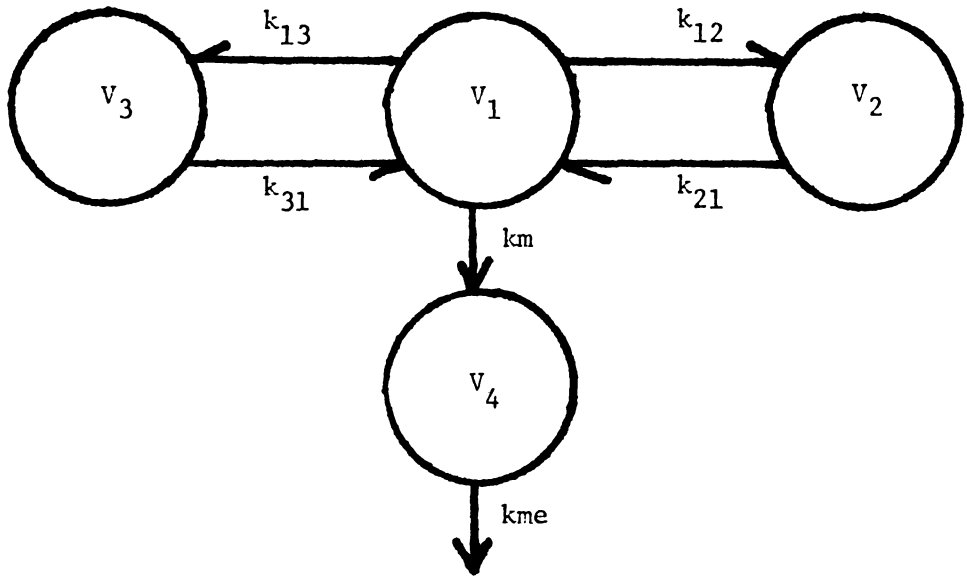
occuring at approximately 6 minutes, is also observed. A high rate of metabolism along with a relatively small volume of distribution for metabolite (compared to parent compound) could explain the higher blood levels observed for diethylstilbestrol monoglucuronide.

Figure 14 also includes a plot of the average biliary excretion rate of diethylstilbestrol monoglucuronide-^{14}C between collection times. Several of the data points are scattered above the curve. It will be demonstrated later that these points are probably due to spontaneous variations in bile flow rate, which will be shown to influence the amount of metabolite excreted per unit time. However, if one accepts the smooth curve as drawn, the parallelism of the terminal portions of all three curves (blood concentration levels of diethylstilbestrol and metabolite, and excretion rate of metabolite in bile versus time) is obvious. Of particular interest is the fact that the projected terminal slope obtained in the fitting of blood data for unchanged diethylstilbestrol (where very low level activity occurs) is seen to parallel the levels of metabolite in blood and bile very closely (where much more confidence in the data is obtained due to the higher levels of activity). This further supports the contention that the three body-compartment model is probably appropriate.

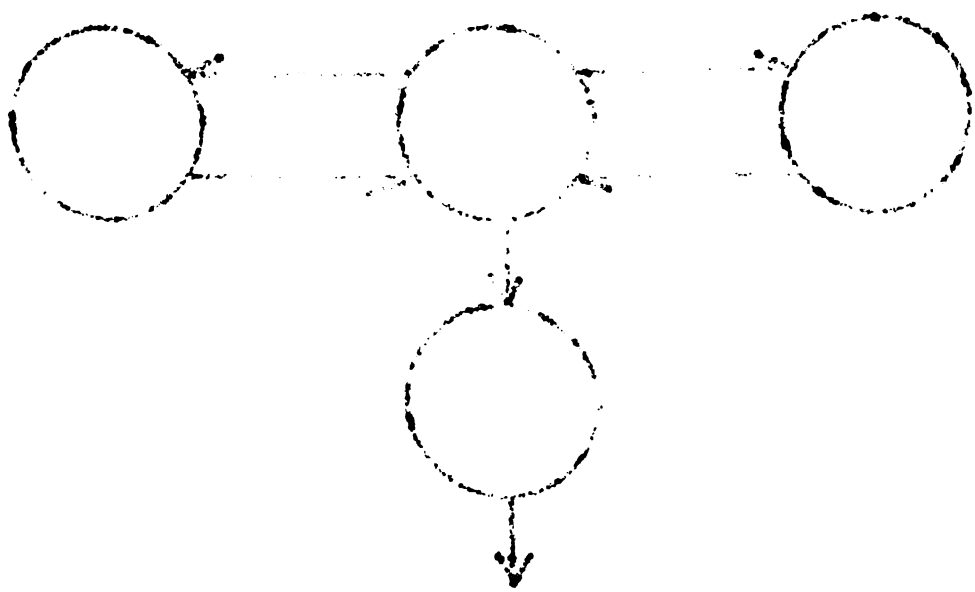
One could also analyze metabolite blood, bile, and urine data on the digital computer and thereby quantitate its pharmacokinetic parameters. The preferred method in doing this would be to first isolate and inject diethylstilbestrol monoglucuronide-^{14}C (of high purity and high specific activity) intravenously. In this manner the disposition characteristics of the metabolite can be determined independently. After a pharmacokinetic model has been obtained for the metabolite one can then use the

computer generated parameters as input in analyzing the complete (pharmacokinetic) model which would describe diethylstilbestrol and metabolite blood levels and metabolite levels in bile and urine.

If these procedures were not carried out and one attempted to fit the data by computer analysis of all the various models which are reasonable, many computational difficulties could occur. Also, interpretation of the statistical information generated for very complex models with a large number of parameters would be complex and based on the statistics generated for diethylstilbestrol-^{14}C blood levels - not particularly discriminating. For example, if one were to assume that diethylstilbestrol monoglucuronide pharmacokinetics could best be described by a one compartment open model, the following overall model would ensue for diethylstilbestrol and metabolite blood levels:



In this four compartment model the integrated equation for diethylstilbestrol blood levels would be the same as previously described. The corres-



ponding equation for metabolite blood levels would be:

$$C_{B_{DESGA}} = \frac{DOSE \cdot km}{V_4} \cdot \left[\frac{(k_{21}-kme)\cdot(k_{31}-kme)}{(\alpha-kme)\cdot(\beta-kme)\cdot(\gamma-kme)} \cdot e^{-kmet} + \right.$$

$$\frac{(k_{21}-\alpha)\cdot(k_{31}-\alpha)}{(kme-\alpha)\cdot(\beta-\alpha)\cdot(\gamma-\alpha)} \cdot e^{-\alpha t} +$$

$$\frac{(k_{21}-\beta)\cdot(k_{31}-\beta)}{(kme-\beta)\cdot(\alpha-\beta)\cdot(\gamma-\beta)} \cdot e^{-\beta t} +$$

$$\left. \frac{(k_{21}-\gamma)\cdot(k_{31}-\gamma)}{(kme-\gamma)\cdot(\alpha-\gamma)\cdot(\beta-\gamma)} \cdot e^{-\gamma t} \right]$$

where km and kme are the apparent first order rate constants for the formation and excretion of diethylstilbestrol monoglucuronide respectively. The term V_4 represents the volume of distribution of diethylstilbestrol monoglucuronide. The other microscopic rate constants are the same as those previously described in the treatment of the pharmacokinetic model for diethylstilbestrol blood levels. The above equation is rather complex and the total number of parameters which would have to be estimated is nine (V_1, V_4, k_{21}, k_{31}, km, kme, α, β, and γ). While one could obtain all the parameter estimates by non linear least squares regression analysis they would probably have large standard deviations and therefore not be particularly informative. Furthermore, in analyzing urine and bile data these problems would be magnified since one may encounter lag times and data which are not as "good" as blood data since excretion rate plots are average rates which are at best crude estimations of instantaneous

rates of excretion. Analysis of the excretion rate data is further complicated in the urine where the presence of multiple metabolites prevent the separate detection and quantitation of the monoglucuronide.

Since the primary purpose of this project was to study the biliary excretion and enterohepatic circulation of diethylstilbestrol and its monoglucuronide, a detailed pharmacokinetic description of drug and metabolite was not persued. However, a pharmacokinetic model for diethylstilbestrol-^{14}C in blood was presented, with a general appreciation of the complexities involved in describing a complete pharmacokinetic model for parent drug and metabolite.

INTRAVENOUS INFUSIONS OF DIETHYLSTILBESTROL-^{14}C

Sodium Taurocholate Induced Alterations of Bile Flow Rate

Since earlier in vitro experiments indicated that an association between bile micelles and sodium diethylstilbestrol monoglucuronide exists, an in vivo experiment was planned to assess how changes in bile salt excretion rate and/or bile flow rate would affect the disposition of this compound in the bile. It was decided that a system at steady state would provide the most appropriate model to examine, since the only variables would be bile salt excretion rate and bile flow rate.

Preparation of Solutions. The intravenous bolus injection solution was prepared in an ethanol (2%) - PEG-400 (50%) - water (48%) system as described in the metabolism section. Exactly 12.294 μCi of radioactive diethylstilbestrol-^{14}C was diluted with 12.375 mg of cold drug resulting in a specific activity of 0.993 μCi/mg. This amount was diluted with the above system to a final volume of 4.5 ml which results in a radioactive drug concentration of 2.732 μCi/ml and a total concentration of 2.75 mg/ml of diethylstilbestrol. After preparation of the solution it was sterilized by filtration through a Millipore filter. The intravenous infusion solution was prepared in an ethanol (1%) - PEG-400 (50%) - water (49%) system. The method of preparation was similar to the above procedure. Exactly 132.24 μCi of radioactive diethylstilbestrol-^{14}C was diluted with 104.00 mg of cold drug resulting in a specific activity of 1.272 μCi/mg. This amount was diluted with the above system to a final volume of 80 ml resulting in a final radioactive drug concentration of 1.653 μCi/ml and a total concentration of 1.30 mg/ml. This solution was again sterilized by filtration prior to administration.

Sodium taurocholate was dissolved in distilled water (44.76 mg/ml) for duodenal infusion during the initial stages of the experiment. Sodium taurodehydrocholate was dissolved in distilled water (37.08 mg/ml) for duodenal infusion during the terminal stage of the experiment. A heparinized saline solution was prepared for intravenous hydration by adding 5,000 units of sterile heparin to 1 liter of sterile normal saline to give a final concentration of 5 units/ml.

Experimental Protocol. Duodenal infusion of sodium taurocholate at the rate of 2.91 μmol/min (0.035 ml/min) was begun 4 hours prior to the start of the experiment to insure that bile flow rate and bile salt excretion rate would reach steady state levels. Intravenous hydration of the monkey with heparinized saline (30 ml/hr) was begun 2 hours prior to starting the experiment and maintained throughout.

Diethylstilbestrol-^{14}C was administered by an intravenous bolus (4.5 ml) followed immediately by an intravenous infusion (t = 0 hours) in order to attain steady state blood levels as rapidly as possible. The infusion was administered at the rate of 0.138 ml/min (0.228 μCi/min) over 480 minutes resulting in a total dose of 66.24 ml which contained 109.49 μCi diethylstilbestrol-^{14}C equivalent to 86.11 mg. Both solutions were administered via the iliac vein and the combined bolus and infusion represent a total dose of 121.78 μCi of diethylstilbestrol-^{14}C equivalent to 98.49 mg. This represents an average specific activity of 1.24 μCi/mg.

First, the intravenous bolus injection was administered, immediately followed by the start of the intravenous infusion of diethylstilbestrol-^{14}C. Sodium taurocholate was infused via the duodenum at an initial rate of 2.91 μmol/min from t = 0 to 3 hours (level 1), and then increased to

13.64 μmol/min for t = 3 to 6 hours (level 2). Then a duodenal infusion of sodium taurodehydrocholate was begun in place of taurocholate at t = 6 to 8 hours at a rate of 9.13 μmol/min (level 3). The substitution of taurodehydrocholate was an attempt to eliminate the micelle forming capacity of bile by replacing taurocholate, a micelle forming bile salt, with taurodehydrocholate, a non-micelle forming choleretic.

Analytical Procedures. Sampling of blood, bile, and urine and assay techniques employed therein are discussed in the metabolism section. The assay procedure for total bile salts in bile is discussed in the analytical methods section.

Results and Discussion. In this experiment 87.3% of the dose could be accounted for with 50.4% appearing in the urine and 36.9% in the bile. As before, not all of the dose could be accounted for during the collection period. Blood concentration (DPM/ml) versus time of diethylstilbestrol-^{14}C and its monoglucuronide are plotted in Figure 15. It is apparent that unchanged drug reaches a steady state concentration in blood with a mean value of 1,654.4 DPM/ml. The total body clearance ($\dot{V}cl$) of diethylstilbestrol-^{14}C may be defined as;

$$\dot{V}cl = \frac{\text{Infusion Rate (DPM/min)}}{\text{Steady State Blood Concentration (DPM/ml)}}$$

$$= \frac{506,306.9 \text{ (DPM/min)}}{1,654.4 \text{ (DPM/ml)}}$$

$$= 306.0 \text{ ml/min}$$

this is close to the clearance value calculated from the intravenous bolus study (342.8 ml/min). In the virtual absence of any excretion of unchanged compound into the bile or urine, this represents the metabolic clearance of diethylstilbestrol.

In contrast to unchanged drug, it appears that metabolite concentra-

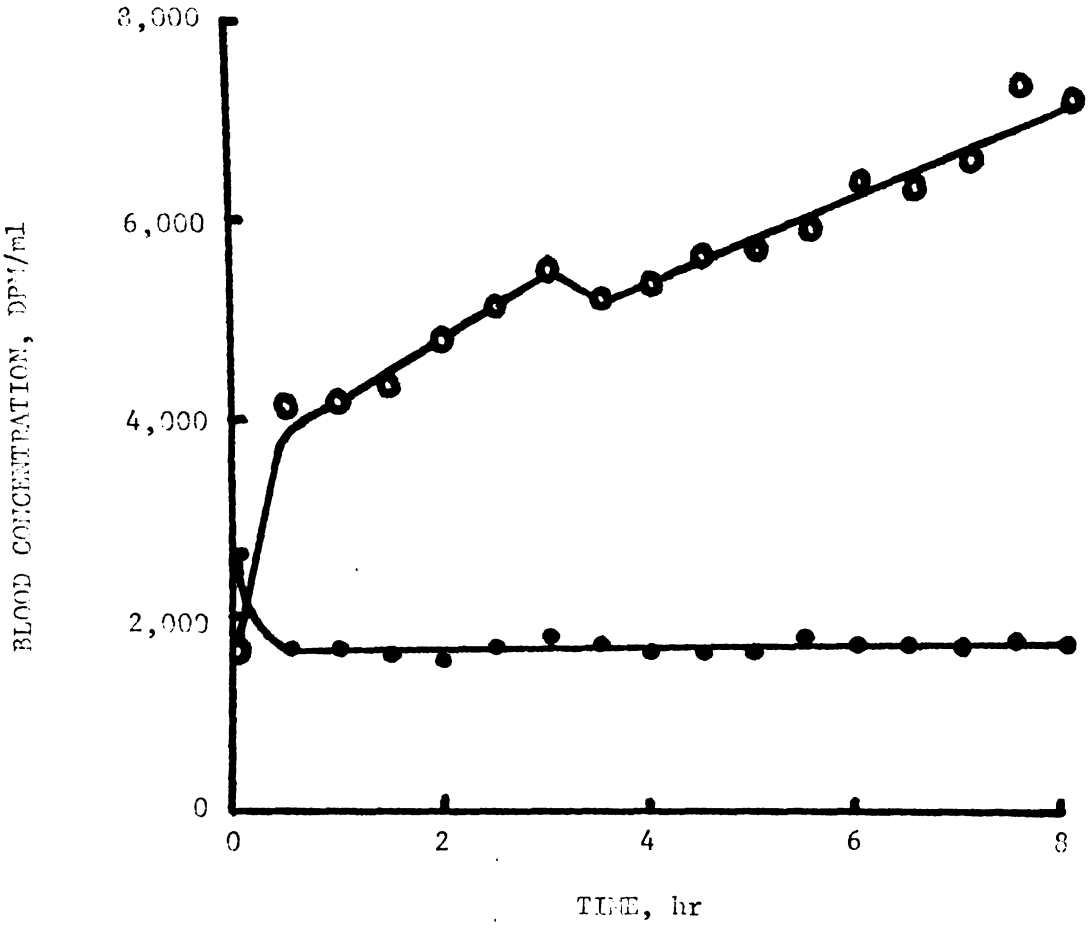


Figure 15 - A plot of the blood levels of diethylstilbestrol-^{14}C (●) and diethylstilbestrol monoglucuronide-^{14}C (◐) obtained during a constant intravenous infusion of diethylstilbestrol-^{14}C. During the experiment a choleresis was maintained by a duodenal infusion of sodium taurocholate. At t = 3 hours, a break in the metabolite blood level curve occurs. This corresponds to an increased bile flow rate which resulted when the taurocholate infusion rate was increased to a higher level.

tions in the blood do not reach a steady state level and appear to be increasing with time. One can also see that there is a sharp break in the curve which occurs at t = 3 hours, at which time the duodenal infusion rate of taurocholate was increased. After this break, the slope of the blood concentration versus time curve decreases. Since blood levels of diethylstilbestrol are constant, there was no change in the rate of glucuronide formation.

As previously noted there are apparently multiple metabolites in the urine. However, if one measures total activity in the urine it is found that during the first three hours the average excretion rate is 272,414 DPM/min while during the 3 to 5 hour interval the excretion rate decreased to 236,938 DPM/min. This corresponds to a 13.0% decrease. During the 5 to 8 hour interval the excretion rate dropped further to 208,443 DPM/min, a decrease of 23.5%. These reductions should cause a net increase in the accumulation of glucuronide in the blood when, in fact, the decrease in slope indicates an actual decrease in the rate of accumulation. Therefore, the change in slope of the blood curve must be due to a net decrease in the rate at which diethylstilbestrol monoglucuronide is being transferred from the biliary system to the blood. This is confirmed by examination of the biliary excretion data which indicates a net increase in the biliary clearance of metabolite during these time intervals. An approximation of the biliary clearance of diethylstilbestrol monoglucuronide can be made using the following formula;

$$\dot{V}cl,\ \text{metabolite, bile} = \frac{\text{Steady State Excretion Rate (DPM/min)}}{\text{Steady State Blood Levels (DPM/ml)}}$$

However, since the blood levels of metabolite are not at steady state, the average blood concentration during level 1 and level 2 was calculated.

The average biliary clearance during level 1 (130,795 DPM/min ± 4,775 DPM/ml) is 27.4 ml/min and during level 2 (210,332 DPM/min ± 5,675 DPM/ml) equals 37.1 ml/min, indicating that excretion into the bile is truly enhanced during choleresis and not simply a reflection of increased blood levels of metabolite.

Figure 16 contains several graphs relating to the biliary excretion of diethylstilbestrol monoglucuronide, including a plot of bile flow rate vs time, metabolite excretion rate vs time and biliary concentration levels vs time. The first three bile collection periods apparently reflect a response to the intravenous bolus of diethylstilbestrol-^{14}C just prior to initiating the constant infusion, resulting in an initial peaking of metabolite excretion rate levels due to the bolus. The latter periods reflect a response to the constant infusion. The samples taken during the second and third hour indicate that an apparent steady state - biliary excretion rate of diethylstilbestrol monoglucuronide has been achieved, even though the blood concentration levels for this metabolite are increasing. One may therefore presume that an apparent transport maximum (Tm) for excretion of diethylstilbestrol monoglucuronide has been attained. After the first three hours, the duodenal sodium taurocholate infusion rate was increased and this leads to a corresponding increase in the rate of diethylstilbestrol monoglucuronide excretion into the bile. While somewhat erratic, the excretion rate of the conjugate apparently also levels off to a new steady state value. It is likely that the perturbations seen in the metabolite excretion rate plot are due to irregularities in the bile flow rate since the peaks and valleys seen in this curve appear to parallel the changes in excretion rate. In fact, this linear dependence of metabolite excretion rate upon bile flow rate is per-

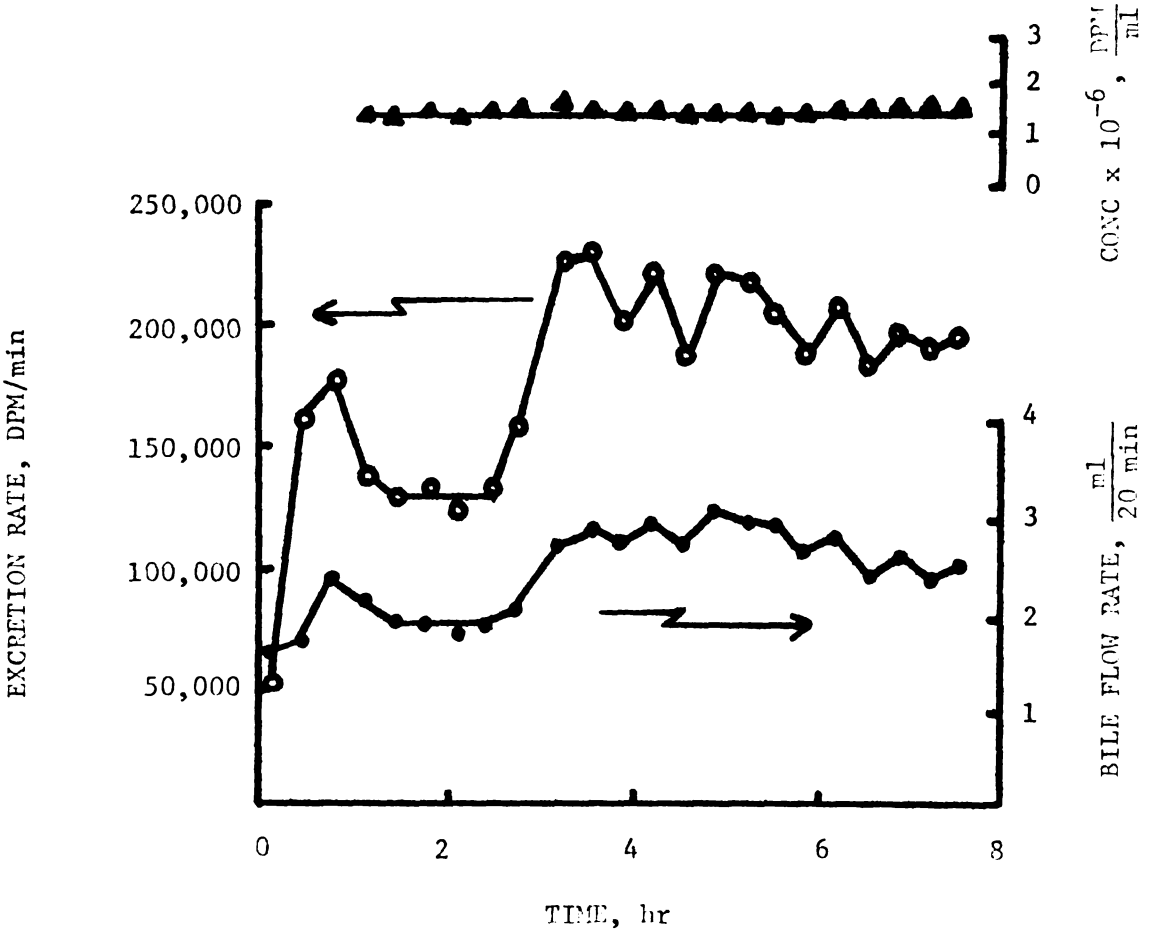


Figure 16 - A plot of bile flow rate (●), sodium diethylstilbestrol monoglucuronide-^{14}C biliary excretion rate (●) and concentration (▲) versus midpoint time. These data were obtained during an experiment in which diethylstilbestrol-^{14}C was administered initially as an intravenous bolus and immediately thereafter as a constant intravenous infusion which was maintained throughout. Bile choleresis was maintained by a duodenal infusion of sodium taurocholate which was infused at a low rate initially and then increased approximately four fold at t = 3 hours.

haps the most striking feature of this figure. If one divides the excretion rate of metabolite by the bile flow rate at each time point one obtains the concentration of metabolite in bile. This results in a horizontal line indicating that the concentration of the conjugate in bile is unaffected by flow rate. When sodium taurodehydrocholate infusion was substituted for sodium taurocholate, no effect was noted at $t > 6$ hours.

Table 9 includes a tabulation of the data obtained from analysis of the bile samples. The linear dependence of glucuronide excretion upon bile flow rate is more clearly demonstrated. Diethylstilbestrol monoglucuronide excretion rate is observed to increase by 60.8% when the sodium taurocholate infusion is increased from level 1 to level 2. Of all the variables listed in the table, it can be seen that only bile flow rate parallels this, increasing by 56.8%. The glucuronide concentration in bile does not change significantly from level 1 to level 2, while bile salt concentration and excretion rate levels increase to a much larger degree than does the glucuronide excretion rate. Therefore, while all three factors may be contributing to the increased glucuronide output into the bile,only bile flow rate exhibits a linear, one to one dependence.

Since the substitution of taurodehydrocholate produced no significant change, other than a slightly diminished bile flow rate, this portion of the experiment is not included in the table. Possible reasons for this lack of effect will be discussed in the next section which deals with another experiment in which taurodehydrocholate is infused intravenously as a choleretic under different experimental conditions.

Since glucuronide excretion into the bile is apparently proceeding at its maximal transport rate during both levels, what we have apparently observed in these experiments is the capacity of a taurocholate induced

Table 9 - The Apparent Steady State Values During a Constant Intravenous Infusion of Diethylstilbestrol-^{14}C With Two Levels of Duodenally Infused Taurocholate.

Conditions		Steady state measurements in bile				
Time interval of measurements (hours)	Taurocholate infusion rate (μmol/min)	Bile salt conc (μmol/ml)	Bile flow rate (ml/min)	Bile salt excretion rate (μmol/min)	NaDESGA[(a)] conc (DPM/ml)	NaDESGA[(a)] excretion rate (DPM/min)
1.1 to 2.5	2.9 - level 1	9.70	0.095	0.922	1,330,291	130,795
3.0 to 6.0	13.6 - level 2	28.3	0.149	4.22	1,425,302	210,332
Ratio (level 2/level 1)		2.92	1.57	4.58	1.07*	1.60
Relative increase (% of level 1)		191.8	56.8	357.7	7.1*	60.8

(a) NaDESGA = sodium diethylstilbestrol monoglucuronide-^{14}C.

* This change in concentration of NaDESGA is not significant at $\alpha = 0.01$. All other measured changes are significantly different at $\alpha = 0.01$.

choleresis to increase the Tm for glucuronide excretion into the bile. A Tm for biliary excretion has been demonstrated for taurocholate (25), bilirubin (26), and bromsulphthalein (BSP) (27). Increasing taurocholate infusion levels were also shown to increase the Tm of BSP into the bile and these workers concluded (27) that the limiting factor for BSP transport was the concentration of BSP in the bile.

In the present experiment one can calculate the average concentration of diethylstilbestrol monoglucuronide in the bile, knowing its specific activity (0.711 μCi/mg or 1,579,555.5 DPM/mg). The average activity for the duration of the 8 hour experiment was 1,455,515.2 DPM/ml (S.D. = 109,488.3) which is equivalent to a concentration of 0.922 mg/ml. This concentration is below the solubility of the conjugate in plain aqueous buffer (1.37 mg/ml, S.D. = 0.17) and well below what one would expect in bile in the presence of micelles (10.40 mg/ml). This apparent inconsistency will be expanded upon in the next section along with a further discussion of the data.

Sodium Taurodehydrocholate Induced Choleresis

In the preceeding section it was observed that the administration of sodium taurodehydrocholate in place of taurocholate did not alter the excretion rate of diethylstilbestrol monoglucuronide into the bile. It was believed that the reason for this lack of an effect could be due to the presence of (micelle forming) taurocholate in the bile which was part of the pool of bile salts not completely depleted during the time course of taurodehydrocholate infusion. Dowling et al. (28) have demonstrated that the bile salt pool size averages about 1 m mole in the rhesus monkey. In addition, they have shown that after complete diversion of the hepatic out-

put of bile salts, it takes about 4.5 hours to deplete this pool. Therefore, an experiment was conducted in which bile return to the duodenum was stopped completely for 9 hours, twice the time necessary to deplete the bile salt pool. An intravenous infusion of sodium taurodehydrocholate was administered to maintain a constant bile flow rate.

In this manner, it was hoped that the normal bile salt concentration would be reduced below that necessary for micelle formation. It is well known that sodium taurodehydrocholate does not form micelles except possibly at very high concentrations. One would expect to see a marked drop in glucuronide excretion into the bile after taurodehydrocholate infusion, if in fact the biliary excretion of diethylstilbestrol monoglucuronide is dependent upon solubilization in bile micelles.

Preparation of Solutions. Both the intravenous bolus and infusion solutions of diethylstilbestrol-^{14}C were prepared and sterilized as indicated in the previous section. The intravenous bolus solution contained 2.65 μCi/ml and was diluted with cold drug to a concentration of 2.17 mg/ml. The total volume of the bolus solution was 4.5 ml. The resultant specific activity of diethylstilbestrol-^{14}C in this preparation was 1.221 μCi/mg. The intravenous infusion solution contained 1.590 μCi/ml and was diluted with cold drug to a concentration of 1.375 mg/ml. The total volume of infusion solution prepared was 80 ml. The resultant specific activity of diethylstilbestrol-^{14}C in this preparation was 1.156 μCi/mg.

Sodium taurodehydrocholate was dissolved in normal saline (7.44 mg/ml) and sterilized by filtration. Sodium taurocholate was also dissolved in normal saline (9.68 mg/ml) and sterilized by filtration. This solution was to be infused during the terminal portion of the experiment in place of taurodehydrocholate. Since both bile salt solutions were to be admin-

istered as intravenous infusions (15 ml/hr) it was not necessary to infuse heparinized saline to maintain adequate hydration and urine output.

Analytical Methods. Blood, bile, and urine were collected and assayed for unchanged and conjugated diethylstilbestrol-^{14}C as previously described in the metabolism section. Additionally, the bile was assayed for total bile salts using the fluorometric technique previously described (see Rapid Spectrofluorometric Assay for Total Bile Salts in Bile) and by an enzymatic method (29). The bile was also subjected to TLC using Hofmanns' (30) solvent system. A 0.1 ml portion of bile was diluted with methanol to 1.0 ml, centrifuged, and a portion of the supernatant was spotted on a silica gel TLC plate. Standards of the sodium salts of taurocholate, taurodeoxycholate, taurochenodeoxycholate, and taurodehydrocholate, in methanol solutions, were also spotted and run concurrently. These samples were developed for 3 hours and air dried. Then the TLC plates were sprayed with phosphomolybdic acid and placed in an oven to reveal the spots. The bile samples were also subjected to analysis by gas liquid chromatography (GLC) using the method of Nair (31).

Experimental Protocol. Bile was completely diverted and collected so that no return of bile salts to the liver could occur. Complete collection of bile was maintained throughout the experiment. An intravenous infusion of sodium taurodehydrocholate (3.5 μmol/min) was started 6 hours after biliary diversion via an external leg vein. The 4.5 ml intravenous bolus containing diethylstilbestrol-^{14}C was administered via the iliac vein, 9 hours after the initiation of complete biliary diversion. Immediately following this (t = 0 hours) a constant infusion of diethylstilbestrol-^{14}C was begun (also via the iliac vein) and continued throughout the experiment. The steady state infusion rate of diethylstilbestrol-^{14}C

was 486,009.2 DPM/min. The duration of the infusion was 8.5 hours and the total dose administered (bolus plus infusion) was 123.83 μCi which was equivalent to 106.54 mg total drug. Hence, the average specific activity of diethylstilbestrol-^{14}C administered to the monkey was 1.162 μCi/mg.

As already mentioned, the taurodehydrocholate infusion (3.5 μmol/min) was started 6 hours after complete biliary diversion. This infusion (level 1) was continued at the same rate until 5 hours after initiation of the steady state infusion of diethylstilbestrol-^{14}C. At t = 5 hours an infusion of sodium taurocholate (4.5 μmol/min) was substituted for taurodehydrocholate. Taurocholate infusion (level 2) was maintained from t = 5 hours to the end of the experiment (t = 8.5 hours). A higher input rate of taurocholate was necessary, to produce the same bile flow rate, since taurodehydrocholate is known to be a more potent choleretic. The diethylstilbestrol-^{14}C infusion rate remained constant throughout the experiment.

Results and Discussion. Only 91% of the dose could be accounted for, with 55.7% being eliminated in the urine and 35.4% being excreted in the bile. Blood concentration levels of diethylstilbestrol-^{14}C and its monoglucuronide versus time are presented in Figure 17. These curves are quite similar to those of Figure 15. Once again it is apparent that diethylstilbestrol-^{14}C blood levels are at steady state, averaging 1,630.4 DPM/ml. The total body clearance can be calculated to be 298.1 ml/min. However, blood metabolite levels are not at steady state and a positive slope of the blood concentration versus time curve is apparent throughout the observation period.

The excretion rate of total metabolites in the urine achieved a

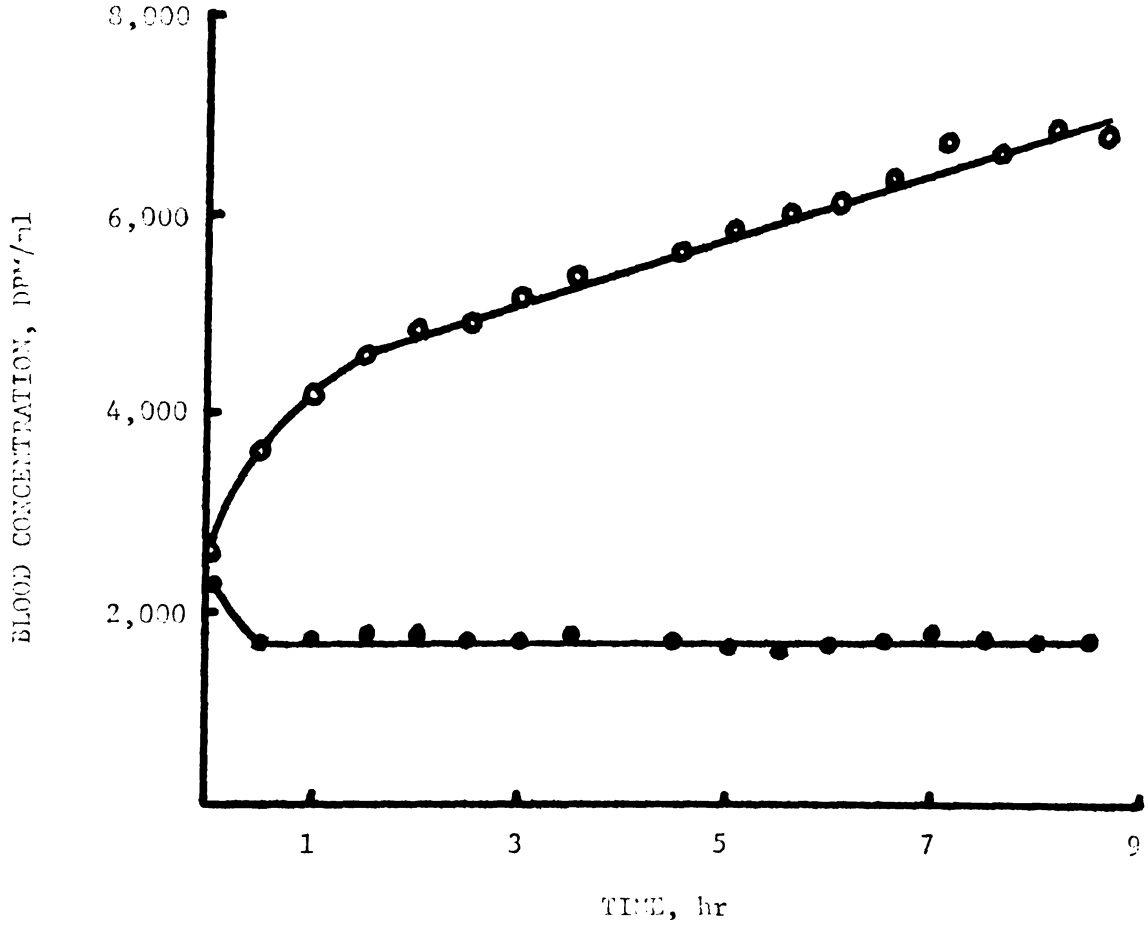


Figure 17 - A plot of the blood levels of diethylstilbestrol-^{14}C (●) and diethylstilbestrol monoglucuronide-^{14}C (◐) obtained during a constant intravenous infusion of diethylstilbestrol-^{14}C. During the experiment a choleresis was maintained by an intravenous infusion of sodium taurodehydrocholate initially (0-5 hrs) and sodium taurocholate terminally (5-8.5 hrs).

steady state and remained relatively constant throughout the entire experimental period at 257,406 DPM/ml (S.D. = 17,084). The excretion rate of diethylstilbestrol monoglucuronide-^{14}C versus midpoint time is plotted in Figure 18. Unfortunately, during the initial portion of the 1 to 2.5 hour interval the taurodehydrocholate infusion was temporarily interrupted due to a jammed infusion pump. This problem was corrected and it is apparent that a steady state excretion rate of glucuronide into the bile is attained at t = 3 hours. During the time interval t = 3 to 5 hours it averaged 177,132 DPM/min. As previously mentioned, at t = 5 hours the taurocholate infusion was substituted for taurodehydrocholate and continued until t = 8.5 hours. During this period the biliary excretion rate for glucuronide achieves a slightly higher level at 189,501 DPM/min. However, there was also an increase in bile flow rate from level 1 (2.43 ml/20 min) to level 2 (2.93 ml/min) and one could conclude that the increased glucuronide excretion rate was primarily due to an increased bile flow rate.

It can be observed in Figure 17 that in spite of the change from the taurodehydrocholate to taurocholate infusion, the metabolite blood level curve shows no break or change in slope and continues to rise linearly with time. This was not unexpected since the excretion rate of glucuronide into the bile (and total metabolites in urine) was not significantly altered. In fact, during the same time interval, the rate of metabolite(s) excretion in the bile and urine appears to be at a steady state. This is consistent with the postulate that a transport maximum (Tm) has been achieved. On the basis of this data, therefore, it seems probable that the urinary excretion of the glucuronide as well as the biliary pathway has been saturated such that a constant rate of glucuronide accumulation

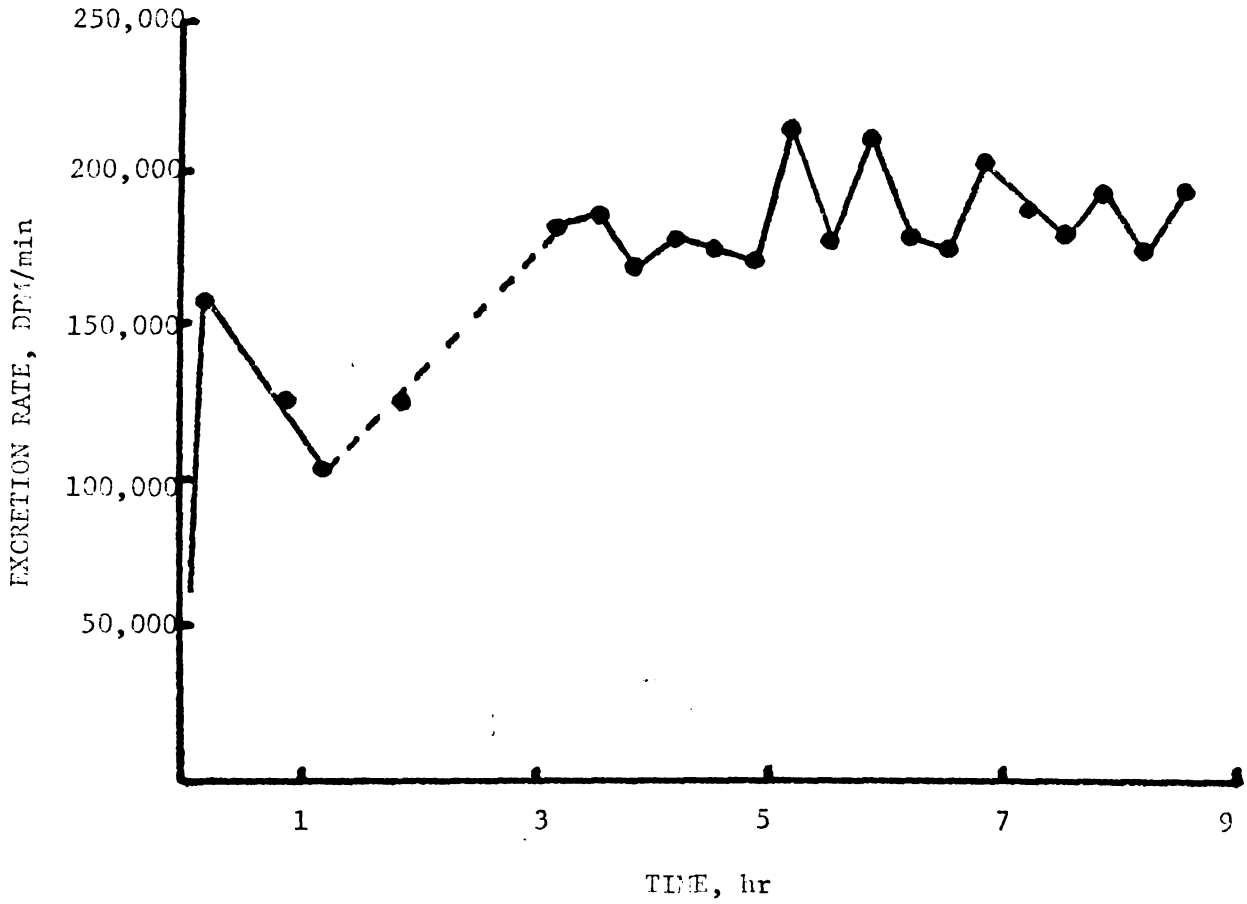


Figure 18 - A plot of the biliary excretion rate of sodium diethylstilbestrol monoglucuronide-^{14}C versus midpoint time. During this experiment diethylstilbestrol-^{14}C was administered initially as an intravenous bolus and immediately thereafter as a constant intravenous infusion which was maintained throughout. Bile choleresis was maintained by an intravenous infusion of sodium taurodehydrocholate initially (0-5 hrs) and sodium taurocholate terminally (5-8.5 hrs). The broken line corresponds to a period during which the taurodehydrocholate infusion was interrupted resulting in a depressed bile flow rate. Bile samples taken during this period were not meaningful and therefore excluded from the figure.

occurs in the blood.

Taurodehydrocholate Effect. The primary goal of this experiment was to investigate the clearance of diethylstilbestrol monoglucuronide via the bile during taurodehydrocholate induced choleresis. However, the anticipated reduction in clearance was not observed. Furthermore, when taurocholate was substituted for taurodehydrocholate essentially no change in the biliary excretion pattern of diethylstilbestrol monoglucuronide was observed except for a slight increase which could be attributed to increased bile flow rate. Therefore a further analysis of the bile samples was undertaken in an attempt to explain these unexpected results.

The bile samples collected during taurodehydrocholate and taurocholate infusion were assayed for bile salts using the fluorometric assay, an enzymatic assay, TLC, and finally GLC. The fluorometric assay is not capable of detecting taurodehydrocholate since the presence of hydroxyl groups are required to produce the fluorescent species. When standard, equimolar solutions of taurocholate and taurodehydrocholate were assayed fluorometrically, the results indicate that taurodehydrocholate produces only 2.7% the fluoresence response of taurocholate. Hence, bile assayed by this method will be sensitive only to di- and tri-hydroxy bile salts. In contrast, the enzymatic assay is sensitive to only those bile salts having a hydroxyl group in the 3-position (29). Obviously taurodehydrocholate would not be detected by this method. However, all trihydroxy bile salts and only some di- and mono-hydroxy bile salts (containing 3-OH groups) would be detected. In the TLC procedure, the plates are treated with phosphomolybdic acid to reveal the spots. Again, taurodehydrocholate is not detected by this treatment.

The results of these various treatments are presented in Table 10.

Table 10 - Determination of Bile Salt Concentration During Taurodehydrocholate and Taurocholate Infusion by Three Different Techniques. Measurements Were Made in Bile at Apparent Steady State Time Periods During the Experiment. See Text and Figure 18 for Further Details.

Conditions	Average bile salt concentration (μmol/ml)		
	Fluorometric assay	Enzymatic assay	TLC[a] assay
Taurodehydrocholate infusion period t = 3.2 to 4.85 hrs	2.9	15.3	+
Taurocholate infusion period t = 5.2 to 8.55 hrs	26.1	29.7	+++

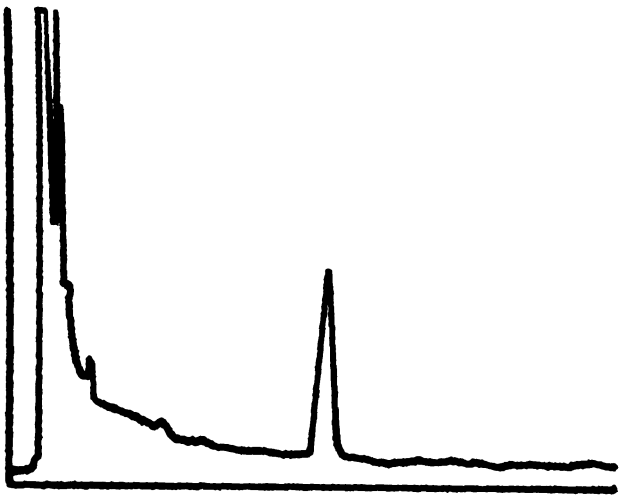
(a) Number of (+) indicates relative intensity of revealed spots corresponding to bile salt standards.

During the terminal taurocholate infusion period the fluorometric and enzymatic assay give approximately the same relative estimate of total bile salts as expected. During the taurodehydrocholate infusion period the enzymatic assay clearly indicates the reduction of at least the 3-keto group to the 3-hydroxy form. The fluorometric data indicate the possibility of even greater reduction to polyhydroxylated derivatives. The TLC data also indicate the presence of polyhydroxylated derivatives.

A more accurate determination of the bile salt constituents can be made by GLC analysis of the bile samples. The data obtained in the GLC analysis of bile during taurocholate and taurodehydrocholate infusion are included in Figures 19a. and b., respectively. The chromatograms indicate (after comparison with standards) that bile collected during taurodehydrocholate infusion contains significant amounts of hydroxylated bile salts which have the same retention times as cholate and chenodeoxycholate. In addition, a large proportion of a "di-keto cholate" derivative is also observed. Non-reduced dehydrocholate was not observed in this study although this compound has a longer retention time and would not appear on the chromatogram as reproduced. The combined hydroxylated bile salts (di- and tri-hydroxylated) correspond to approximately 25% of the total "bile salt" output. Bile collected during taurocholate infusion shows only a single cholate peak when subjected to GLC indicating no metabolism of this compound. Hence, we have conclusive evidence from GLC that significant amounts of (micelle forming) di- and tri-hydroxy bile salts are formed and excreted into the bile during taurodehydrocholate infusion.

These data are consistent with at least two mechanisms. The first, is that taurodehydrocholate is metabolized on passage through the liver

(a)



(b)

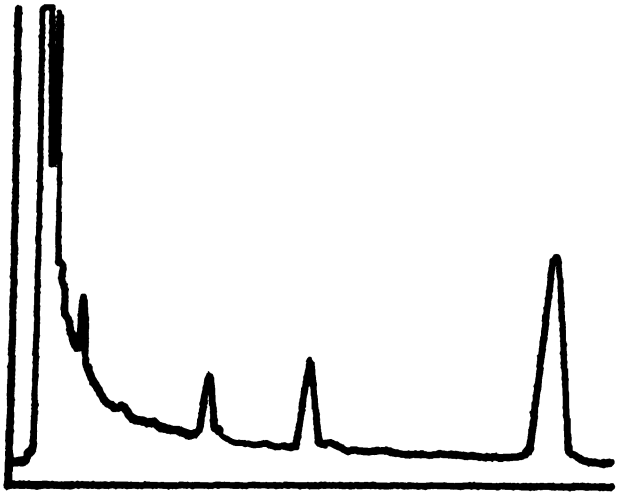


Figure 19 - Gas-liquid chromatograms of bile samples obtained during sodium taurocholate infusion (a) and sodium taurodehydrocholate infusion (b). During taurocholate infusion a single peak corresponding to cholic acid is obtained. During taurodehydrocholate infusion two early peaks with the same retention times as chenodeoxycholic and cholic acid standards are obtained. In addition to these a peak with a much longer retention time is also obtained which represents a di-keto derivative of cholic acid.

(reduction of keto functions) to variously hydroxylated products. That this occurs with the unconjugated dehydrocholate in both man (32) and rat (33) has been established. The latter reference specifically points out that dehydrocholate infused bile has 10 to 17 per cent of the cholesterol solubilizing capacity of taurocholate (equimolar) infused bile. Hence the metabolites of dehydrocholate were shown to have a significant micelle forming capacity. It was initially assumed that dehydrocholate in conjugated form (taurodehydrocholate) would not be as extensively metabolized. It is also possible that the liver is producing hydroxylated bile salts endogenously. Dowling and co-workers (28) have demonstrated that the metabolic synthesis and secretion of normal bile salts after complete interruption of their EHC can approach approximately 7.1 per cent of the bile salt secretion rate under normal conditions (intact EHC). It is likely that both of these processes are in effect in the present experiment. Hence the original goal in the taurodehydrocholate infusion experiment of producing micelle free bile could not be achieved and the hypothesis that micelle carrier structures are necessary for the excretion of diethylstilbestrol monoglucuronide into the bile could not be definitively tested in vivo.

However, if one combines the data of this and the previous experiment several observations can be made. The primary determinant of diethylstilbestrol monoglucuronide clearance into the bile appears to be bile flow rate. It would seem that the concentration or excretion rate of bile salts in bile bears no simple, one to one, relationship to glucuronide excretion into the bile. It was noted that the concentration of diethylstilbestrol monoglucuronide in sampled hepatic bile remained within very narrow limits throughout a given experiment. Furthermore, the average

concentration in the present experiment was 0.932 mg/ml while in the previous experiment it averaged 0.922 mg/ml. Hence, the concentration of glucuronide appears to be invariant once the Tm for excretion into the bile has been established. Therefore, any alteration of bile flow rate produces a corresponding change in glucuronide excretion rate. While bile flow rate seems to be the driving force for glucuronide excretion into bile, it would be incorrect to say that there is no dependence upon bile salt excretion rate, since the latter has been shown to be the driving force for bile flow rate.

An interesting similarity exists between the in vitro solubility data and the in vivo data. In the in vitro taurocholate-lecithin system it was observed that as the taurocholate concentration increased from 2.5 to 7.7 mM the solubility of diethylstilbestrol monoglucuronide increased from 2.99 to 6.05 mg/ml. This represents a taurocholate concentration increase of 3.1 fold while glucuronide solubility increased by only 2.0 fold. Hence, the in vitro system does not demonstrate a one to one relationship between solubilizer and solubilized moieties. In the in vivo data, shown in Table 9, as the total bile salt concentration in bile increased by a factor of 2.9 (level 1 to level 2) the glucuronide excretion rate increased by a factor of 1.6 which is comparable to the in vitro data. It would seem that while a direct relationship between total bile salt concentration and glucuronide excretion rate is to be expected, this need not be a simple one to one relationship. One cannot make a similar analysis in the present experiment since the exact nature and concentration of all the bile salt moities are not known (during taurodehydrocholate infusion).

As previously discussed, the association between bile micelles and

diethylstilbestrol monoglucuronide is unusual in that a significant amount of the glucuronide is dissolved in the aqueous environment. This rather unique interaction wherein a solubilized molecule has appreciable water solubility helps to explain the in vivo data obtained with regard to the fact that sampled hepatic bile contains much less glucuronide than it could maximally solubilize, based on the in vitro studies. If one assumes that bile was saturated with diethylstilbestrol monoglucuronide at the canalicular level, one could further hypothesize that as the bile traveled down the ductules a portion of the glucuronide (freely dissolved in water) could be reabsorbed. This would cause more glucuronide to transfer into the aqueous environment which could also be reabsorbed until a new equilibrium is established between the micelle, the free drug and the blood into which it is transferring. As this process continued throughout the biliary ductules it would be possible to deplete the bile micelles of much of their solubilized glucuronide. Therefore, by the time one samples the hepatic bile, glucuronide concentrations can be well below the theoretical maximum calculated using in vitro models. Even so, this miceller solubilization appears to be important, for without it the glucuronide would very likely be totally reabsorbed long before bile reaches the extra hepatic collecting ducts.

This model is theoretical and one would have to sample canalicular bile in order to substantiate it. This may be accomplished using micopuncture techniques or possibly in vitro liver preparations. Another method which could be of value is retrograde injection of solutions containing both solubilized and freely dissolved glucuronide (no micelles present) in order to determine the actual capacity of the ductules to reabsorb diethylstilbestrol monoglucuronide under both conditions.

DUODENAL ADMINISTRATION OF DIETHYLSTILBESTROL-^{14}C IN SOLUTION

A major goal of this project was to determine the potential for an enterohepatic circulation of diethylstilbestrol and/or its metabolites. It was decided to initiate our absorption studies with the parent compound. Since the monkey was already prepared with a duodenal catheter the dose could be administered via this route.

Preparation of Solutions. The drug was administered in solution (4.5 ml) containing 1 mg (41.118 μCi) of diethylstilbestrol-^{14}C in 1% ethanol, 50% PEG-400, and 49% distilled water. The preparation of this solution is the same as previously described for the intravenous bolus solutions. The preparation and administration procedures of the sodium taurocholate and heparinized saline solution are described in the metabolism section.

Experimental Protocol. Food was withheld for 4 hours prior to the experiment but water was given ad libitum. The semi-aqueous vehicle containing the drug was administered as a duodenal bolus followed by 2 ml of blank bile. Urine, blood, and bile were sampled for 12 hours after administration of the dose and, additionally, feces was collected for two days.

Analytical Procedures. Blood, bile, and urine were processed and assayed as described in the metabolism section. Feces was collected and placed in a freezer. After two days, the feces samples were combined and eluted with methanol. A fine suspension in methanol was prepared by mixing and sonicating the feces-methanol mixture. After centrifugation, an aliquot of the supernatant was assayed for total activity as previously described, assuming 100% recovery.

Results and Discussion. Approximately 78.9% of the total dose administered could be accounted for, with 29.7% appearing in the bile, 35.2% in the urine, and 13.9% in the feces. Adding the recoveries in urine and bile gives the total amount absorbed which totals 64.9%. The rest of the dose could probably be accounted for if the feces were collected for a longer period of time.

Figure 20 is a plot of the blood levels of diethylstilbestrol-^{14}C and its monoglucuronide vs time along with the biliary excretion rate data for the monoglucuronide. Even though unconjugated diethylstilbestrol-^{14}C was administered, only a small fraction of the unconjugated form was detected in the blood. This is seen in the bottom curve of Figure 20. The fraction of the administered dose which is absorbed, in unmetabolized form, into the blood can be estimated (35) by the following relationship,

$$F(\%) = \frac{(\int_0^\infty Cpdt)\ \text{oral}}{(\text{DOSE})\ \text{oral}} \times (\dot{V}cl)_{\text{i.v.}} \times 100$$

$$= \left(\frac{18{,}138\ \frac{\text{DPM}\cdot\text{min}}{\text{ml}}}{9.13\times10^{7}\ \text{DPM}}\right) \cdot \left(300\ \frac{\text{ml}}{\text{min}}\right) \cdot (100)$$

$$= 5.96\%$$

The area under the blood concentration vs time curve of diethylstilbestrol-^{14}C was determined graphically using the trapezoid rule. The mean clearance was estimated in the previous sections where diethylstilbestrol-^{14}C was administered by constant intravenous infusion to steady state blood levels. Since only 64.9% of the administered dose was absorbed, a correction can be made to convert the unmetabolized fraction absorbed to a per cent of the actual total amount which entered the fluids of distribution,

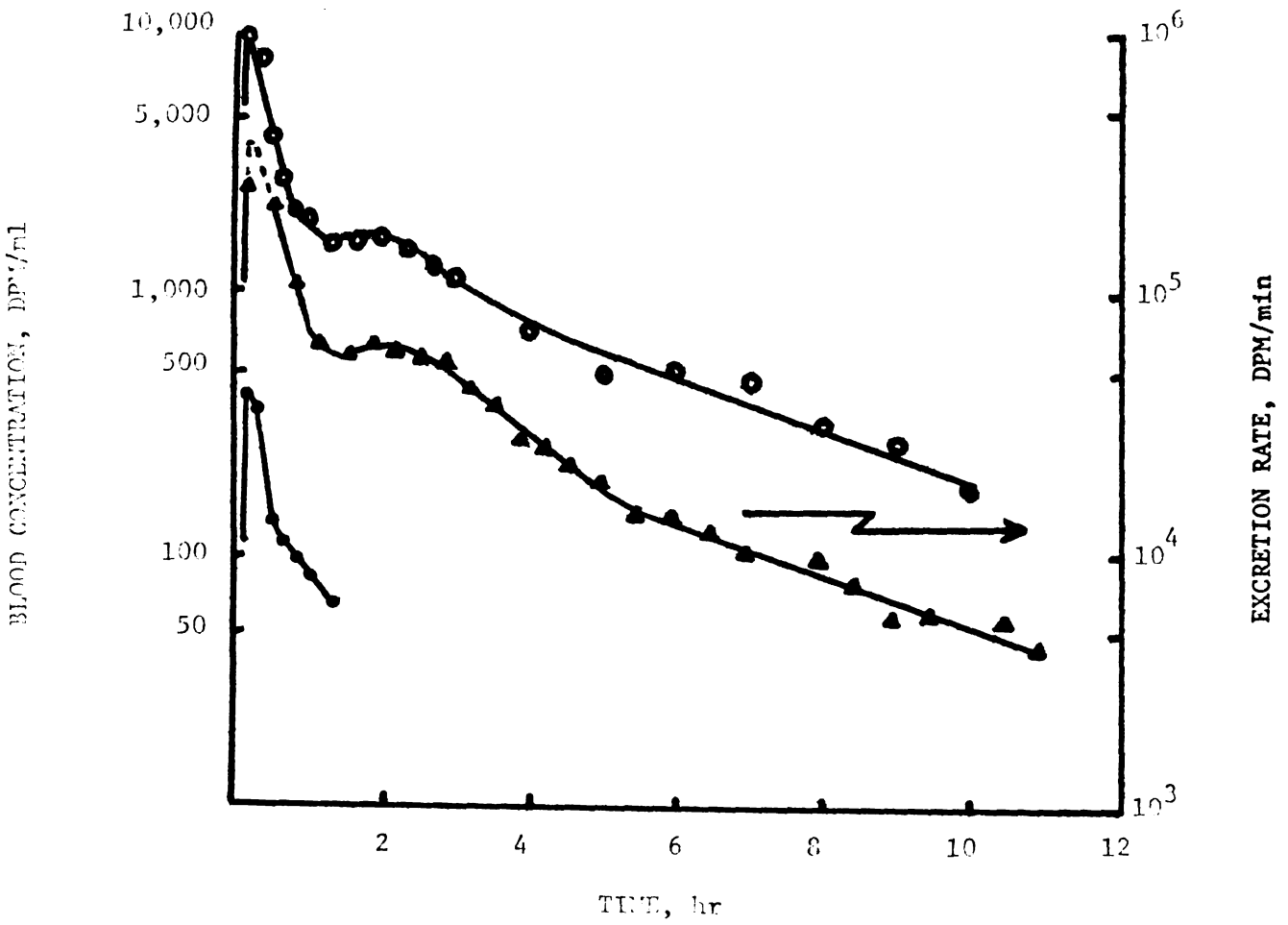


Figure 20 - A semilogarithmic plot of the blood concentration levels of diethylstilbestrol-^{14}C (●) and diethylstilbestrol monoglucuronide-^{14}C (⊙) versus time along with the biliary excretion rate data of diethylstilbestrol monoglucuronide-^{14}C (▲) versus midpoint time - obtained after a duodenal bolus of diethylstilbestrol-^{14}C in solution.

$$F \text{ corrected } (\%) = \frac{(\int_0^\infty Cpdt)\ \text{oral}}{\text{DOSE absorbed}} \cdot \dot{V}cl \cdot 100$$

$$= \left(\frac{18{,}138\ \frac{\text{DPM}\cdot\text{min}}{\text{ml}}}{5.92 \times 10^7 \text{DPM}}\right) \cdot \left(300\ \frac{\text{ml}}{\text{min}}\right) \cdot (100)$$

$$= 9.18\%$$

Apparently the remaining 90.8% of the absorbed dose was conjugated in transit through the gut wall and the liver in the "first pass", prior to reaching the blood and fluids of distribution. The metabolite blood and bile curves, of course, reflect the much larger percentage of the dose converted to the glucuronide. One may also take note that the peak blood levels for metabolite and unchanged compound occur at approximately the same time.

These observations correlate well with earlier studies indicating a very high metabolic clearance of diethylstilbestrol with a rapid rate of formation and an early peaking of the conjugate after an intravenous dose. It can be seen that at all time points, the blood levels of metabolite greatly exceed those of unchanged diethylstilbestrol. If one calculates the area under the blood concentration-time curve (AUC) for both compounds it is found that the AUC for metabolite (705,664 DPM·min/ml) exceeds that of unchanged compound (18,138 DPM·min/ml) by a factor of approximately 39 to 1. This again indicates a significant first pass effect, since after an intravenous dose of diethylstilbestrol the corresponding ratio is about 3 to 1.

Since systemic arterial blood is being analyzed it is impossible to separate metabolism which is occuring in the intestinal mucosa from that in the liver or other metabolizing organs. It is obvious from previous

experiments that a large portion must be metabolized in the liver. However, it is well known that the alimentary tract does have the capacity for glucuronide formation (36). In fact, it has been demonstrated that diethylstilbestrol can be conjugated with glucuronic acid by everted intestinal sac preparations from the rat (37). Hence, a combination of gut wall and liver metabolism is a very likely explanation for such a huge first pass effect and the almost simultaneous peaking of diethylstilbestrol and metabolite blood levels.

A secondary peak, occuring at approximately 2 hours, is seen (Figure 20) in both the metabolite blood level curve and also the biliary excretion rate curve for metabolite. It is apparent that there is a general parallelism for metabolite blood levels and biliary excretion rate throughout the time course of this experiment. This secondary peaking is not observed after an intravenous bolus of diethylstilbestrol-^{14}C as shown in Figure 14. It would therefore seem to be a direct result of the present experimental conditions.

An explanation of this delayed peak can be offered. If one were to assume that glucuronide formation does occur in the gut wall, it is possible that only a portion of the conjugated metabolite leaves the mucosal cell by transferring into hepatic portal blood, while the rest "spills" back into the intestinal lumen. Once in the gut lumen the glucuronide would not be readily absorbed due to its polar, anionic nature and therefore passes down the gut to the bacteria rich environment of the large intestine. Here bacterial enzymes can hydrolyze the glucuronide to free diethylstilbestrol which is readily absorbed. However, during gut wall and liver transit diethylstilbestrol is re-conjugated, predominately in the liver, prior to reaching the systemic blood and fluids of distribution.

The appearance of this additional "bolus" of glucuronide gives rise to the secondary peak observed. The 2 hour time lag is probably due to the passage of metabolite from the duodenum to the large intestine.

Some evidence for the above hypothesis can be found in a study of oestriol and oestradiol in human subjects (38). This study clearly demonstrated that after duodenal or jejunal intubation of oestriol to a patient with complete biliary fistula, glucuronide conjugates could be isolated in the intestinal contents, intestinal wall and also in the effluent venous blood. Furthermore, the estriol glucuronide conjugates, once formed and deposited into the duodenal lumen, were not absorbed as such. One would not expect that the conjugates would be hydrolyzed in the duodenum since no bacteria are found in the normal human small intestine. It was further shown that the glucuronide conjugating capacity of the gut wall is greatest in the proximal small intestine and virtually absent in the large intestine. The above findings correlate well with the present interpretation of blood and bile levels of diethylstilbestrol monoglucuronide after duodenal administration of diethylstilbestrol, a compound very similar to the naturally occuring estrogens.

DUODENAL ADMINISTRATION OF BILE CONTAINING SODIUM DIETHYLSTILBESTROL MONOGLUCURONIDE-^{14}C

An investigation into the potential for an enterohepatic recycling of diethylstilbestrol monoglucuronide-^{14}C contained in a bile sample was studied by instillation of the radioactive bile solution directly into the duodenum. This is an ideal way to study the absorption characteristics of the glucuronide, since after excretion into the bile it would normally be emptied directly into the duodenum. It is possible that if this compound was given orally and allowed to pass through the stomach, an altogether different picture might ensue. For example, the acid pH of the stomach could cause hydrolysis of the labile glucuronide with subsequent rapid absorption of free diethylstilbestrol. Since the glucuronide in bile would not normally reach the acid environment of the stomach, such results would be the artifact of an incorrectly designed experiment.

Procedure. Radioactive bile was obtained from a previous experiment in which diethylstilbestrol-^{14}C was administered as an intravenous bolus and collected bile was frozen as soon as possible thereafter. A total dose of 4.72 ml of pooled radioactive bile was administered containing 5.53 μCi total activity. Of this activity, 5.7% (6.8 μg) consisted of free diethylstilbestrol and 94.3% (184.6 μg) diethylstilbestrol monoglucuronide. This dose was washed into the duodenum with about 2 ml of blank bile. An ostensibly constant bile flow rate was maintained with a duodenal infusion of sodium taurocholate. The intravenous hydration with normal saline and other conditions of the experiment were the same as described in the previous section. Blood and urine sampling and total bile collections were continued for 8 hours post administration of the dose. Feces was collected for 24 hours and pooled. The processing and assay of

blood, bile, urine, and feces was the same as described in the previous section.

Results and Discussion. Sixty one per cent of the administered dose could be accounted for with 25.2% appearing in the bile and 16.2% in the urine, indicating a total absorption of 41.4% of the dose. Feces collected over a 24 hour period contained 19.7% of the administered dose. It is likely that a larger portion of the dose would be accounted for if the fecal collection was carried out for a longer period of time. Although some unconjugated diethylstilbestrol was present in the administered dose, it accounted for only 5.7% of the total activity. Since 41.4% of the dose was absorbed, at least 35.7% of the glucuronide initially present in the bile was "absorbed". One can compare this to the previous experiment in which 64.9% of a duodenal dose of diethylstilbestrol was absorbed. It is apparent that when diethylstilbestrol is administered in conjugated form it is much less available for absorption than the free form.

Figure 21 includes the blood concentration curve for diethylstilbestrol monoglucuronide after the administration of radioactive bile. This is seen as the bottom curve (closed triangles). Also included in the same figure is the glucuronide blood level curve after an intravenous bolus of diethylstilbestrol-^{14}C, seen as the middle curve (closed circles). This is redrawn from Figure 14. Finally, the glucuronide blood level curve (redrawn from Figure 20) obtained after the duodenal administration of diethylstilbestrol-^{14}C in solution is seen as the top curve (open circles). In all three experiments newly formed hepatic bile was collected precluding the possibility of recycling. However, a taurocholate solution (containing no drug) was infused via the duodenum to maintain a con-

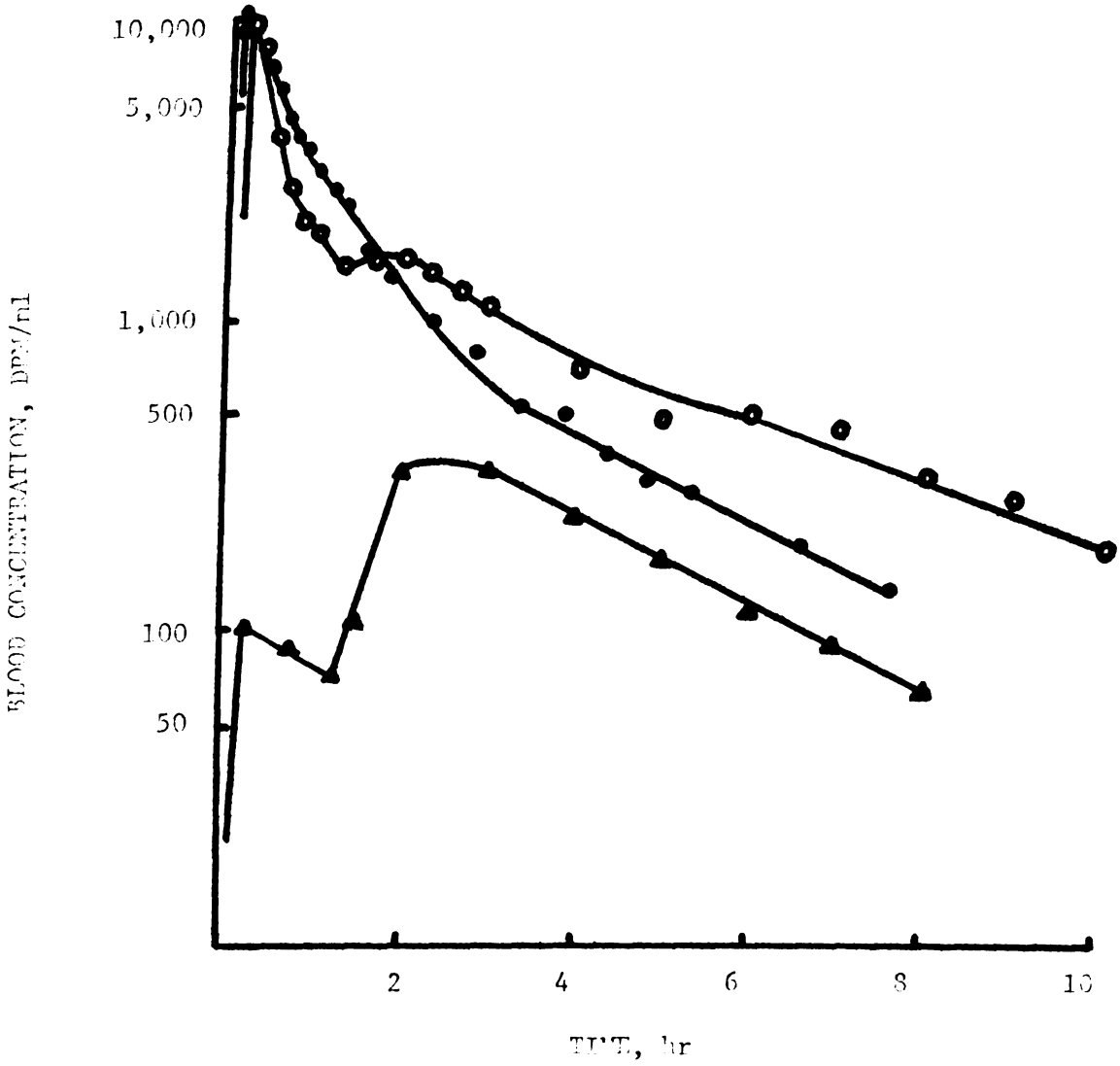


Figure 21 - A semilogarithmic plot of diethylstilbestrol monoglucuronide-^{14}C blood levels (versus time) obtained after: a duodenal bolus of bile containing diethylstilbestrol monoglucuronide-^{14}C (▲), a duodenal bolus of diethylstilbestrol-^{14}C in solution (◎), and an intravenous injection of diethylstilbestrol-^{14}C (●).

stant bile salt pool.

After an intravenous bolus of diethylstilbestrol-^{14}C one sees only a single peak in the metabolite blood level curve (closed circles) at about 6 minutes post administration. This is, of course, due to metabolic conversion. After this peak the blood level curve decays exponentially with no indication of secondary peaks.

After a duodenal bolus of radioactive bile, no unchanged diethylstilbestrol could be detected in the blood. This is probably due to the small amount absorbed in unchanged form as well as the lack of sufficient sensitivity of the assay method. The metabolite blood level curve (closed triangles) contains an early and delayed peak similar to that observed after a duodenal bolus of diethylstilbestrol-^{14}C (open circles). Close examination of these curves brings out several interesting points. After the duodenal bolus of radioactive bile (closed triangles) the early peak in blood occurs at about 15 minutes. It is proposed that this early peak is due to absorption (and subsequent conjugation) of the small amount of free diethylstilbestrol originally present in the radioactive bile sample. This can be compared to the duodenally administered bolus of free diethylstilbestrol-^{14}C (open circles) which results in an early metabolite blood level peak at about 10 minutes. The secondary peak after duodenal administration of radioactive bile occurs at about 2.4 hours, while after duodenal administration of free diethylstilbestrol it occurs at about 2.0 hours. The time at which the secondary peak occurs is proposed to be a function of the transit time for glucuronide to pass from the small to the large intestine which would be affected by changes in intestinal motility. In spite of this the secondary peak times are relatively close for both experiments. **While the dose administered in each experiment was not equivalent, comparisons made on the basis of the shape of the curves are still valid.**

The terminal portions of the metabolite blood level curves are parallel following an intravenous bolus of diethylstilbestrol-^{14}C (closed circles) and duodenal administration of radioactive bile containing diethylstilbestrol monoglucuronide (closed triangles). This indicates that hydrolysis of the conjugate and absorption of the liberated diethylstilbestrol are not rate limiting after the conjugate reaches the bacterial rich environment of the large intestine. In contrast, the blood level curve for metabolite after the duodenal bolus of diethylstilbestrol-^{14}C (open circles) exhibits a large degree of scatter during the terminal phase and one could propose that a third peak occurs at approximately 6 hours. This may be due to variations in the peristaltic movements of the intestine which in this particular experiment emptied the glucuronide into the large intestine in discrete portions rather than as a single bolus. Another possibility which cannot be excluded is that diethylstilbestrol, being a very insoluble substance, precipitates from solution after introduction into the duodenum. Absorption (and subsequent conjugation) would then become a function of dissolution which can be prolonged and quite variable, thus giving rise to a corresponding variability in the metabolite blood level curve as well as a prolonged terminal slope. However, since a taurocholate infusion was administered, intraduodenally, subsequent to the administration of diethylstilbestrol, precipitation may have been prevented.

However, the overall results are compatible with the concept that when diethylstilbestrol monoglucuronide appears in or is delivered to the intestinal lumen it must be cleaved to free diethylstilbestrol (probably by bacterial enzymes in the large intestine) prior to absorption, reconjugation, and subsequent appearance in the systemic blood and fluids

of distribution. Since it is known that the gut wall of the large intestine has a relatively smaller capacity for glucuronidation than duodenal tissue (38), a greater proportion of free diethylstilbestrol will transit the large intestinal gut wall and arrive at the liver. It is likely that the liver will clear most of the free diethylstilbestrol, converting it to the glucuronide. After conjugation, a portion of the metabolite is then transferred into the systemic circulation while the rest appears in the bile.

Thus, after an intravenous dose of diethylstilbestrol, the following events would take place in the intact rhesus monkey:

i) Diethylstilbestrol is conjugated with glucuronic acid in the liver and the conjugate is partly excreted via the bile into the duodenum while the remainder passes into the systemic circulation and is excreted via the urine.

ii) Diethylstilbestrol monoglucuronide from bile apparently transits the small intestine unaltered until it reaches the large intestine where at least a portion will be hydrolyzed by bacterial enzymes. The released diethylstilbestrol is available for absorption into the hepatic-portal blood stream while the unhydrolyzed glucuronide will be excreted in the feces.

iii) During absorption, diethylstilbestrol is re-conjugated in part during transit through the gut wall but predominantly in the liver. A portion of the newly formed glucuronide is re-excreted into the bile while the rest is transferred to the systemic circulation and excreted in the urine.

iv) That portion of the dose retained in the bile will be emptied into the gut lumen and repeat the cycle.

v) During each succeeding enterohepatic circulation a progressively smaller amount of the dose is "trapped" in the cycle until all is recovered either in the urine or feces.

It should be emphasized that efficient enterohepatic circulation of a compound depends upon the stability of its metabolite(s) in the gut. Compounds excreted into the bile as stable metabolites will be excreted in the feces since most polar, ionic metabolites are poorly absorbed, as such, from the gut. Diethylstilbestrol monoglucuronide is quite easily hydrolyzed, apparently by bacterial enzymes, thereby making it a good candidate for an extensive enterohepatic circulation.

REFERENCES

1. Small, D.M., Size and Structure of Bile Salt Micelles, Adv. in Chem. Series, 84, 31 (1968).

2. Small, D.M., Bourges, M.C. and Dervichian, D.G., The Biophysics of Lipidic Associations - I. The Ternary Systems Lecithin-Bile Salt-Water, Biochim. et Biophys. Acta, 125, 563 (1966).

3. Bickel, M.H. and Minder, R., Metabolism and Biliary Excretion of the Lipophilic Drug Molecules, Imipramine and Desmethylimipramine in the Rat - II (Uptake into Bile Micelles), Biochem. Pharmcol., 19, 2437 (1970).

4. Hardison, W.G.M. and Apter, J.T., Micellar Theory of Biliary Cholesterol Excretion, Am. J. Physiol., 222, 61 (1972).

5. Swell, L., Entenman, C., Leong, G.F., and Holloway, R.J., Bile Acids and Lipid Metabolism IV. Influence of Bile Acids on Biliary and Liver Organelle Phospholipids and Cholesterol, Am. J. Physiol., 215, 1390 (1968).

6. Gartner, L.M., Lane, D.L., and Cornelius, C.E., Bilirubin Transport by Liver in Adult Macaca Mulatta, Am. J. Physiol., 220, 1528 (1971).

7. Boyer, J.L., Scheig, R.L. and Klatskin, G., The Effect of Sodium Taurocholate on the Hepatic Metabolism of Sulfobromophthalein Sodium (BSP). The Role of Bile Flow, J. Clin. Invest., 49, 206 (1970).

8. Carey, M.C. and Small, D.M., Micelle Formation by Bile Salts, Arch. Intern. Med., 130, 506 (1972).

9. Deardorff, D.L., Ophthalmic Solutions in "Remington's Pharmaceutical Sciences" 13th Ed., Edited by E.W. Martin, Mack Pub. Co., Easton, Penn., 1965, p. 468.

10. Slater, T.F. and Griffiths, D.B., Studies on the Enzymic Composition of Rat Bile Following the Administration of Various Toxic Substances, in "The Biliary System" edited by W. Taylor, Blackwell Scientific Pub., Oxford, 1965, pp. 431-38.

11. McBain, M.E.L. and Hutchinson, E., Data and Facts of Solubilization, in "Solubilization and Related Phenomena" Edited by E. Hutchinson, Academic Press Inc., New York, 1955, pp. 63-78.

12. Millburn, P., Factors in the Biliary Excretion of Organic Compounds, in "Metabolic Conjugation and Metabolic Hydrolysis" Vol II, Edited by W. Fishman, Academic Press, New York, 1970, pp. 1-74.

13. Javitt, N.B., Bile Salt Regulation of Hepatic Excretory Function, Gastroent. 56, 622 (1969).

14. Shaffer, C.B., Critchfield, F.H., and Nair, J.H., The Absorption and Excretion of a Liquid Polyethylene Glycol, J. Am. Pharm. Assoc., Sci. Ed., 39, 340 (1950).

15. Smyth, H.F., Carpenter, C.P., and Weil, C.S., The Toxicology of the Polyethylene Glycols, J. Am. Pharm. Assoc., Sci. Ed., 39, 349 (1950).

16. Jayle, M.F. and Pasqualini, J.R., Implication of Conjugation of Endogenous Compounds - Steroids and Thyroxine in "Glucuronic Acid". Edited by G.J. Dutton, Academic Press, New York and London, 1966, pp 519-21.

17. Adlercreutz, H., Oestrogens in Human Bile in "The Biliary System". Edited by W. Taylor, Blackwell Sci. Pub., Oxford, 1965, pp. 369-84.

18. Sheppard, C.W., "Basic Principles of the Tracer Method", Wiley and Sons, Inc., New York, N.Y. 1962 p. 44.

19. Riegelman, S., Loo, J.C.K. and Rowland, M., Shortcomings in Pharmacokinetic Analysis by Conceiving the Body to Exhibit Properties of a

Single Compartment, J. Pharm. Sci., 57, 117 (1968).

20. Benet, L.Z., General Treatment of Linear Mammillary Models with Elimination from any Compartment as Used in Pharmacokinetics, J. Pharm. Sci. 61, 536 (1972).

21. Nagashima, R., Levy, G., and O'Reilly, R.A., Comparative Pharmacokinetics of Coumarin Anticoagulants IV, J. Pharm. Sci. 57, 1888 (1968).

21a. Boxenbaum, H.G., Riegelman, S., and Elashoff, R.M., Statistical Estimations in Pharmacokinetics, J. Pharmacokin. Biopharm., In press.

21b. Loo, J.C.K. and Riegelman, S., Assessment of Pharmacokinetic Constants from Postinfusion Blood Curves Obtained After I.V. Infusion, J. Pharm. Sci., 59, 53 (1970).

21c. Riegelman, S., Loo, J., and Rowland, M., Concept of a Volume of Distribution and Possible Errors in Evaluation of This Parameter, J. Pharm. Sci., 57, 128 (1968).

22. Reuning, R.H., Sams, R.A., and Notari, R.E., Role of Pharmacokinetics in Drug Dosage Adjustment. 1. Pharmacologic Effect Kinetics and Apparent Volume of Distribution of Digoxin, J. Clin. Pharmcol., 13, 127 (1973).

23. Ketterer, S.G., Wiegand, B.D., and Rapaport, E., Hepatic Uptake and Biliary Excretion of Indocyanine Green and its Use in Estimation of Hepatic Blood Flow in Dogs, Am. J. Physiol., 199, 481 (1960).

24. Wan, S.H. and Riegelman, S., Renal Contribution to Overall Metabolism of Drugs I: Conversion of Benzoic Acid to Hippuric Acid, J. Pharm. Sci., 61, 1278 (1972).

25. Wheeler, H.O., Mancusi-Ungaro, P.L. and Whitlock, R.T., Bile Salt Transport in the Dog, J. Clin. Invest., 39, 1039 (1960).

26. Arias, I.M., Johnson, L. and Wolfson, S., Biliary Excretion of Injected Conjugated and Unconjugated Bilirubin by Normal and Gunn Rats, Am. J. Physiol., 200, 1091 (1961).

27. O'Maille, E.R.L., Richards, T.G., and Short, A.H., Factors Determining the Maximal Rate of Organic Anion Secretion by the Liver and Further Evidence on the Hepatic Site of Action of the Hormone Secretin, J. Physiol., 186, 424 (1966).

28. Dowling, R.H., Mack, E. and Small, D.M., Effects of Controlled Interruption of the Enterohepatic Circulation of Bile Salts by Biliary Diversion and by Ileal Resection on Bile Salt Secretion, Synthesis, and Pool Size in the Rhesus Monkey, J. Clin. Invest., 49, 232 (1970).

29. Palmer, R.H., The Enzymatic Assay of Bile Acids and Related 3-Hydroxysteroids. In "Methods in Enzymology" Vol 15. Edited by S.P. Colowick, Academic Press, New York, 1969, pp 280-88.

30. Hofmann, A.F., Thin-Layer Adsorption Chromatography of Free and Conjugated Bile Acids on Silicic Acid, J. Lipid Res., 3, 127 (1962).

31. Nair, P.P. and Garcia, C., A Modified Gas-Liquid Chromatographic Procedure for the Rapid Determination of Bile Acids in Biological Fluids, Anal. Biochem., 29, 164 (1969).

32. Soloway, R.D., Hofmann, A.F., Thomas, P.J. and Schoenfield, L.J., Dehydrocholic Acid: Hepatic Metabolism and Effect on Biliary Secretion in Man, Gastroent., 60, 754 (1971).

33. Hardison, W.G., Metabolism of Sodium Dehydrocholate by the Rat Liver: Its Effect on Micelle Formation in Bile, J. Lab. Clin. Med., 77, 811 (1971).

34. Levi, A.J., Gatmaitan, Z. and Arias, I.M., Two Hepatic Protein Fractions, Y and Z, and Their Possible Role in the Hepatic Uptake of

Bilirubin, Sulfbromophthalein, and Other Anions, J. Clin. Invest., 48, 2156 (1969).

35. Rowland, M., Influence of Route of Administration on Drug Availability, J. Pharm. Sci., 61, 70 (1972).

36. Dutton, G.J., The Biosynthesis of Glucuronides in "Glucuronic Acid" Edited by G. J. Dutton, Academic Press, New York and London, 1966, Ch 3 pg 220.

37. Fischer, L.J. and Millburn, P., Stilboestrol Transport and Glucuronide Formation in Everted Sacs of Rat Intestine, J. Pharmcol. Exptl. Ther., 175, 267 (1970).

38. Diczfalusy, E., Franksson, C. and Martinsen, B., Oestrogen Conjugation by the Human Intestinal Tract, Acta Endocrin., 38, 59 (1961).

SUMMARY AND CONCLUSIONS

The purpose of this section is to summarize and discuss the various conclusions and hypotheses made within the body of the Dissertation. Those areas where more than one explanation of the data are plausable will be emphasized. Finally, future experiments or treatments of the data will be suggested which might answer some of the questions which still remain.

Metabolism. An important goal in the study of any drug intended for human or animal use is to determine the metabolic fate of the drug. In the present study it was concluded that diethylstilbestrol was completely metabolized and virtually no diethylstilbestrol was excreted unchanged in the urine or bile. This conclusion might be questioned since diethylstilbestrol was found in small quantities (<5%) in both urine and bile. However, we believe that the presence of unchanged compound was probably due to hydrolysis of labile metabolites. The hydrolysis occuring within the urinary and biliary organs cannot be prevented but could be minimized if suitable catheters, which allow rapid drainage, were employed. In addition, the urinary and biliary samples could have been collected in an ice bath or immediately frozen on collection, by immersing the collecting vessels in dry ice. Neither of these alternatives were considered necessary in our studies since we believed that they would have a negligible effect on the conclusions drawn and impose only minor qualitative changes in the results.

The presence of multiple metabolites in the urine was demonstrated by TLC. However, separation and identification of these metabolites was not attempted. This observation led to the unproven postulate that the kidney was an additional metabolizing organ. Other sites of metabolism are

possible. For example, the entire gastro-intestinal tract also has significant metabolic activity and those additional metabolites found in the urine may just as well have been formed here. In fact, it is possible that the liver could form several metabolites of diethylstilbestrol, only one of which (diethylstilbestrol monoglucuronide) is efficiently excreted into the bile while the rest are cleared by the kidney. However, if this were so one would also expect to see these additional metabolites in the blood, yet this was not the case.

In order to ascertain the complete metabolic picture it will first be necessary to separate, identify, and quantitate all those metabolites which are formed from diethylstilbestrol. After this has been accomplished one would then attempt to determine which organs and to what extent they are involved in the metabolism of diethylstilbestrol. A number of experimental techniques are possible such as isolated liver, kidney, and intestinal perfusion studies. Another method would be to remove these organs entirely and perform metabolic studies in vitro using tissue slices or homogenates. However, the preferred technique would be to implant permanent intravascular catheters at strategic locations in a restrained monkey for chronic use. For example, one could separate out gastro-intestinal metabolism from hepatic metabolism (after an oral dose) by catherizing the portal vein, which conveys blood from the gastro-intestinal organs into the liver. In addition to this, a catheter inserted into the hepatic vein, which conveys blood from the liver into the inferior vena cava, would allow one to quantitate the relative metabolic activity of these two organs. Knowing the exact fraction of metabolic activity occuring in the liver and intestine, suitable experiments could then be designed which would allow one to quantitate metabolism which is occuring in the kidney. Experiments

such as these are quite feasible and monkeys have been prepared in such a manner in our laboratories for chronic use. In the study of diethylstilbestrol, which is probably metabolized in the liver, intestine, and kidney, such a preparation would be invaluable. However, this is a major project and would require a full time effort.

Mechanism of Biliary Excretion. It has been experimentally shown that the biliary excretion of diethylstilbestrol monoglucuronide is bile flow dependent. Further, it was maintained that an association complex of this metabolite with the micellar structures of bile could explain this phenomenon. In vitro solubility studies led to the conclusion that diethylstilbestrol monoglucuronide can interact strongly with bile salt micelles. A theoretical discussion regarding the nature of the complex formed and how such an interaction could explain the observed data was offered. Essentially, one could conceive of a saturated (with diethylstilbestrol monoglucuronide) bile solution being formed at the canalicular level which can be depleted of much of its solubilized glucuronide by the time bile reaches the external hepatic ducts. However, incontrovertible proof of this proposed mechanism for the excretion of sodium diethylstilbestrol monoglucuronide into the bile was not offered.

Other explanations may have equal validity. For example, one can consider that bile is not saturated with diethylstilbestrol monoglucuronide at any level of the biliary tree. In those studies involving intravenous infusions of diethylstilbestrol-^{14}C (pgs. 110 & 119) it was observed that the biliary concentration of diethylstilbestrol monoglucuronide remains within relatively narrow limits throughout a given experiment and hence shows a marked dependence upon bile flow rate. This behavior is also compatible with an osmotically driven mechanism of glucuronide excretion into

the bile. As the bile salts are transported into the canalicular space, water is also carried along and the possible mechanisms of this have been discussed (see Mechanism of Bile Formation). Various inorganic solutes can also pass into the canaliculi by osmotic diffusion and it is conceivable that organic solutes such as diethylstilbestrol monoglucuronide may pass into the canaliculi by osmotic diffusion as well. If this were true it would explain why the biliary glucuronide excretion rate is linearly dependent upon bile flow rate.

This latter explanation is also consistent with the observations of the taurodehydrocholate experiment (pg 119). During both the taurodehydrocholate and taurocholate infusion periods the bile concentration of diethylstilbestrol monoglucuronide and the bile flow rate were about the same. Hence, diethylstilbestrol monoglucuronide excretion rate was not significantly different during the two periods. However, the bile salt concentration, as indicated in Table 9, changed considerably. This would suggest that an osmotic effect is controlling glucuronide excretion rather than an interaction with the bile salts in bile. However, even if this were the case, the bile salts present in the bile above the CMC may be responsible for retaining the glucuronide in bile by incorporating at least a portion of it into the micelle, thereby reducing its escaping tendency and retaining it in hepatic bile. Other organic compounds which can not strongly interact with the bile micelles are reabsorbed (from the hepatic ductules) back into the systemic circulation and finally excreted via the urine.

Therefore, at least two mechanisms of diethylstilbestrol monoglucuronide excretion into the bile can reasonably explain the data. Both are

equally plausable but there may be other explanations as well.

Additional experiments can be carried out which would also aid in deciding what the correct mechanism is. For example one could establish the site of glucuronide excretion into the bile by the administration of secretin as a choleretic. It is well known that secretin is a hydrocholeretic. That is, it produces choleresis by adding water and electrolytes to the bile at the ductular level and the end result is a diluted bile. In this experiment one would not expect a secretin induced choleresis to result in a corresponding increase in biliary glucuronide excretion rate if diethylstilbestrol monoglucuronide is being added to the bile at the canalicular level. Another experimental technique of value would be retrograde infusion experiments. Essentially, a series of diethylstilbestrol monoglucuronide solutions could be prepared in plain buffer and in micellar systems. These solutions would be gently infused via the common bile duct into the liver while blood and urine samples are being taken. If bile micelles can truly retard the reabsorption of the glucuronide such an experiment would give the most direct measurement of this.

Before even considering these additional experiments, however, one could do further studies on the bile samples obtained in the various experiments already completed. Since these bile samples were found to contain glucuronide in concentrations well below the theoretical maximum (in a micellar system) one could question whether any association at all had occured. It should be emphasized that although the glucuronide concentration may be low, this does not necessarily mean that the glucuronide is unassociated with the bile micelles. Even in a dilute solution, an equilibrium of the glucuronide between the micelle and the aqueous environment is

possible. This could be tested by a number of methods which include equilibrium dialysis, ultrafiltration and ultracentrifugation. Of the three, ultracentrifugation is probably the most appropriate since both dialysis and ultrafiltration could disrupt the micelles which are equilibrium systems as well. The ultracentrifugation experiments, however, could answer the question of whether or not an association between bile micelles and sodium diethylstilbestrol monoglucuronide exists in endogenously produced bile. This would go far in supporting either of the two mechanisms proposed.

Transport Maximum. Two separate experiments in which diethylstilbestrol-^{14}C was administered as a constant infusion (pgs 110 and 119) indicated that a transport maximum exists for the excretion of diethylstilbestrol monoglucuronide into the bile. This conclusion was based on the non parallel behavior of metabolite blood and bile levels. However, an alternative explanation of the data might be offered. For example, one could propose that the continuous increase of metabolite blood levels in the face of steady state blood levels of parent compound is due to the metabolite having a longer biologic half-life than parent compound. If this were true, one would expect that a longer period of time would be required before steady state blood levels for metabolite are attained. Such an explanation was considered but not accepted for the following reasons. After an intravenous injection of diethylstilbestrol-^{14}C (Figure 14) the terminal (blood level) slopes of diethylstilbestrol and diethylstilbestrol monoglucuronide are the same, hence there are no grounds for assuming that the biologic half-lives of these compounds would be different. Furthermore, the metabolite blood levels also parallel metabolite biliary excretion rate levels (Figure 14). Therefore, unless the biliary excretion of metabolite has been saturated one should observe this metabolite parallel-

ism (during the constant infusion of diethylstilbestrol-^{14}C) in blood and bile. However, while metabolite blood levels are increasing (Figures 15 and 17) the bile data (Figures 16 and 18) indicate that a steady state metabolite excretion rate has been attained. Also, in urine, the "total metabolites" excretion rate either remained relatively constant or decreased slightly. Finally, if one examines Figures 15 and 17 it is apparent that metabolite blood levels are increasing linearly (except for the initial time periods). There is no indication of curvature which would be expected (on cartesian-coordinate graph paper) if the metabolite blood levels were increasing exponentially. In light of these observations it is concluded that diethylstilbestrol monoglucuronide blood levels reflect a zero order increase indicative of saturation of the biliary transport mechanism.

The above conclusion is given further support in Figures 15 and 16. Assuming that bile flow rate provides the driving force for glucuronide excretion one would expect that an increase in bile flow rate would cause an increase in the clearance of metabolite from the blood. This is evidenced by the break in the metabolite blood level curve which occured at the same time that the bile flow rate was increased. This increased bile flow rate was accompanied by an increased metabolite excretion rate (Figure 16). After the initial increase in bile flow rate (which was then maintained for the duration of the experiment) the slope of the metabolite blood level curve decreases and further details of this are discussed in the relevant sections. These experiments indicate that bile flow rate controls metabolite clearance from the blood and the higher the flow the greater the biliary clearance. This observation lends support to the concept of the bile micelles acting as carriers of diethylstilbestrol monoglucuronide which are saturated at the canalicular level. Therefore, the

increased bile flow rate induced by taurocholate, provides more carrier micelles which can transport more glucuronide from the blood.

This type of data would probably be amenable to a Michaelis-Menton kinetic treatment. If one were to employ a large range of diethylstilbestrol infusion levels it would be possible to define this transport maximum quantitatively. Other studies which would allow one to assess the maximum metabolite "carrying" capacity of bile could be done. For example, one could infuse diethylstilbestrol at a level known to exceed the transport maximum for the metabolite and then increase the bile flow rate until steady state blood levels of metabolite are attained. Studies of this type would clarify the inter-relationships between metabolite levels in blood and bile, and bile flow rate. Such studies might also help define the mechanism(s) controlling diethylstilbestrol monoglucuronide excretion into the bile.

Enterohepatic Recycling. A series of experiments were carried out which led to a hypothesis describing the enterohepatic circulation of diethylstilbestrol and diethylstilbestrol monoglucuronide. Essentially, it was found that diethylstilbestrol monoglucuronide is deposited into the intestine via the bile and must then travel down to the large intestine where bacterial enzymes cleave the glucuronide prior to reabsorption, thus setting up an enterohepatic circulation. Blood level and biliary excretion data, obtained after a duodenal bolus of both diethylstilbestrol and its monoglucuronide support this hypothesis. However, additional experiments could be carried out which would go a long way in the support or negation of the above hypothesis. For example, if it is true that bacterial enzymes are responsible for the cleavage of metabolite and that this is necessary for an enterohepatic circulation of drug and metabolite, one

should be able to prevent the cycle by removing the bacteria. This could be done by pre-treating the monkey orally with antibiotics such as neomycin or kanamycin which sterilize the entire gastrointestinal tract. If metabolite is then introduced into the intestine none should be reabsorbed since cleavage to the unconjugated form would be prevented. Hence, the secondary peaks observed in the blood and bile after duodenal administration of free drug and metabolite would not be seen in an antibiotic-treated monkey. Of course, suitable experiments would have to be done to rule out the possibility that the antibiotic itself affected the absorption characteristics of drug or metabolite.

Pharmacokinetics. A three compartment open model for diethylstilbestrol blood levels has been proposed. Along with the kinetic treatment of parent drug a discussion of the theoretical models which would also include metabolite were considered. The proposed model was useful in explaining why diethylstilbestrol may be retained in the body long after administration of the dose and indicated that tissue uptake was very significant, resulting in a large volume of distribution for this drug. A very high clearance of diethylstilbestrol was also observed and this finding explained the huge first pass effect that was seen when diethylstilbestrol was administered duodenally. However, the model has several limitations as pointed out, since only one animal and one dose level were studied.

The inability to account for 100% of the dose within 24 hours after intravenous administration was ascribed to a deep peripheral compartment which was able to sequester a significant portion of the dose and release it slowly,back into the systemic circulation. However, other explanations can also be offered. There may be other routes of excretion such as the

saliva, skin, lungs, or intestine. Since bile was collected totally during all experiments the feces were not collected for analysis. It is possible that a portion of the drug is excreted by the cells of the gastrointestinal tract and may be excreted via the feces as a result. The skin and saliva are also significant routes of excretion for a number of compounds but neither of these were analyzed. The lungs are the primary route of excretion for many of the volatile anesthetic gases but would probably be insignificant for a compound such as diethylstilbestrol, having an aromatic ring structure not likely to be broken down in vivo. Other explanations are also possible, however, approximately 90% of the dose was accounted for and even if the other 10% could be recovered it probably would not significantly alter the conceived model or any of the conclusions which were drawn from the data.

It was emphasized earlier that the pharmacokinetic model presented applies only to a monkey whose bile is completely collected, thus preventing an enterohepatic circulation of drug and metabolite. The next step would be to allow return of bile to the intestine removing only a portion for analysis. This could be done using a commercially available instrument (stream splitter) which has the capacity to separate and collect any desired fraction of the bile while returning the rest to the intestine. This experimental model would allow one to quantitatively assess the effect of an enterohepatic circulation on the pharmacokinetics of diethylstilbestrol and diethylstilbestrol monoglucuronide. One could also study an intact monkey but here the problem of uncontrolled bile flow rate and variable emptying of the gall bladder would complicate interpretation of the data.

Another factor which could play a large role in the pharmacokinetics

of a drug is protein binding. While a study of the protein binding characteristics of diethylstilbestrol and its monoglucuronide was not undertaken, it would be surprising if this drug did not bind extensively to proteins in plasma and tissue. Strong interactions with proteins can have a profound effect on drug distribution and elimination. Unfortunately, complex binding characteristics can also introduce mathematical computation problems which render such models unsolvable. Even so, this aspect of the drugs overall fate in vivo should be considered since protein binding also affects biologic activity. However, if a drug is only weakly bound to plasma protein one can assume that binding is a linear process and from a pharmacokinetic point of view, it can be neglected. In either case the protein binding characteristics of a drug and its metabolites are an integral part of the study of a drugs fate in vivo.

The above considerations point out that there is much more to be done in the study of diethylstilbestrol and its metabolites. However, it is probably true for many research projects that additional questions and problems are generated in the pursuit of ones initial goals. In fact, it is not unlikely that many significant contributions of the scientific community are the result of research projects which deviated from that which was anticipated. Indeed, if the answers were already known and the experimental results always predictable there would be no incentive to continue in research.