# THE METABOLISM OF DIETHYLSTILBESTROL

Author: **Manfred Metzler**
Institute of Toxicology
University of Würzburg
Federal Republic of Germany

Referee: L. J. Fischer,
Department of Pharmacology
College of Medicine
University of Iowa
Iowa City, Iowa



## I. INTRODUCTION

Diethylstilbestrol (DES, Figure 1) is a stilbene derivative with an estrogenic activity comparable to that of the natural estrogen, estradiol-17β. Its synthesis by Dodds and co-workers[29,30] in 1938 was the result of a search for an inexpensive and orally effective estrogen. DES met both of these goals, and it came into clinical use by the end of 1938 in America and in Europe.[28] The major indications for DES were estrogen deficiencies, e.g., in hormonal dysgenesis, and the treatment of prostatic tumors.

In 1946, it was first reported that DES might prevent miscarriage.[151-153] Although its efficacy for this indication was seriously questioned,[24,38] DES became widely accepted as a therapy for threatened abortion. It has been estimated that a total of 500,000 to two million pregnant women in the U.S. were treated with DES.[134] Later, DES became popular as a postcoital contraceptive, although the extent of this usage remains largely unknown.

Aside from its use in human medicine, DES found widespread utilization for agricultural purposes. In 1943, Lorenz observed that DES had a fattening effect in chickens,[95,96] and from 1947 on, DES implants were used in poultry until this application was stopped in the U.S. by the FDA in 1959, when DES residues were found in chicken livers.[75] In beef cattle and sheep, DES treatment by implantation or in the diet promotes growth, as was first reported by Dinusson et al.[27] Therefore, DES was extensively used in the cattle industry until it was banned by the FDA in 1979, and it is estimated that more than 80% of the cattle raised in the U.S. have received DES.[106] This application caused a considerable release of DES into the environment, either directly through contamination on the feedlots or indirectly through the cattle waste. According to an analysis of the FDA Bureau of Veterinary Medicine, 27,648 kg of DES were used for feedlot cattle in 1970.[107]

Although several reports on the observations of tumors in various animal species after exposure to DES began appearing in the literature as early as 1938 (for review see References 106 and 109), major concern about a possible health hazard was raised only in 1971. In that year, Herbst et al. reported a possible association of DES with the vaginal and cervical carcinomas observed in eight young women whose mothers had been treated with this synthetic estrogen during their pregnancy.[69] This association was soon confirmed and extended,[106] and today there are more than 300 cases of these previously rare tumors in young women, at least half of which have an established history of *in utero* exposure to DES.[67] Moreover, nonmalignant abnormalities of the genital tract appear to occur at a higher incidence in "DES daughters" as compared to nonexposed women, e.g. cervical hoods, transverse ridges and adenosis,[68] and an abnormal shape of the uterus.[79] Teratogenic effects were also observed in the genital tract of the male offspring of women treated with DES during pregnancy. These in-

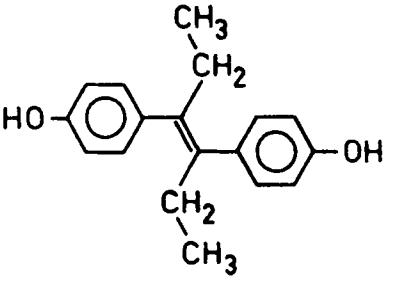



FIGURE 1. Structure of diethylstilbestrol (DES).

clude epididymal cysts, testicular and sperm abnormalities,[11,12,52] undescended testes, and meatal stenosis.[21]

Despite its widespread use and its unique toxicological features, relatively little is known about the fate of DES in both the mammalian organism and in the environment. The toxic effects of DES give rise to several questions, such as the mechanism of transplacental toxicity and the risk provided by other estrogenic compounds, e.g. from oral contraceptives.

An important consideration for understanding both the fate and the mechanism of toxicity of a compound is its metabolism. This paper is an effort to review the data on the biotransformation of DES and to deduce some implications for the toxicity of this synthetic estrogen.

## II. METABOLITES OF DIETHYLSTILBESTROL (DES)

Interest in the metabolism of DES arose almost simultaneously with its synthesis, since it was hoped that the metabolism might provide a clue for understanding the much higher effectiveness of synthetic estrogens after oral administration over the natural estrogens estrone and estradiol.[159,178] For a long period of time the primary interest focused on the formation of conjugates and only recently has oxidative metabolism been studied. Due to the transplacental effects of DES, attention is now also being paid to the biotransformation of DES in the perinatal organism, and to the biological activity of the metabolites.

### A. Conjugation Reactions of DES

#### *1. Glucuronide Formation In Vivo and In Vitro*

The first experiments on the metabolism of DES were published by Stroud in 1939, shortly after the synthesis of the compound had been reported. Stroud[159] compared the fate of DES and of two other synthetic estrogens, hexestrol and dienestrol, with that of estrone by measuring the amount of estrogen excreted in rabbit urine. The substances were quantitated by the estrous response in ovariectomized female rats, isolated in crystalline form, and identified by mixed melting point with the parent compound. In all the compounds under investigation, a part of the estrogen, the free form, could be extracted from the urine with benzene, but another part remained in the aqueous phase and could only be extracted with benzene after acidic hydrolysis. This was thought to indicate a conjugated form. For DES, the ratio of free to conjugated form was approximately 1:1. In 1942, the water-soluble metabolite of DES was isolated from rabbit urine in crystalline form by Mazur and Shorr.[105] Elementary analysis, optical activity, equivalent weight determination by titration with alkali of the conjugate, and its acid hydrolysis into DES and glucuronic acid (identified by a positive naphthoresorcinol test) were used to assign the structure of DES monoglucuronide

Table 1
PHYSICAL DATA OF SYNTHETIC MONO- AND DIGLUCURONIDE (FREE ACID FORM) OF DES ACCORDING TO KROHN[85]

| | Monoglucuronide | Diglucuronide |
|---|---|---|
| Solvent for crystallization | Acetone/benzene | Acetone/diethylether |
| Melting point | Sintering at 65—75°C melting at 175°C | Decomposition at 130°C |
| $[\alpha]_D^{20}$ (ethanol) | −58.2° | −64.4° |
| Infrared spectrum (KBr, $cm^{-1}$) | 3400,1732,1610,1508 | 1730,1610,1508 |
| UV spectrum (methanol) $\lambda max$ (log ε) | 237 (4.21) 280 sh (3.70) | 225, 280 sh |

From Krohn, K., *Hoppe Seyler's Z. Physiol. Chem.*, 358, 1551, 1977. With permission.

(DESG) to the conjugate. Of a total dose of 3 g of DES, administered over a period of 2 weeks, 1.5 g of DESG (corresponding to 30% of the dose) were isolated from urine. This indicated that glucuronidation was a major conjugation pathway for DES in the rabbit.

The findings of Mazur and Shorr were eventually confirmed in 1948 by Dodgson et al.[31] who showed an even higher yield (71% of a dose of 150 mg or DES/kg) of DESG from rabbit urine, and by Simpson and Wilder-Smith.[14] For many years, the urine of rabbits dosed with DES served as a source of DESG, until finally in 1977 the chemical syntheses of both DES monoglucuronide and DES diglucuronide were reported.[85] These two conjugates could be separated due to the different solubility of their sodium salts, and were obtained in crystalline form. The physical data of the synthetic DES monoglucuronide (Table 1) agree well with those reported for the biochemically obtained DESG.[31] Also in 1948, DESG was crystallized as the benzylamine salt from the urine of two women, who each received 5 × 20 mg of DES within 24 hr. Again, a high yield (35% of the dose) was obtained.[32]

Subsequent investigations demonstrated that DESG is also formed from DES in other species in vivo (Table 2). DESG was not isolated in pure form in any of these studies. Instead, other methods were used for its identification, such as cochromatography with DESG from rabbit urine in paper and thin-layer chromatography, and hydrolysis to DES by β-glucuronidase. For the separation of DESG from the biological source, usually urine or bile, a method already introduced by Mazur and Shorr[105] was often used and this was later expanded upon in greater detail by Teague and Brown.[162] In this method, the free and monoglucuronide form of DES could be extracted jointly from the aqueous biological sample with diethylether at pH 2 to 3. DESG was then removed from the ether extract with saturated sodium bicarbonate solution and recovered into ether after acidification of the aqueous phase. In more recent studies, chromatographic methods were preferred for the separation of DES and DESG, e.g., column chromatography on florisil,[45] porapak Q,[7] or neutral alumina.[111,112]

The synthesis of radio-labeled DES, first reported in 1951,[62,63,164] greatly facilitated the quantitation of DESG from the excretions of the different species listed in Table 2. For example, by using $^{14}C$-DES, Hanahan et al.[62] and Fischer et al.[44] demonstrated that more than 60% and 72%, respectively, of the radioactivity found in rat bile was DESG. Similarly, Mitchell et al.[126] identified 90% of the radioactivity found in the

## Table 2
## IDENTIFICATION OF DES MONOGLUCURONIDE (DESG) IN DIFFERENT SPECIES IN VIVO

| Species | Label of DES | DESG found in | Methods used for the identification of DESG | Ref. |
|---|---|---|---|---|
| Rabbit | None | Urine | Isolation of DESG in crystalline form, identification through chemical analysis | 31,105,147 |
| | None | Urine | Isolation of DESG in crystalline form | 76,175 |
| Rat | $^{14}C$ | Bile | Fractionate extraction,* followed by acid hydrolysis to DES, which was identified by reverse-isotope-dilution | 62,63 |
| | $^{14}C$ | Bile | Identical retention value (in PC ?) with DESG from rabbit urine | 44 |
| | $^{14}C$ | Bile | TLC comparison with DESG isolated from rabbit urine. | 122 |
| | $^{14}C$ | Bile<br>Plasma | Water soluble, not extractable into chloroform | 81 |
| | $^{3}H$ | Bile | Chromatography on alumina, hydrolysis with β-glucuronidase | 111,112 |
| | $^{14}C$ | Intestine | Chromatography on florisil columns | 91 |
| Mouse | $^{14}C$ | Plasma<br>Liver | Chromatography on florisil, hydrolysis with β-glucuronidase | 146 |
| | $^{14}C$ | Urine | Chromatography on alumina, hydrolysis with β-glucuronidase | 115 |
| | $^{14}C$ | Urine | Chromatography on LH 20, hydrolysis | 65 |
| Hamster | $^{3}H$ | Urine<br>Bile | Chromatography on alumina, hydrolysis with β-glucuronidase | 55,111,112 |
| Chicken | $^{14}C$ | Liver<br>Bile | Solubility characteristics before and after acid hydrolysis | 72 |
| | $^{14}C$ | Urine<br>Feces<br>Bile | TLC chromatography and enzymic hydrolysis | 138 |
| Guinea pig | $^{14}C$ | Urine | Chromatography on alumina, hydrolysis with β-glucuronidase | 181 |
| Sheep | $^{3}H$ | Urine | Fractionate extraction,* hydrolysis with β-glucuronidase | 70 |
| Steer | $^{3}H$ | Urine<br>Bile | Fractionate extraction* | 126,127 |
| | $^{3}H$ | Urine | Fractionate extraction,* hydrolysis with β-glucuronidase | 70 |
| | $^{14}C$ | Urine | Chromatography on porapak Q, hydrolysis with β-glucuronidase/ arylsulfatase | 7,8, |
| Rhesus monkey | $^{14}C$ | Bile<br>Blood<br>Urine | TLC comparison with biosynthetic DESG from rabbit urine | 128,129 |
| | $^{14}C$ | Urine | Chromatography on alumina, hydrolysis with β-glucuronidase | 121 |
| | $^{14}C$,$^{3}H$ | Urine | Chromatography on LH 20, hydrolysis with β-glucuronidase | 66 |
| Chimpanzee | $^{14}C$ | Urine | Chromatography on alumina, hydrolysis with β-glucuronidase | 121 |
| | $^{14}C$ | Urine | Chromatography on LH 20, hydrolysis with β-glucuronidase | 66 |
| Human | None | Urine | Isolation of the benzyl salt of DESG and comparison with DESG from rabbit urine | 32 |
| | $^{3}H$ | Urine | PC comparison with biosynthetic DESG from rabbit urine, hydrolysis with β-glucuronidase | 46 |
| | $^{14}C$ | Urine | Chromatography on alumina, hydrolysis with β-glucuronidase | 112 |

* According to Teague and Brown.[142]

bile and 73% of that found in the urine of steers after oral administration of $^{3}$H-DES as DESG. These and the other studies listed in Table 2 indicated that DESG is a major in vivo metabolite of DES in the rabbit, rat, mouse, hamster, chicken, guinea pig, sheep, steer, rhesus monkey, chimpanzee, and man.

The in vivo studies were supplemented by several investigations reporting the formation of DESG by various tissues in vitro. Thus, Zimmerberg,[177] after incubating rat liver slices, found water-soluble conjugates of DES, which according to their hydrolytic behavior were believed to be the glucuronide and the sulfate of DES. Whereas the sulfate hydrolyzed easily under acidic conditions, autoclaving was required to split the glucuronide. Gabaldon et al.[50] provided additional evidence that the DES conjugate formed in rat liver slices and homogenate was DESG. This was done through its fractionated extraction (according to the procedure of Teague and Brown, see above) and thin-layer cochromatography with DESG isolated from rabbit urine.

In 1959, Hartiala and Lehtinen[64] reported that slices from rat duodenum also formed a water-soluble conjugate of DES, which could be split by β-glucuronidase. By using the everted-sac-technique and $^{14}$C-DES, Fischer and Millburn[43] confirmed that the rat intestine can form DESG, which was identified by thin-layer cochromatography and reverse isotope dilution with biosynthetic DESG from rabbit urine, and by hydrolysis with β-glucuronidase to DES. Recently, absorption and glucuronidation of $^{14}$C-DES by segments of rat small intestine were studied *in situ*.[91] DESG accounted for almost 50% of the radioactivity appearing in the intestinal venous blood, and no difference in DESG formation was observed between intestinal segments located near the pyloric sphincter or near the ileo-cecal junction.

Liver homogenates from chicken and hamster have also been demonstrated to form DESG. After incubation of $^{14}$C-DES with minced chicken liver,[73] 75% of the radioactivity remained dissolved in the aqueous system after extraction with ether, but became ether-extractable after hydrolysis with β-glucuronidase and was then shown by paper chromatography to cochromatograph predominantly with DES. In studies with hamster liver homogenates,[48] DES consumption was measured with and without uridine-diphosphoglucuronic acid (UDPGA), and the DESG formed was characterized by its cochromatography on thin-layer plates with biosynthetic DESG from rabbit urine and its hydrolysis to DES by β-glucuronidase, which could be inhibited with saccharolactone. Lacomba and Gabaldon[88] also reported DESG formation in vitro in kidney of hamster and rat, and found the glucuronyltransferase activity for DES to be five times higher in hamster kidney homogenates as compared to rat kidney. Chronic treatment with DES leads to a decrease in hepatic DES-glucuronyltransferase activity in the hamster,[88] whereas other compounds such as Triton X-100® and adenosine triphosphate (ATP) together with UDPGA increased this enzyme activity.[49]

Extensive glucuronidation of $^{14}$C-DES in vitro was also demonstrated in sheep by using an isolated perfused liver preparation.[74] After 1-hr perfusion, 99% of the radioactivity in the perfusate, 95% of that in the liver, and 97% of that in the bile was DESG. Everted intestinal sacs and rumen mucosa incubations were also shown to form DESG in this study.

Finally, there is evidence that DES can be glucuronidated by liver and intestinal tissue of rodents during the perinatal period, as will be discussed in a later section (II.D.)

All these studies indicate that the glucuronidation of DES is quantitatively a very important pathway in the metabolic disposition of this compound. It should be noted that this also appears to be true for most of its oxidative metabolites, which generally have been detected as glucuronides (see II.B.1.).

Since DES has two hydroxyl groups, a diglucuronide could be formed. The observation of DES diglucuronide has occasionally been suspected. For example, Fischer et

al.[44] have reported finding among the biliary metabolites of $^{14}$C-DES in the rat a metabolite which has also been found in the bile of rats injected with DES-monoglucuronide, and which on incubation with β-glucuronidase gives DES. Similarly, Millburn et al.[122] after injection of $^{14}$C-DESG into rats with biliary fistulas have noted that, other than DESG, only one metabolite has been excreted in the bile which on hydrolysis yielded DES. However, since pure DES diglucuronide has not been available as a reference substance until recently,[85] these findings cannot be taken as conclusive evidence, and the possibility remains that the observed metabolite is a glucuronide of an oxidative metabolite of DES or a mixed glucuronide/sulfate conjugate of DES. Several oxidative metabolites of DES have been found in rat bile (see II.B.1.). These are difficult to separate from DES, and β-glucuronidase is invariably contaminated with traces of arylsulfatase.

*2. Enterohepatic Circulation of DES and Its Glucuronide*

Hanahan et al.[62] were the first to suggest that DES undergoes an enterohepatic circulation in the rat. Enterohepatic circulation refers to the excretion of a compound from the liver into the intestine via the bile where it is reabsorbed and returned to the liver via the portal system. Such a recirculation had been well-known for the bile acids and a number of other endogenous and exogenous compounds. In the case of DES, the initial observation was that 6 hr after subcutaneous administration of $^{14}$C-DES to adult female rats, 41 to 76% of the injected radioactivity was found in the intestinal tract and the bile was detected as the major route of excretion, containing 64 to 90% of the administered radioactivity.[63] When bile from rats dosed with $^{14}$C-DES was infused in the duodenum of other rats, 62 to 99% of the infused radioactivity was found in the bile of the recipient animals, indicating reabsorption of the radioactive material from the intestine.[62] Since the DES excreted in the bile of rats was mainly in the monoglucuronide form,[44,62] the question arose as to whether the DESG was reabsorbed as such or was first hydrolyzed to DES. After intraduodenal infusion into biliary cannulated rats, $^{14}$C-DESG was excreted into the bile much more slowly than $^{14}$C-DES (21% vs. 79% of the administered radioactivity in the 4-hr-bile). The intestinal contents and the portal blood 5.5 hr after intraduodenal infusion of $^{14}$C-DESG contained both DESG and free DES.[44] These findings indicated that the recirculation was at least partially dependent upon the hydrolysis of DESG to DES in the intestine.

The importance of intestinal hydrolysis for the reabsorption of DESG was further clarified through in vitro studies by Fischer and Millburn.[42,43] Applying the everted-sac-technique, the passage of $^{14}$C-DES and $^{14}$C-DESG through the intestinal wall was found to differ considerably. $^{14}$C-DES readily entered the inverted intestinal sac, resulting in a high concentration in the serosal fluid (up to eight times that remaining in the mucosal solution). By contrast, the sacs were relatively impermeable to $^{14}$C-DESG, and a ratio of serosal to mucosal concentration of less than 0.3 was obtained. The transport of DES was higher in sacs from the colon or from distal segments of the small intestine as compared to the proximal small intestine, and was drastically reduced under anaerobic conditions or in the presence of 2,4-dinitrophenol, sodium azide or l-tryptophan. When the serosal and mucosal solution and the intestinal tissue were analyzed in the experiments with $^{14}$C-DES, considerable amounts of DESG were found, particularly in the serosal fluid (89 to 94% of the radioactivity in this solution). This proved the ability of the intestinal tissue to glucuronidate DES and explained the finding that large amounts of glucuronide conjugates were found in the portal blood shortly after the intraduodenal administration of DES to rats.[44]

The poor absorption of DESG from rat intestine and its hydrolysis in the intestine under normal conditions was further demonstrated by Clark et al.[18] When $^{14}$C-DESG was infused intraduodenally together with glucaro-1,4-lactone, a β-glucuronidase in-

hibitor, the rate of absorption of radioactivity was decreased by 90% compared with that in the absence of the inhibitor. Clark also showed that the β-glucuronidase for DESG hydrolysis was mainly provided by intestinal bacteria, since after pretreatment of rats with antibiotics, such as neomycin or kanamycin, which suppressed the intestinal bacteria, the rate of intestinal absorption of radioactivity after intraduodenal injection of $^{14}$C-DESG was 50 times less than in untreated rats. The rate of absorption of free $^{14}$C-DES was not affected by neomycin treatment.

Walsh et al.[169] confirmed the role of intestinal bacteria for the enterohepatic circulation by showing that pretreatment of rats with lincomycin reduced the amount of radioactivity excreted in the urine after s.c. injection of $^{14}$C-DES, but led to a pronounced excretion of DESG in the feces. In the control animals, almost no conjugate was found in the feces. Moreover, the biliary excretion of cecally administered radioactive material that had been collected from the bile of rats injected with $^{14}$C-DES was drastically reduced in lincomycin-treated rats.

Fischer et al.[41] injected $^{14}$C-DES and $^{14}$C-DESG into the lumen of closed intestinal loops of 25-day-old rats *in situ* and determined the rate of disappearance and the nature of the absorbed radioactivity. When $^{14}$C-DESG was introduced into a proximal intestinal loop, approximately 80% of the radioactivity was still present after 2 hr and consisted of 95% conjugate form. With $^{14}$C-DES, only 25% of the dose was recovered from the loop after 20 min, part of which was DESG. This indicated the preferred absorption of free DES and the ability of the intestinal mucosa to glucuronidate DES. The differences in the rate of absorption between DES and its glucuronide were also seen in the distal portion of the small intestine, but not in the cecum. This was explained by the rapid hydrolysis of DESG by cecal bacteria and, indeed, only unconjugated DES was found 20 min after injection of $^{14}$C-DESG into the closed loop of this part of the intestine. The rate of absorption, however, was much slower in the cecum than in the small intestine, and this difference was attributed to the higher amount of fecal material hindering the contact of DES with the mucosal surface of the large intestine. Elimination of bacterial β-glucuronidase by pretreatment with lincomycin almost completely eliminated the hydrolysis of DESG in the cecum. In 5-day-old rats, glucuronidation of DES appeared to occur in the proximal and distal small intestine, since the majority of the radioactivity found 60 min after injection of $^{14}$C-DES into closed loops of these parts of the intestine was $^{14}$C-DESG. In contrast to the adult rat, however, a considerable absorption of intact DESG occurred in the distal portion of the small intestine in the 5-day-old rats.[41]

A striking toxicological implication of the biliary excretion and enterohepatic circulation of DES in the rat was demonstrated by Klaassen.[80] In bile-duct-ligated rats, the 24 hr $LD_{50}$ of DES, administered i.p. in dimethylsulfoxide, was only 0.75 mg/kg as compared to 100 mg/kg for sham-operated control rats. This 140-fold increase in toxicity was accompanied by a much slower plasma disappearance of DES in the bile-duct-ligated rats.[81]

The clear picture of the fate of DES in the adult rat derived from these data is somewhat complicated due to the fact that, aside from glucuronidation and hydrolysis, oxidative metabolism takes place in the liver and the intestine (see II.B.), and the bile of DES-treated rats contains several metabolites other than DESG.[44,111,112] Although there are some data showing that the rate of intestinal absorption of these metabolites is less than one tenth that of DESG,[18] their importance for the final elimination of DES remains to be elucidated. At present, no thorough analysis of the fecal metabolites of DES in the rat appears to have been undertaken.

Recent data show that biliary excretion and enterohepatic circulation of DES also takes place in the rhesus monkey. A male monkey with indwelling arterial-venous catheters and permanent catheters to the common bile duct and duodenum was used in

these studies by Mroszczak and Riegelman.[128,129] Following i.v. administration of $^{14}$C-DES, the compound was rapidly eliminated from the blood with a half-life of 55 min. Of the dose, 23 to 37% appeared in the bile and 54 to 62% in the urine. The radioactive material in bile was more than 95% conjugated, and was tentatively identified as DESG by thin layer chromatography (TLC). This method, however, does not allow distinction between the glucuronides of DES and certain oxidative metabolites with a similar polarity, such as methoxy-DES (see II, B.). Therefore, the nature of biliary DES metabolites in the rhesus monkey needs further investigation. The same criticism applies to the metabolites found in monkey blood, which, according to TLC, consist of DES (about 30%) and DESG (70%).

After the administration of $^{14}$C-DES into the duodenum of the monkey, 30% of the dose was found in bile and 35% in urine. Analysis of the blood level of conjugated DES showed an early peak at about 10 min and a lower and broader peak after 2 hr. The first peak represents DES absorbed from the small intestine and conjugated in the intestinal wall and during the first passage through the liver, whereas the second peak was attributed to DES glucuronidated in the intestinal mucosa but returned into the lumen. The conjugate apparently had to reach the large intestine for bacterial hydrolysis and subsequent absorption.

This interpretation was consistent with findings obtained when monkey bile containing 94% DESG and 6% DES was intraduodenally introduced. In this case the late blood level peak at approximately 2 hr was predominant. Since 25% of the administered radioactivity was recovered in the bile and 16% in the urine, a significant portion of the conjugate was hydrolyzed in the cecum. Virtually no conjugate was found in the feces of intact rhesus monkeys after oral administration of $^{14}$C-DES.[121] This could mean that the hydrolysis of DESG is completed in the large intestine or, alternatively, after expulsion of the feces.

The intestinal absorption of DES and DESG in humans was investigated in an elegant study by Fischer et al.[46] By using $^{3}$H-labeled DES and $^{14}$C-labeled DESG, both compounds were ingested simultaneously and their plasma levels and excretion kinetics determined. $^{3}$H-DES appeared to be rapidly absorbed from the proximal portion of the GI tract since peak concentrations of radioactivity in the plasma were found between 20 and 40 min after ingestion. In contrast, radioactivity from $^{14}$C-DESG gave a lower and broader plasma peak concentration between 3 and 6 hr after ingestion, suggesting that absorption occurred in a more distal portion of the intestine. In agreement with the fast absorption of DES, urinary excretion of $^{3}$H-radioactivity started rapidly and was essentially completed after 35 hr, whereas $^{14}$C-radioactivity from DESG appeared later in the urine and disappeared only after 60 hr. Analysis of the radioactivity in blood and urine indicated very little unconjugated material. No major difference was observed in the pattern of urinary conjugates after DES and DESG ingestion: 58 to 81% of the radioactivity in urine was identified as glucuronides, half of which was DESG as determined by paper chromatography and reverse-isotope-dilution analysis of the aglycon. Similar data for the pattern of urinary conjugates after ingestion of $^{14}$C-DES were reported by Metzler.[112] Employing radio gas chromatography (GLC) and gas chromatography-mass spectometry (GC/MS) techniques, DES (68%) and two oxidative metabolites (32%; see II, B.) were found in the glucuronide fraction, which comprised 87% of the total urinary radioactivity.

Fischer's study of the fate of DES and DESG in man, together with the data obtained from monkeys and rats, imply that the enterohepatic circulation of DES is a general phenomenon in mammals. A quantitative comparison of the data, however, clearly shows that this circulation is more pronounced in the rat as compared to the primates. Among the factors involved, the excretion of glucuronidated DES into the bile appears to be the primary reason for the species difference, whereas differences

in the formation of DESG, its bacterial hydrolysis and reabsorption from the intestine may be less important. The extent of biliary excretion of organic molecules is known to depend on the molecular weight. In order to achieve good excretion into bile, a compound has to exceed a certain molecular weight.[1,122] This threshold is species-specific and assumed to be approximately 320 for the rat and 470 for the human. Thus DESG with a molecular weight of 444 is exceptionally well suited for biliary excretion in the rat, but only moderately so in the primates, where a considerable portion of the DESG is passed from the liver into the blood circulation and, therefore, subject to excretion through the kidneys. Species differences in the elimination pathways will be considered in more detail in II.C.1.

*3. Other Conjugates of DES*

The preceding chapters have emphasized the glucuronidation of DES and the enterohepatic circulation of this conjugate. The possibility exists that other conjugates may play a role in DES metabolism. However, from the scant data available to date, it appears that their importance for the elimination of this compound is rather limited.

Due to its phenolic nature, it was expected that DES would form an ethereal sulfate. However, Teague reported in an abstract[161] in 1945 that DES causes no significant increase in the output of ethereal and neutral sulfur in the urine of rabbits and rats, thus indicating that little or no ethereal sulfate and mercapturic acid were excreted by these species. This was confirmed for the rabbit by Dodgson et al.[31] and Starnes and Teague.[156] On the other hand in rats, cats, and dogs, Starnes and Teague[156] found a small increase of ethereal sulfate excretion in urine after the i.m. administration of large doses (450 to 2400 mg) of DES, but the results varied in different experiments and cannot be considered as conclusive evidence for the formation of DES sulfate. A different method was used by Simpson and Wilder-Smith[148] for the separation and assay of DES sulfate. Extraction of the acidified urine with ether removed DES and DES glucuronide, and the remaining urine thought to contain the DES sulfate was hydrolyzed by acid and the liberated DES determined by bioassay. Based on this method, which does not appear to be very specific and sensitive, it was claimed that DES sulfate occurred in small amounts in the urine of rabbits (0.04 to 0.4% of the dose) and of humans (0.1 to 9%).[148,173]

In their analysis of urine from humans who had ingested $^{3}$H-DES and $^{14}$C-DESG, Fischer et al.[46] considered 6 to 16% of the urinary radioactivity to be DES sulfate. This was determined by a comparative hydrolysis with β-glucuronidase and with a β-glucuronidase-arylsulfatase mixture, followed by extraction with ether. The increase in extractable radioactivity after hydrolysis with the enzyme mixture over that released by β-glucuronidase alone was considered to represent sulfate conjugates. However, this technique could not be expected to provide accurate data since hydrolytic procedures are rarely complete, and β-glucuronidase usually contains traces of sulfatase, giving rise to overlapping hydrolysis.

Chromatographic procedures appear to be more suited for unequivocal identification and quantitation than fractionated extraction or hydrolysis of conjugates. A column chromatographic method which consisted of subsequent elution of unconjugated material, sulfates, and glucuronides from neutral alumina by means of 95% ethanol, water, and a phosphate/citrate buffer pH 6 was utilized to analyze the pattern of urinary DES conjugates in rats and hamsters,[111,112] mice,[115] rhesus monkeys and chimpanzees,[121] and humans.[112] In the urine of all of the species mentioned above, some radioactivity was found in the sulfate fraction, ranging from 32% of the urinary radioactivity in the male rat down to 3% in the female chimpanzee (see also II.C.2. for these species differences). Although mercapturic acids would have appeared in the same fraction, sulfates must be considered the predominant or sole metabolites in this

fraction, since more than 80% of the radioactivity became ether extractable after hydrolysis with steroid sulfatase.

The largest amount of DES monosulfate claimed thus far was for the hen. This metabolite accounted for up to 33% of the urinary radioactivity and 17% of the fecal radioactivity after $^{14}$C-DES was i.v. injected into hens with exteriorized ureters.[138] The 12-hr bile obtained from bile duct cannulated hens, contained 40% of the administered radioactivity, 52% of which was DES monosulfate. This conjugate was identified by a combination of extraction procedures, TLC in four systems, and hydrolysis, and it was stated by the authors that in their chromatography systems monosulfates, disulfates, monoglucuronides, diglucuronides, and unconjugated material could be differentiated.

A few in vitro studies demonstrated the capability of mammalian organs to form DES sulfates. Thus Zimmerberg has shown as early as 1946 that the amount of DES conjugated by rat liver slices is decreased when $MgSO_4$ is replaced by $MgCl_2$ in the incubation medium.[177] In 1963, Payne and Mason found that the mono- and disulfate of DES are formed in a microsome-free fraction of rat liver.[136] In their study, $K_2{}^{35}SO_4$ was used as a source of sulfate and the radioactive products were extracted with *n*-butanol and separated by paper chromatography. DES disulfate was identified through cochromatography with the synthetic reference compound and by its lack of free hydroxy groups. The two monosulfates detected were attributed to the *cis* and *trans* form of DES, because they could be converted to DES disulfate by repeated sulfation. Conversely, partial hydrolysis of the DES disulfate gave products cochromatographing with the suspected monosulfates. In addition, a kinetic study demonstrated that the two monosulfate esters appeared prior to the disulfate, and all three sulfate esters of DES increased progressively in intensity during the incubation. Schneider and Dingell recently reported in an abstract[145] that sulfate conjugates were formed on incubation of DES with the soluble fraction of rat and guinea pig liver or brain and $^{35}$S-phosphoadenosine phosphosulfate, the "active sulfate". The activity of the sulfotransferase in both organs was even higher for DES (3- to 40-fold) than for estradiol-17$\beta$.

Thus there is some evidence from in vivo and in vitro studies that sulfation is indeed a conjugative pathway utilized by DES, although the amounts of DES sulfate excreted appear to be very low as compared to DES glucuronide. However, sulfation could be of importance for the metabolic fate of DES in the body. Since no data are available on the concentration of DES sulfate in the blood and organs of animals and man, it cannot be said whether sulfation is as important for the intermediary metabolism and storage of DES as it is for steroidal estrogens.[16]

Recent studies[9,59] have demonstrated that DES mono- and disulfate were not metabollically inert in vivo. Both compounds underwent desulfation in the rat, with the liberation of inorganic sulfate in the urine. When $^{35}$*S*-labeled DES disulfate was injected i.p., 58 to 68% of the administered radioactivity was recovered in the 48 hr urine as inorganic sulfate, and no parent DES-disufate was found.[9] Between 14 and 24% of the dose appeared in the feces, but was not identified. When DES disulfate was injected i.v. into bile duct cannulated rats, 31 to 33% of the radioactivity was excreted in the 6-hr urine, predominantly as inorganic sulfate, and 16 to 18% in the bile. Of the biliary radioactivity, 90% was represented by one metabolite, which could be identified as DES monosulfate monoglucuronide. When $^{35}$*S*-DES monosulfate was administered to the bile duct of catheterized rats, a large proportion, 47 to 49%, of the dose appeared in the bile and consisted almost exclusively of DES monosulfate monoglucuronide. This double conjugate proved to be metabolically stable because after reinjection into the rat, 88 to 96% of the dose was excreted unchanged in the 2-hr bile. The rapid desulfation of DES disulfate and DES monosulfate and the partial glucuronidation of the monosulfate were also shown to occur in the perfused rat liver

in this study, and a microsomal arylsulfatase was suggested as the hydrolyzing enzyme.

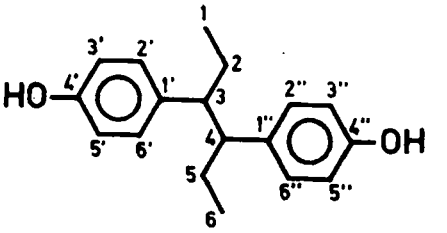
Another conjugation reaction, which plays an important role in the metabolism of steroid estrogens, is methylation. No evidence is available to date that methyl ethers of DES itself occur as metabolites in vivo, and an in vitro system which is comprised of *S*-adenosyl-(³H-methyl)-L-methionine (the "active methyl") and the soluble fraction of rat liver has failed to methylate DES.[155] However, when NADPH and the 9000-g supernatant or microsomes are included in the incubation, a methylation product is obtained and considered to be the *O*-methyl catechol of DES. Apparently, the oxidation of DES to its catechol is a prerequisite for the methylation. These findings are in agreement with results previously reported by Daly et al.,[22] who have studied the substrate specificity of catechol-*O*-methyltransferase (COMT) and have found that a methoxy derivative is formed in vitro when DES is incubated with microsomal hydroxylase and COMT from rabbit liver. Studies on the oxidative metabolism of DES (see Section II.B.1.) have revealed that, indeed, methoxy-derivatives of DES and subsequent oxidation products are major metabolites of DES in vivo.

Conjugation with glucose, a reaction not commonly observed in mammals, but rather in insects and plants, has also been studied with DES.[86,87] Incubation of rabbit or mouse liver microsomes with UDP-(6-³H)-glucose has revealed the DES monoglucoside, characterized by TLC and enzymic cleavage to DES by almond emulsin. Compared to the extent of glucuronidation, however, only small amounts of the DES-glucoside are formed in these experiments and, so far, no evidence exists to show that glucosidation of DES plays a role in vivo.

### B. Oxidative Metabolism of DES

Interest in the oxidative biotransformation of DES is of relatively recent origin, and prior to 1975, the only metabolites of DES with established structures were its conjugates. Several reasons may have contributed to this fact. First, it was thought that the extensive glucuronidation of DES might exclude this compound from oxidative metabolism. Indeed, the structure of DES with its two-parent hydroxy groups implied that no phase I metabolism is required. This teleological view was supported by several early findings that the only compound released from the glucuronide was unchanged DES. For example, Hanahan et al.[62] reported that 96% of the radioactivity liberated from the biliary glucuronide fraction of ¹⁴C-DES-treated rats cocrystallized with unlabeled DES in a reverse-isotope-dilution analysis. Moreover, attempts to find $^{14}CO_2$ in exhaled air after administration of ¹⁴C-DES were negative for mice,[164] chickens,[71] sheep,[4] steers,[144] and rats,[62,63] or only marginally positive (less than 0.4% of the dose) for the rat in other reports.[51,164] This was also taken as an indication that oxidation of DES did not occur to a significant extent in vivo, seemingly supporting an earlier conclusion made by Zimmerberg in 1946, who stated that conjugation was the preferred means of metabolism of DES, and oxidation occurred only when the conjugation capacity of the tissues was exceeded.[177]

However, several other studies provided evidence for the in vivo formation of metabolites of DES other than conjugates. When randomly labeled ³H-DES was given to a young rat and its body water analyzed for radioactivity, 5.2% of the dose was found as tritiated water, which indicated some oxidative attack on the DES molecule.[51] The same authors found four radioactive compounds with a polarity similar to unconjugated DES in the ether extract of the hind leg muscles of rats injected with ¹⁴C-DES. Similarly, in whole-body extracts of 25-day-old rats 4 hr after injection of ¹⁴C-DES, only 38% of the unconjugated radioactivity was DES, the rest being attributed to nonpolar metabolites.[45] A similar proportion of unchanged DES and metabolites was noted in the glucuronide fraction of these rats. The portion of DES metabolites clearly increased with the age of the animals and the duration of the experiment. In several other studies on the disposition of radiolabeled DES in vivo, the observation was made




DES: diethylstilbestrol (one double bond between C-3 and C-4)

ψ-DES: pseudo diethylstilbestrol (one double bond between C-2 and C-3)

DIES: dienestrol (one double bond between C-2 and C-3, and one double bond between C-4 and C-5)

HES: hexestrol (no olefinic double bond)

FIGURE 2. Nomenclature for DES metabolites. For the unambiguous designation of different oxidative metabolites, each position of the DES molecule is numbered and different abbreviations are used for compounds with different number or position of double bonds. (According to Metzler, M. and McLachlan, J. A., *J. Environ. Pathol. Toxicol.*, 2, 579, 1978. With permission.)

that after appropriate hydrolysis of conjugated material, only part of the radioactivity cocrystallized with DES in a reverse isotope dilution analysis. This was found for radioactivity obtained from the bile and GI tract of steers,[7] from steer liver,[7,8,142] and from steer urine.[8]

These hints for the formation of DES metabolites were supported by observations made in in vitro studies. Hopwood et al.[73] while studying the glucuronidation of DES by chicken liver, noted small amounts of two other metabolites in the glucuronide fraction. Five separate ether-soluble metabolites were observed by Jellinck and Lucieer[78] when $^{14}$C-DES was incubated with rat liver postmitochondrial supernatant.

### *1. Nature of Oxidative Metabolites*

Since 1975, the independent efforts of two laboratories have resulted in the assignment of structures to the nonpolar DES metabolites. Somewhat different approaches have been taken by the two groups. While Engel has been primarily interested in the DES metabolites formed in vitro, Metzler's work focuses on the in vivo oxidative metabolism of DES. In their methods for elucidating the structures, both laboratories make extensive use of mass spectrometry, particularly taking advantage of the "twin ion technique". This term means that through the use of stable isotopes, a characteristic isotopic pattern will appear in the mass spectrum of the labeled compound, and also in the spectra of its metabolites, thereby distinguishing the metabolites from other compounds present in a biological sample. Moreover, the isotopic pattern can help in elucidating the structure, as will be discussed in more detail below. For the use of the twin ion technique in DES metabolic studies, Marshall and Engel[102] have synthesized DES with a perdeuterated methyl group, while Metzler[113] has prepared pentadeuterated DES, with the label located in one ethyl group.

The discussion of the structures of individual metabolites can be greatly facilitated by using a recently proposed nomenclature.[118] Thus the different carbon atoms of the DES molecule are numbered and abbreviations are used according to Figure 2. Furthermore, the stereoisomerism caused by the olefinic double bonds is accounted for by using the E,Z nomenclature (Figure 3).

Tentative structures for the in vivo oxidative metabolites of DES were first obtained from a study of the biliary and urinary excreted radioactivity after i.p. administration of labeled DES to female rats and male hamsters.[111,112] A 1:1 mixture of tritium- and deuterium-labeled DES was used: here the tritium is generally distributed in the molecule at a relatively low specific radioactivity, whereas the deuterium is located in one

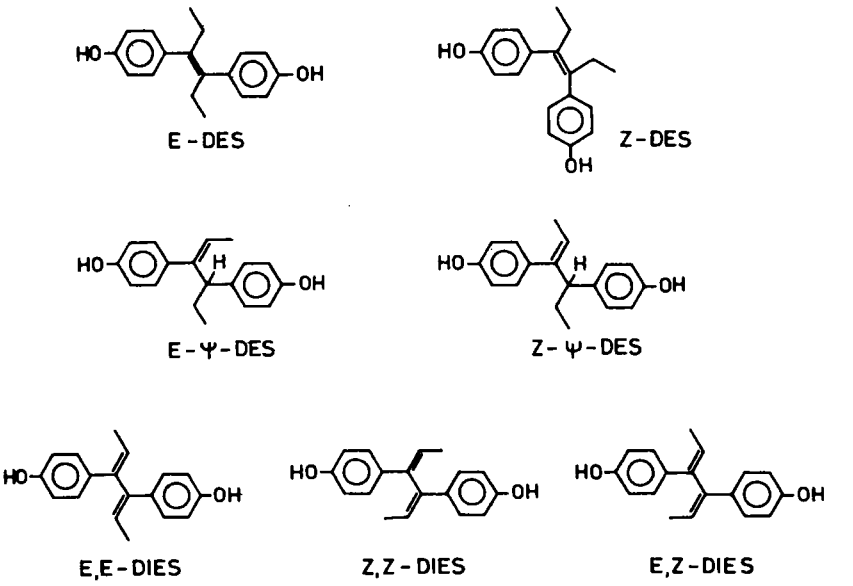



FIGURE 3. Double-bond stereoisomers of diethylstilbestrol (DES), pseudo diethylstilbestrol (ψ-DES), and dienestrol (DIES).

ethyl group at practically 100 atom per cent. In both the urine and the bile from both species, 60 to 80% of the recovered radioactivity is in the glucuronide fraction, which is obtained through chromatography on an alumina column of the ether/ethanol (3:1) extract from the ammonium sulfate saturated excreta. The glucuronides are hydrolyzed by β-glucuronidase and the deconjugated radioactivity is extracted with ether and analyzed by radio-GLC. Apart from the E- and Z-form of DES, several other radioactive peaks are observed in the chromatograms, and their mass spectra obtained by GLC-mass spectrometry.

Due to the mixture of pentadeuterated and nondeuterated DES used, two molecular ions with a mass difference of 5 are observed in the spectra of DES and of those metabolites where the ethyl groups are not affected, such as 3′-methoxy-DES (Figure 4) and 3′,3″-dimethoxy-DES (spectrum not shown). A second series of DES metabolites is characterized by a decrease of two mass units (of the nondeuterated molecules), and the complete loss of one deuterium atom, indicating that dienestrol (DIES) is the parent structure of this type of metabolites. A more complicated situation is encountered in the spectra of those compounds where hydroxylation of the terminal methyl group (ω-hydroxylation) has occurred (1-hydroxy-DES and 1-hydroxy-z,z-DIES; see Figures 5 and 6), and this is discussed in more detail by Metzler.[112]

In addition to the metabolites mentioned so far, several others have been found in subsequent studies. For example, 4′-hydroxypropiophenone (HPP) is detected among the urinary glucuronides of male rats,[112] 1-hydroxy-ψ-DES as a urinary glucuronide of female mice,[115] the catechol 3′-hydroxy-DES and the cyclic compound, indenestrol A, as glucuronide in rat bile and mouse urine, respectively,[181] and 1-hydroxy-3′-methoxy-ψ-DES in hamster urine.[55]

All the oxidative in vivo DES metabolites identified to date are arranged in a metabolic scheme in Figure 5 and their mass spectra are listed in Figure 6. A brief account of the present evidence available for confirming their structure might be appropriate before discussing the metabolic pathways in more detail.

Of the three possible stereoisomers of DIES, only one is predominantly found as a DES metabolite. It has been noted earlier[111] that this is not the isomer used as an estrogenic drug, which is known as α-dienestrol,[30] and for which an E,E-configuration

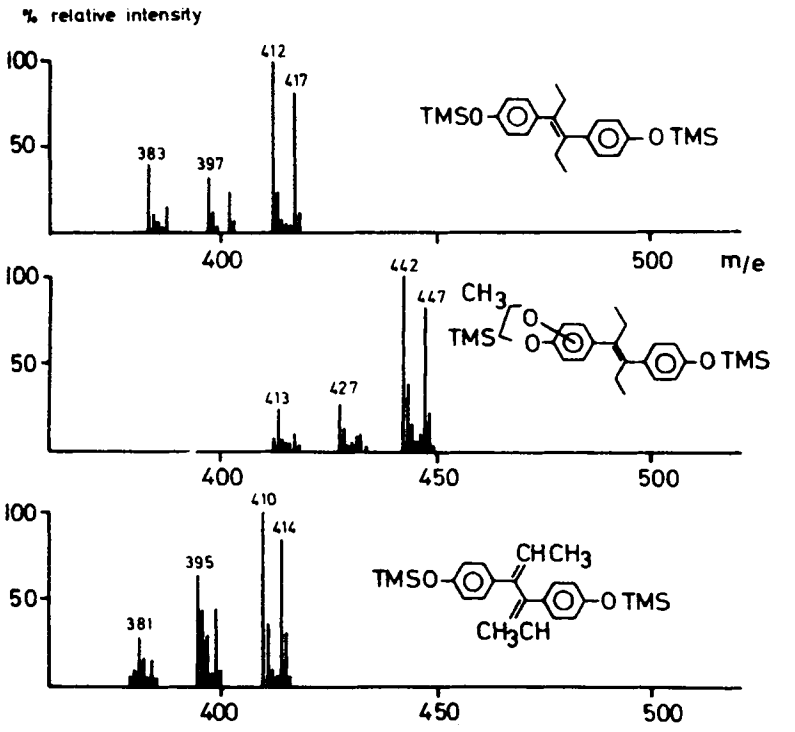



FIGURE 4. Electron-impact mass spectra (70 eV) of some DES metabolites (TMS derivatives) obtained by the "twin ion technique". This means that a certain isotopic pattern is caused in the mass spectrum by employing compounds labeled with stable isotopes. In this particular case, unlabeled DES was mixed with DES pentadeuterated in one ethyl group. The "twin ions" with a mass difference of 5 will appear unchanged in all metabolites where the label is not affected, or, alternatively, indicate the number of isotopes lost during metabolism.

has been established through X-ray crystallography.[34] Therefore, the other two stereoisomers of dienestrol have been synthesized,[111] and the metabolic DIES has proven to cochromatograph in GLC with the isomer formerly named β-dienestrol[89] and having the Z,Z-configuration.[118]

The 1-hydroxy-DIES (Figure 5) has also been chemically synthesized and been shown by nuclear magnetic resonance spectrometry to be Z,Z-configurated.[114] The identity of metabolic and of authentic 1-hydroxy-DIES has been established through cochromatography in TLC and GLC and by cocrystallization of the triacetates.[114]

Authentic reference compounds have also been available for 3′-hydroxy-DES, indenestrol A, and HPP. Synthetic 3′-hydroxy-DES and indenestrol A have been cochromatographed in radio GLC with their metabolic counterparts obtained from $^{14}$C-DES, and also show identical mass spectra.[161] Cochromatography in GLC and TLC and reverse-isotope-dilution analysis have been used to identify HPP.[112]

For the other DES metabolites shown in Figure 5, no reference compounds are yet available. In addition to their mass spectra (Figure 6), however, some evidence supports the proposed structures. 1-hydroxy-DES and 1-hydroxy-ψ-DES, upon catalytical hydrogenation, yield the same products as obtained from 1-hydroxy-DIES, namely threo- and erythro-1-hydroxy-hexestrol.[115] Hydrogenation of the olefinic double bond of 3′,3″-dimethoxy-DES yields the correspondingly substituted hexestrol. Because the mass spectra of hexestrol and its derivatives exhibit a pronounced cleavage of the molecules between the two benzylic carbon atoms, the possibility can be ruled out that the

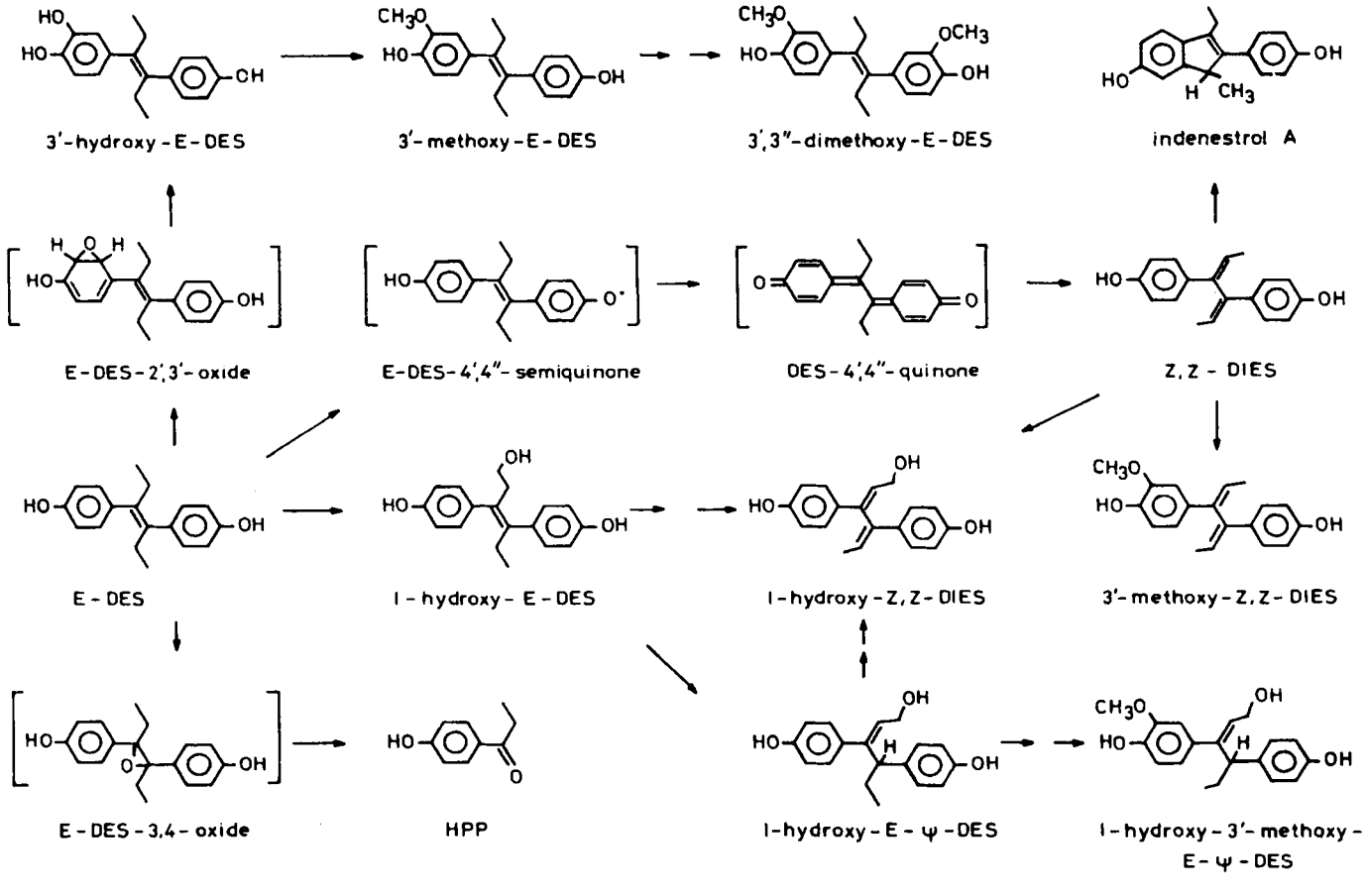



FIGURE 5. Oxidative pathways in the metabolism of diethylstilbestrol (DES). Compounds in parentheses are probable metabolic intermediates.

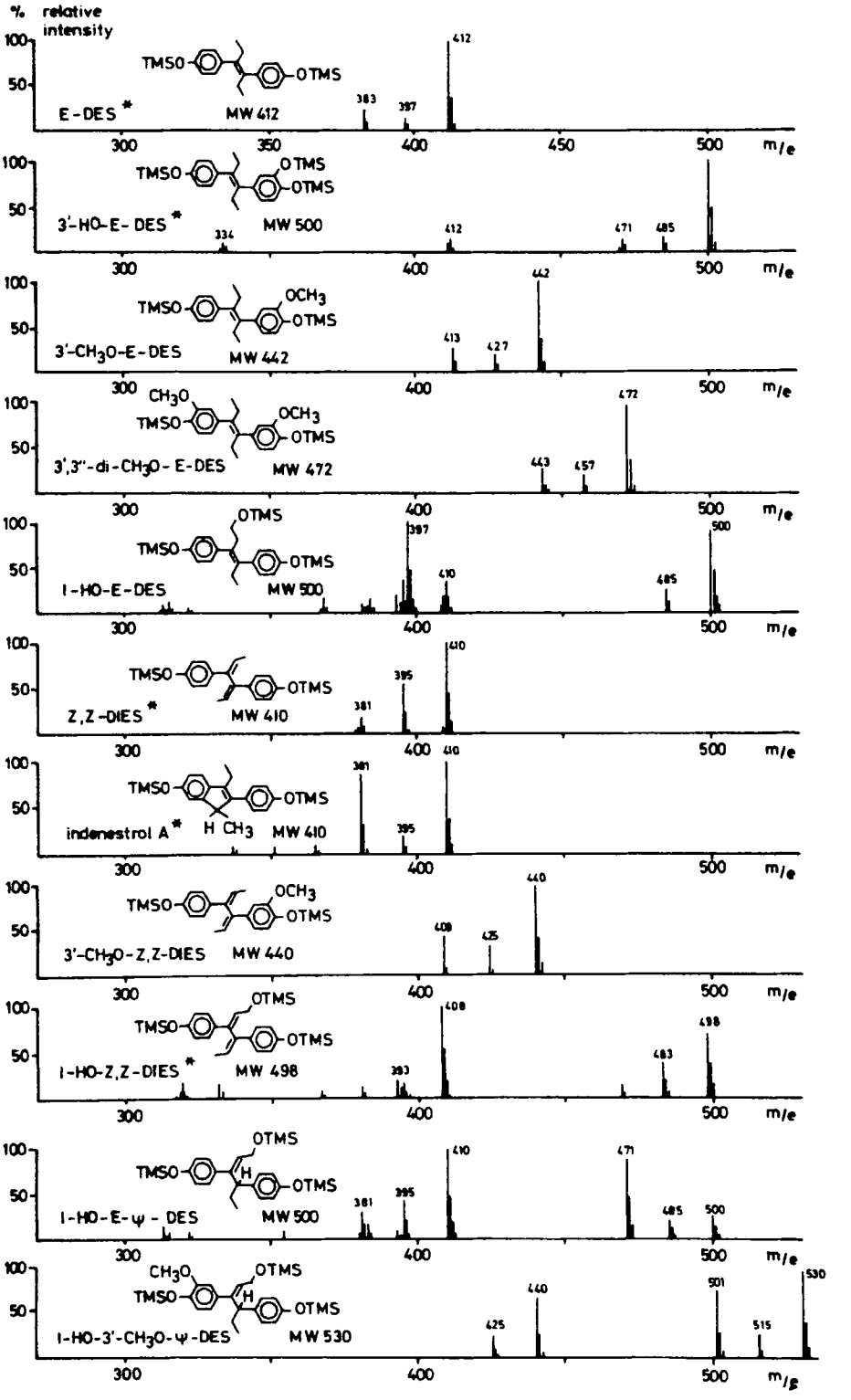



FIGURE 6. Electron-impact mass spectra (70 eV) of major oxidative DES metabolites (TMS derivatives). For compounds marked with an asterisk, authentic reference compounds were available.

two methoxy groups are in the same ring.[111,112] The only question which is not yet completely settled with respect to the methoxy-metabolites, is the isomerism, i.e., whether the second oxygen is in ortho- or meta-position to the 4′-oxygen, and whether the methyl group is attached to the parent 4′-oxygen or to the newly introduced oxygen. However, several lines of evidence from in vitro studies (see II.A.3.) are in favor of the structures shown in Figure 5. DES itself is not methylated by the soluble fraction of rat liver, but methylation is obtained after hydroxylation of DES.[155] The fact that the methylation reaction is achieved by catechol-O-methyltransferase (COMT)[24] implies the catechol nature of the hydroxylation product and also makes the 3′-position of the methyl group more likely than the 4′-position.

From the end products of metabolism excreted in urine and bile and shown in Figure 5, several metabolic pathways must be concluded. First, hydroxylation of the aromatic ring, leading to 3′-hydroxy-DES and, subsequently, to 3′-methoxy-DES. This sequence of reactions can obviously take place in both aromatic rings prior to excretion of the metabolite, thus giving rise to 3′,3″-dimethoxy-DES. Secondly, cleavage of the DES molecule leading to HPP. The third metabolic route comprises dehydrogenation of DES to DIES, which, to a small extent, can cyclize to indenestrol A. Finally, hydroxylation of the terminal methyl groups entails the 1-hydroxy-derivatives of DES, DIES and ψ-DES. All these metabolic end products can be excreted as glucuronides. The probable intermediates of the metabolic pathways will be discussed later.

Several in vitro studies are in agreement with these metabolic routes of DES. Engel et al.[35] have incubated double labeled ($^{14}C$ and $^{2}H$) DES with rat liver homogenates and rat liver microsomes. In addition to water-soluble metabolites, which are presumed to be conjugated forms and have not been further investigated, ether-soluble metabolites are observed. On paper chromatography, these metabolites are separated into compounds more polar and products less polar than DES. The addition of *S*-adenosyl-L-methionine (SAM, "active methyl") to the liver homogenate shifts the ratio of nonpolar to polar ether-soluble metabolites in favor of the nonpolar products. Omission of NADPH from the microsomal incubations, or anaerobic conditions, greatly diminishes the formation of ether-soluble metabolites. In the presence of NADPH and $O_2$, polar ether-soluble metabolites are predominantly formed, whereas additional presence of SAM favored the formation of nonpolar ether-soluble metabolites. Polar ether-soluble metabolites obtained from microsomal incubations of $^{14}C$-DES, upon incubation with partially purified catechol-*O*-methyltransferase from rat liver and (methyl-$^{3}H$)-SAM, are converted to double-labeled nonpolar compounds, indicating the incorporation of the methyl group from SAM. Mass spectrometric analysis of the polar ether-soluble metabolites gives evidence for a dihydroxy-derivative of DES and of DIES, while in the fraction of nonpolar metabolites, a monomethoxy-derivative of DES is found.

These studies confirm the formation of DIES and show that aromatic hydroxylation, followed by methylation constitutes an important pathway in DES metabolism. The experiments with catechol-*O*-methyltransferase already indicate that a catechol results from the aromatic hydroxylation. In a recent study from the same laboratory,[170] the catechol nature of the intermediate has been further substantiated. The product formed through microsomal oxidation of DES is found to be identical with that arising through oxidation of DES with mushroom tyrosinase (polyphenol oxidase) in the presence of NADPH, an enzymic method known to specifically yield catechols. The identification is accomplished through mass spectrometric analysis of a stable phenazine derivative (Figure 7) obtained after oxidation of the catechols with periodate and reaction of the resulting ortho-quinone with ortho-phenylenediamine. The same product is obtained through oxidation of DES with potassium nitrosodisulfonate (Fremy's salt).

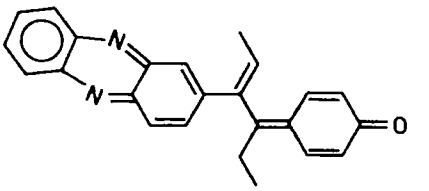


FIGURE 7. Phenazine derivative of the catechol 3′-hydroxy-DES.

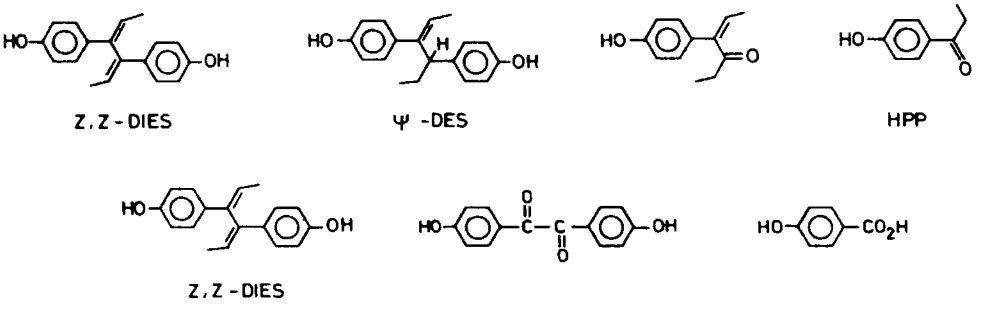



FIGURE 8. Some compounds formed from DES upon exposure to bacteria (upper row) and air (lower row).

It was shown in 1940 by Westerfeld[171] that tyrosinase destroys the estrogenic activity of DES, but the products of this reaction have not been identified. Similarly, Jellinck[77] has found that tyrosinase attacks $^{14}$C-DES with the formation of products that bind to protein, but the structures of the products have remained unclarified. Based on present knowledge it must be assumed that the binding metabolite is the ortho-quinone derived from 3′-hydroxy-DES.

The formation of 3′-hydroxy-DES and 3′-methoxy-DES in vitro upon incubation of $^{3}$H-DES with liver slices or microsomes from rats and other laboratory animals has also been studied in Masaracchia's dissertation.[104]

Other in vitro studies on the metabolic alteration of DES have involved peroxidases and bacterial enzymes. Liao and Williams-Ashman[94] have found that incubation of DES with horseradish peroxidase and $H_2O_2$ results in the formation of Z,Z-DIES (β-dienestrol, isodienestrol). The same reaction has been studied with peroxidase from mouse uterus by Metzler and McLachlan.[117,120] For the intermediates of this oxidation and its possible implication for the toxicity of DES see Sections II.B.2. and II.B.4., respectively.

Bacterial metabolism of DES other than the hydrolysis of its glucuronide has been studied very little so far, despite the extensive enterohepatic circulation of this compound (II.A.3.) and its considerable release into the environment (see Section V). Certain microorganisms cause a loss of the estrogenicity of DES.[179] When $^{14}$C-DES is added to fresh ruminant feces at ambient temperature, less than 5% is unchanged DES after 7 days.[5] More than 50% of the radioactivity was not characterized, but the identified products (Figure 8) comprised DIES ("nonestrogenic isomer", presumably Z,Z-DIES), 3,4-di(*p*-hydroxyphenyl)-2-hexene (ψ-DES), 3-(*p*-hydroxyphenyl)-2-hexen-4-one, *p*-hydroxypropiophenone (HPP), acetic acid and propionic acid. A similar pattern of degradation products is observed when $^{14}$C-DES is administered to ruminants, and their feces are analysed after keeping them at ambient temperature for 7 days.[6] Decomposition, however, is markedly less extensive in air-dried feces as compared to wet feces.

Finally, oxidation of DES is also observed after prolonged exposure to air, particularly in alkaline solution. Following earlier observations that DES in solution loses estrogenic activity in the course of several weeks,[154,173,174] Vanderlinde et al.[167,168] have been able to isolate Z,Z-DIES (isodienestrol, β-dienestrol) with a yield of 17 to 33% from a solution of DES in 0.02 *N* NaOH through which oxygen is bubbled periodically over a period of 2 weeks. From more dilute DES solutions in NaOH, small amounts of three additional products have been isolated, two of which could be identified as 4,4′-dihydroxy-benzil and 4-hydroxy benzoic acid (Figure 8).

*2. Intermediates of Oxidative Pathways*

The intermediates of the metabolic routes of DES (Figure 5) are of interest primarily because they may constitute reactive compounds implicated in the toxic effects of DES (see Section IV). To date, however, only scant information is available on their chemical nature, and some of the intermediates shown in Figure 5 are still speculative.

This applies particularly to the aromatic hydroxylation pathway of DES. No data appear to exist in support of either an arene oxide intermediate or, alternatively, a direct hydroxylation reaction. Although arene oxide formation is generally considered as the mechanism of aromatic hydroxylation, some cases of direct oxygen insertion are known.

Another interesting question is the mechanism of DIES formation from DES (Figure 5). It has previously been proposed that this reaction might proceed through an epoxide of the olefinic double bond of DES, followed by hydrolysis to the respective diol and loss of water.[111] Studies with $^{14}$C-DES-3,4-oxide, however, have cast some doubt on this sequence.[115] After i.v. and i.p. administration of this hypothetical intermediate to female mice, analysis of the glucuronide fraction from the 24-hr urine, which contains approximately 20% of the dosed radioactivity, has failed to give any indication for DIES under conditions where 0.1% of the dose should have easily been detected. Instead, HPP has been found in this fraction and, even more pronounced, in the sulfate fraction, together with 3,3-di(*p*-hydroxyphenyl)-hexan-4-one, a compound arising from the epoxide through chemical rearrangement. Also, no DIES is found under the same conditions when 3,4-dihydroxy-hexestrol is given to intact female mice.

Therefore, it presently appears more likely that DIES formation proceeds through another intermediate, the DES-4′,4″-quinone (Figure 5). This compound has been proposed in 1962 by Liao and Williams-Ashman[94] as an intermediate of the oxidation of DES by horseradish peroxidase and $H_2O_2$. It can be chemically synthesized, e.g., through oxidation of DES with lead tetraacetate,[36] and tautomerizes easily to Z,Z-DIES. None of the other isomers of DIES are formed in significant amounts upon the tautomerization of DES-4′,4″-quinone, nor in the peroxidase-mediated oxidation of DES in vitro.[117] This specificity of formation of a particular isomer strongly recommends the quinone as the intermediate in DIES formation in vivo. Peroxidases are found in several mammalian organs, particularly in estrogen target organs.[98,99] Moreover, it is known that some other hemoproteins, such as hemoglobin and cytochrome P-450, have peroxidase activity.[139] The contribution of different peroxidatic enzymes to the amount of DIES formed in vivo remains to be clarified.

Another question not yet completely settled is the origin of HPP found in rat urine in vivo.[112] A probable precursor of that metabolite is the DES-3,4-oxide, which has been shown (see above) to be metabolically unstable and is excreted in high yields as HPP when administered to intact mice.[115] Since, however, HPP was also found as a product of bacterial metabolism of DES,[5] and DES is known to undergo an extensive enterohepatic circulation in the rat (II.A.2.), the HPP found as in vivo metabolite in the rat might well be the product of intestinal bacteria. Additional studies, including in vitro studies with rat liver fractions, are necessary to clarify this point.

*3. Induction of Oxidative Metabolism*

A few earlier reports indicated that the metabolism of DES in rodents can be influenced by pretreating the animals either with known inducers of hepatic microsomal enzymes, such as phenobarbital, or with DES itself. As early as 1943, Zondek et al.[180] reported on an "attempt to train liver to inactivate DES". For this purpose, adult female rats were injected daily with 1 mg DES for 10 days, and the livers were tested 1 week later for their capacity to inactivate DES in vitro, i.e., to decrease the estrogenic activity in a bioassay with spayed female mice. It was found that liver homogenate from animals pretreated with DES inactivated DES more effectively than liver from untreated rats. Of course, it was not determined at that time as to whether the inactivation of DES is due to an increase in oxidative metabolism or to glucuronidation. Later, Levin et al.[92] found that pretreatment of immature female rats with phenobarbital for 4 days inhibited the uterotropic action of DES. Microsomes prepared from the livers of pretreated animals were found to metabolize DES in vitro in the presence of a NADPH-generating system to a greater extent than control microsomes, whereas glucuronidation of DES in vitro was not enhanced. Thus the decrease in uterotropic potency of DES in phenobarbital-treated rats was due to an induction of oxidative DES metabolism. Similarly, a decrese in the uterine wet weight (reduced uterotropic activity) and increase in liver weight (enzyme induction) was found in adult female mice treated with phenobarbital and physiological doses of DES as compared to animals receiving only DES.[37]

That DES had a potent inducing effect on hepatic monooxygenase activity in the rat was reported only recently in an abstract by Allaben and Gass.[2] DES was fed at 250 ppb for 1, 2, 4, and 8 weeks, and the hepatic monooxygenase activity measured by *O*-demethylation of *p*-nitroanisole and hydroxylation of aniline and benzo(*a*)pyrene. These enzyme activities were already significantly increased (50 to 150%) after the first week, and a corresponding increase was noted in microsomal protein content. Interestingly, estradiol-17$\beta$, estrone, progesterone, and testosterone did not significantly alter hepatic monooxygenase activity and microsomal protein content, even when fed at 1000 ppb.

## C. Species Differences in DES Metabolism

The fate of DES has probably been studied in more different species than any other compound. This is due to the fact that it has been used as a model compound for investigating species differences in the biliary excretion of foreign compounds;[1] it is used both in medicine and in agriculture; and, more recently, its unique toxicological properties have evoked interest in its disposition in susceptible and nonsusceptible animals. This has led to a considerable amount of data concerning both the routes and rates of excretion and the formation of conjugated and oxidative metabolites.

*1. Absorption, Distribution, and Excretion of DES and its Metabolites*

For pharmacokinetic studies of DES, methods must be available for its quantitative determination. Aside from the use of radioactively labeled DES, colorimetric methods, assays measuring estrogenic activity, chromatographic procedures, and radioimmunoassay can be used for the determination of DES and its metabolites.

The two most frequently used colorimetric reactions were the Dingemanse reaction, which is based on the development of a red color by antimony pentachloride,[25,26,163] and the Malpress reaction, which measures the yellow color obtained by nitration followed by neutralization.[100,101] Examples for the use of the hormonal assay, which is based on the uterine weight response of immature mice, are the determinations of DES in the excretions of cattle[110,157] and sheep[157,158] and also in the edible tissue of steers.[166]

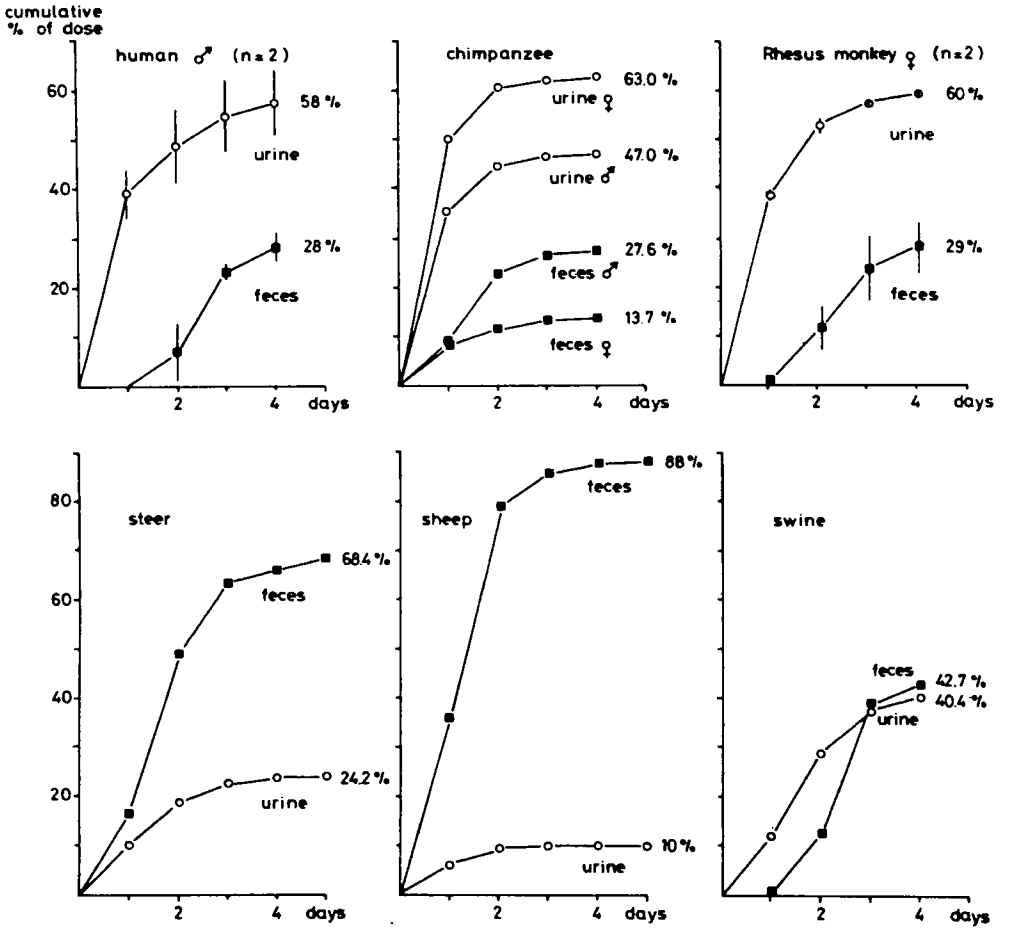



FIGURE 9A.

Several chromatographic procedures have been reported which have a sensitivity comparable to the hormonal assay (about 1 ppb). Gas chromatographic methods for the determination of DES by measuring its trifluoroacetate or dichloroacetate[33] have so far been mostly used for demonstrating the absence or presence of DES residues in animal tissues. The specificity of the latter method was further increased by using gas chromatography-mass spectrometry techniques.[23] A fluorometric procedure based on the conversion of DES to a phenanthrene derivative by irradiation with UV light, followed by thin-layer chromatography for confirmation, has also been proposed.[137] Very recently, the development of a radioimmunoassay has been reported,[90] which allows the detection of DES in muscular tissues at a sensitivity of 0.1 ppb.

For studies on the excretion of DES, these methods are only of limited value. Both the colorimetric methods and the hormonal assay are not specific for DES-related compounds and, moreover, have different sensitivity towards various DES metabolites (see Section III.A.) This was, for example, demonstrated in chickens,[71] where 74% of the radioactivity was recovered in the feces-urine, but only 35% of the expected hormonal activity was found in the excreta. Chromatographic procedures, on the other hand, are specific for unchanged DES and, consequently, the oxidative metabolites of DES will be missed when these methods are used. Reliable quantitative data on the excretion of DES and its metabolites, therefore, have only been obtained with radioactively labeled DES.[62,63,164]

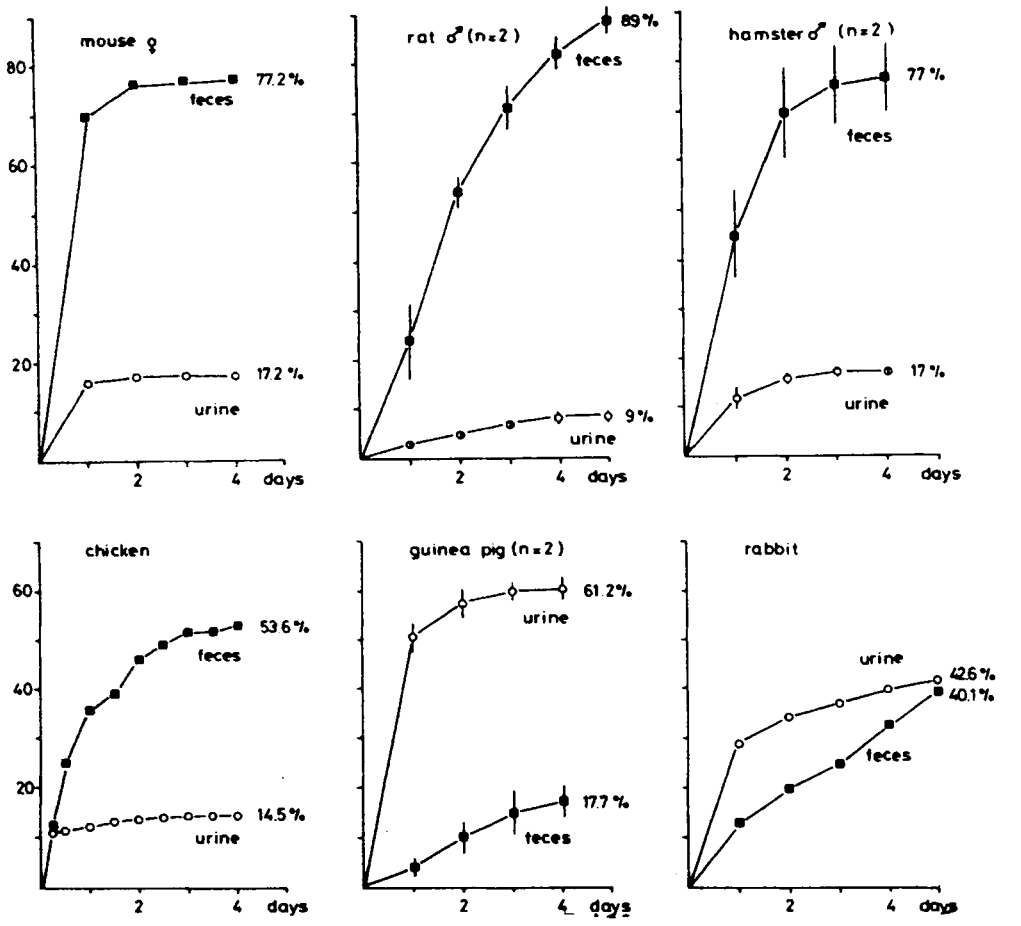



FIGURE 9B.

FIGURE 9 (A and B). Cumulative excretion of total radioactivity in urine and feces of different species after a single dose of radioactively labeled DES.[4,5,7,112,121,138,164,181]

Excretion of radioactivity in the urine and feces after the administration of a single dose of radio-labeled DES has been determined in man,[46,112] chimpanzees,[66,121] rhesus monkeys,[61,121,128,129] steers,[7] sheep,[4] swine,[5] chickens,[138] rabbits,[181] guinea pigs,[181,182] rats,[44,111,112] hamsters,[111,112] and mice.[65,146,164] The excretion curves for these twelve species are shown in Figure 9. It is apparent that considerable differences exist. Whereas urinary excretion of DES and its metabolites predominates in man, chimpanzees, rhesus monkeys, and guinea pigs, the other species studied so far, including the rat, hamster, and mouse which are more commonly used in laboratory research, prefer the fecal route of excretion. In swine and rabbits, renal and fecal excretion are about the same. There is a slight discrepancy in the data for the excretion of DES by humans, since Fischer et al.[46] have recovered only 40% in urine and 50 to 55% in the feces, whereas Metzler[112] has found 55 to 65% in urine and some 30% in feces. However, this difference may be due to the large difference in the dose employed (3.7 ng/kg vs. 0.5 mg/kg). For the data given in Figure 9, the dose of DES is in the same order of magnitude.

The reason for the species difference in urinary vs. fecal excretion of DES appears to reside in the capability of different species for biliary excretion. All data available to date suggest that DES and its metabolites are rapidly conjugated in the intestinal

wall and liver and are excreted into the bile mainly as glucuronides (see Section II.A.1.). There is, however, a species-dependent threshold for the molecular weight which must be exceeded for good biliary excretion. Abou-el-Makarem et al.[1] have determined the amount of radioactivity excreted into the 3-hr bile of different species after i.v. administration of 10 mg/kg $^{14}$C-DES-monoglucuronide and have found 95% of the dose for the rat, 93% for the hen, 77% for the cat, 65% for the dog, 32% for the rabbit, and 20% for the guinea pig. Thus biliary excretion of glucuronidated DES is very efficient in the rat and hen, and these species, therefore, prefer the fecal route of excretion (Figure 9). On the other hand, the rabbit and the guinea pig, which are poor biliary excretors of DESG, have to utilize the renal elimination. Similarly, in the rhesus monkey, only 23 to 37% of an i.v. dose of $^{14}$C-DES is found in the 24-hr bile.[129] For ruminants, biliary secretion of DES has not been quantitated; however, the high amounts of radioactivity in the feces (Figure 9) after an oral dose of $^{14}$C-DES suggest that ruminants are good biliary excretors. Based on the above data for the biliary excretion of DESG, the dog and cat can be expected to prefer fecal excretion of DES over renal elimination.

Data on the plasma and tissue levels of radio-labeled DES are also available for different species. A comparison of these studies, however, is seriously hampered by the different modes of administration of the compound and large differences in the doses employed.

In male humans, the plasma levels reach a peak concentration between 20 and 40 min after ingestion of DES, indicating a rapid absorption from the proximal portion of the gastrointestinal tract.[46] After an initial rapid decrease, the plasma concentration of radioactivity levels off after 3 to 6 hr, and a half-life of 2 to 3 days can be estimated for the later phase of excretion.[112]

In an older study,[39] $^{14}$C-DES diphosphate has been administered i.v. by injection or drip infusion to male patients suffering from cancer of the prostate. The plasma level of the administered compound and of free and conjugated have been determined, as well as the distribution of free DES in several tissues. It was found that the phosphorylated DES is rapidly hydrolyzed and vanishes from the blood within 3 hr. After 6 hr, plasma levels of free DES were very low, and the conjugated form (the nature of which was not stated) accounted for over 96% of the circulating drug. When the concentration of free DES in prostatic tissue was determined at 0.5, 2, and 4 hr after injection of the DES diphosphate, the peak concentration was noted at 0.5 hr. Only at this time was the concentration of free DES in the prostate higher by a factor of 5 to 7 than in muscle and fat, whereas no such difference was seen at 2 and 4 hr. After i.v. drip infusion, prostatic tissue did not contain a higher level of free DES than muscle tissue after 4 to 5 hr and was even lower than body fat.

In sheep, the concentration of radioactivity in plasma after oral administration of $^{14}$C-DES reaches its maximum level within 16 hr, and then declines with an approximate half-life of 16 to 20 hr during the next two days.[4] Similarly, in steers, a maximum plasma concentration of $^{14}$C around 12 hr after oral dosing of $^{14}$C-DES was observed, followed by a rapid decline to about 1/30 of the peak concentration after 4 days.[7]

After i.v. administration of radioactive DES, plasma levels have also been determined for the bile-duct cannulated rhesus monkey[129] and the intact pregnant mouse.[146] In the latter, disappearance of the radioactivity from the plasma is very rapid during the first 30 min (with half-lives of 4.4 sec, 1.1 min and 12.9 min), but becomes very slow (half-life 13.6 hr) after 1 hr.[146] A special situation prevails when DES is continuously fed or implanted as a pellet. This is particularly relevant for meat-producing animals treated with DES to promote growth or fattening. Studies on the plasma level and tissue residues under these conditions of DES exposure have been conducted with steers[8,61,142-144] and chickens[72] and are reviewed, in part, by Aschbacher.[5]

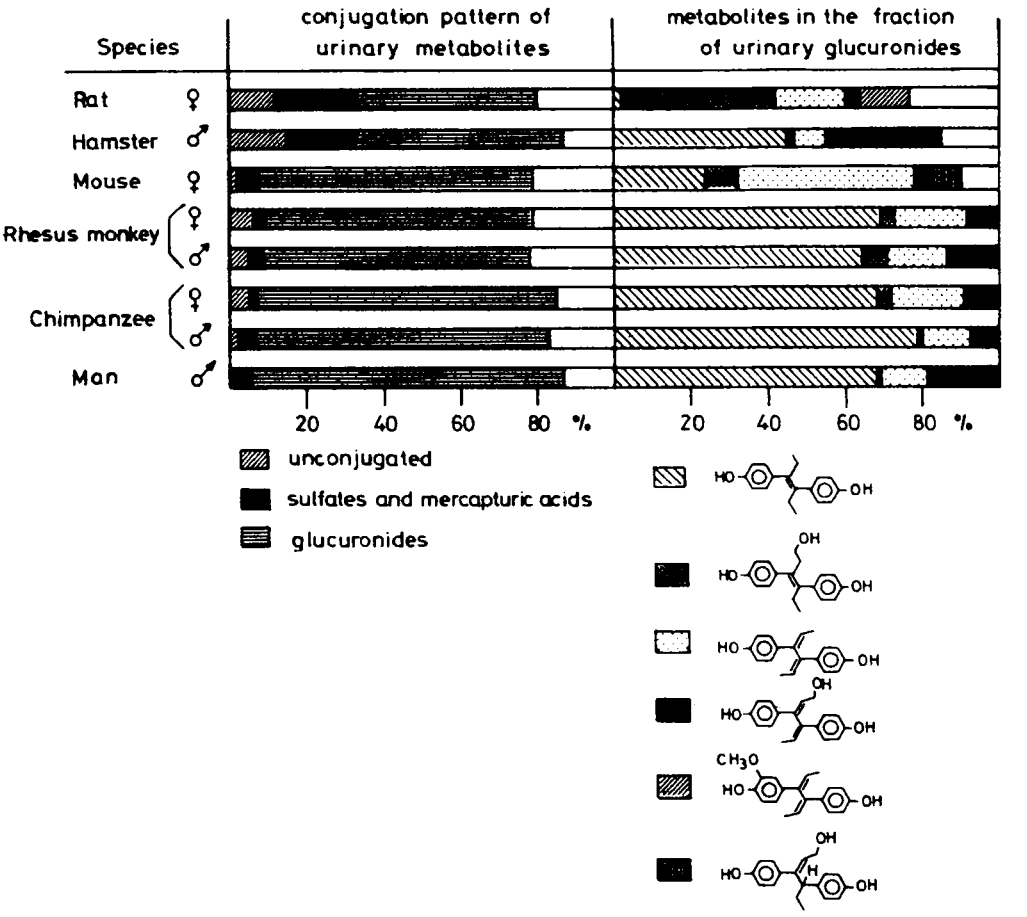



FIGURE 10. Species differences in DES conjugation and oxidative metabolism, as reflected by the pattern of conjugated metabolites in urine (left) and the composition of the glucuronide fraction from urine (right).[111,112,115,123,181]

*2. Species Differences in Conjugative and Oxidative Metabolism of DES*

It has been repeatedly mentioned that the major conjugates excreted in vivo after administration of DES are glucuronides. This is also apparent from Figure 10, where the patterns of conjugates found in the urine in different species are compared. All data are obtained from the same laboratory, utilizing chromatography on neutral alumina for the separation of conjugates. This involves subsequent elution of unconjugated metabolites, sulfates, and glucuronides with 95% ethanol, water, and a phosphate/citrate buffer pH 6, respectively. In control chromatographies with DES and its synthetic mono- and disulfates[9] and glucuronides,[85] a complete separation is obtained.[55]

Figure 10 shows that glucuronides of DES and its metabolites are particularly prevalent in the urine of man and the nonhuman primates, whereas significant amounts of sulfates are found in rat and hamster urine. Recent studies with $^{14}$C-DES given orally to guinea pigs and rabbits[181] show that these species also excrete DES predominantly as glucuronide (76 and 68%, respectively, of the urinary radioactivity). Similarly, more than 85% of the urinary radioactivity after an oral dose of $^{14}$C-DES to steers is found to be glucuronides.[7] When radio-labeled DES is injected into chicken with an exteriorized ureter, 58% of the radioactivity excreted in urine is glucuronides.[138]

Similar or even higher amounts of glucuronides than in the urine appear to be excreted in bile, as indicated by studies with rhesus monkeys,[128,129] rats,[44,112] and hamsters.[112] For chicken dosed with $^{14}$C-DES, Shimoda[149] has found that 73% of the biliary radioactivity is water soluble, but becomes ether extractable after enzymic hydrolysis with β-glucuronidase, while Quamme et al.,[138] based on chromatographic methods, claim that in chicken bile DES monosulfate exceeds the glucuronide (52 vs. 19%). This discrepancy, which may be due to methodological differences, remains to be clarified.

Some pronounced species differences among rodents are observed in the pattern of oxidative DES metabolites excreted as urinary glucuronides (Figure 10). The most striking examples are the rat, where practically no unchanged DES is found as glucuronide in the urine, and the mouse, which excretes significant amounts of 1-hydroxy-ψ-DES, a metabolite of only minor importance in other species. In primates, hamsters, and also the guinea pig (data not shown), DES predominates, and small amounts of Z,Z-DIES together with 1-hydroxy-Z,Z-DIES are invariably found.

Data on the pattern of oxidative metabolites in the urine of other species, such as ruminants and chicken are not yet known. The same applies for biliary and fecal metabolites of most species with the exception of the rat and hamster, where several ring-hydroxylated and methoxylated products have been demonstrated in the bile.[111,112] In the feces of humans and rhesus monkeys, unconjugated DES accounted for virtually all the radioactivity and no oxidative metabolites are found in the studies of Metzler[112] and Metzler et al.[121] Recently however, Helton et al.[66] have obtained evidence for DIES, and additional, as yet unidentified metabolites in rhesus monkey feces. In the feces of mice, only DES is found.[65] In the feces of steers given $^{14}$C-DES, 64% of the radioactivity is DES, and 23% is identified as 3-(*p*-hydroxyphenyl)-2-hexen-4-one (Figure 8), which probably arises through the bacterial metabolism of DES.[6]

Virtually nothing is known about oxidative DES metabolites present in the blood except in the case of the mouse, where DES and Z,Z-DIES are found in a ratio of about 1:1 in the glucuronide fraction of plasma taken 3 hr after oral administration of $^{14}$C-DES.[115]

It is apparent that the data on the oxidative metabolites of DES in blood, bile, and feces are too scant for demonstrating species differences.

Interesting effects caused by the dose and by the route of administration on DES metabolism in the rat have been observed by Neumann.[131,132] The pattern of biliary metabolites is determined over a dose range from 0.1 to 10 mg $^{3}$H-DES (per 200 g rat). While glucuronides constitute 70 to 90% of the biliary radioactivity for all doses, a change in the nature of the oxidative metabolites present in the glucuronide fraction is noted.[131] At lower doses, nonpolar metabolites such as DES, DIES, 3′-methoxy-DES and 3′,3″-dimethoxy-DES prevail, but at higher doses, the percentage of the polar, as yet unidentified metabolites increases at the expense of the nonpolar products.

The route of administration also affects DES metabolism in the rat, and different patterns of metabolites are found in the blood after oral, i.v., and i.p. administration of $^{3}$H-DES.[132] For example, unconjugated polar metabolites predominate after oral uptake of DES, unconjugated nonpolar metabolites after i.v. injection, and glucuronides of nonpolar metabolites after i.p. administration. These differences in plasma metabolites are explained by intestinal metabolism of DES and a liver first-pass effect.

### D. Placental Transfer and Perinatal Metabolism of DES

Perinatal exposure to DES has been associated with teratogenic and carcinogenic alterations of the genital tract in humans. Some of these lesions can be induced in the mouse and hamster by treatment with DES shortly before or after birth (for review, see Reference 106). In order to understand the susceptibility to DES toxicity in the perinatal period, it is important to know the disposition of the compound by the fetal

and neonatal organism. This includes both the passage of DES or its maternal metabolites into the fetus and the capability of the fetal and neonatal animal to metabolize DES.

The first observations of a placental transfer of DES were obtained for the mouse. By using $^{14}$C-DES and whole-body autoradiography, Bengtson and Ullberg[10] found that radioactivity reached the mouse fetus and that concentrations in the fetus were highest in the liver, gall bladder, and small intestine. Interestingly, the amount of radioactivity passing into the mouse fetus was much lower after DES than after estradiol-17$\beta$ and estrone, which indicated a partial placental barrier for DES or its metabolites. In agreement with this hypothesis, Shah and McLachlan[146] found that after i.v. injection of $^{3}$H-DES into near-term pregnant mice, 10 to 30 min was required to reach a maximum of radioactivity in the fetus, while in the maternal organs liver, muscle, and uterus, peak concentrations of radioactivity were observed after 1 to 5 min. Concentrations in fetal organs after 30 min were highest in liver, followed by intestine and plasma. Most interestingly, the fetal reproductive tract contained radioactivity levels similar to that of fetal plasma, indicating that DES indeed reached its target organ for the toxic effect. Separation of this radioactivity into unconjugated and conjugated material revealed that the reproductive tract had a threefold higher concentration of unconjugated radioactivity as compared to fetal plasma. Conjugated material was found in all fetal organs studied, including fetal plasma, and was particularly high in the fetal liver with a ratio of conjugated to unconjugated radioactivity of about ten, while in the placenta, conjugates were relatively low and were exceeded by unconjugated material during the first 4 hr. This may indicate that conjugates were formed in the fetus. Indeed, a significant level of UDP glucuronyl transferase activity (approximately one tenth of the maternal liver value) was found with DES as a substrate in the fetal liver, while activities were about one order of magnitude lower in the fetal intestine, placenta, and maternal uterus.[146]

In vivo studies with neonatal mice[115] confirmed the ability of the mouse to glucuronidate DES early in life. $^{14}$C-DES was injected i.p. at a dose of 10 $\mu$g/g into neonatal mice 1, 4, and 8 days old, a whole-body-homogenate prepared after 2 hr, and the extracted radioactivity analyzed for unconjugated radioactivity, glucuronides, and other conjugates. Even in 1-day-old mice, only one third of the injected dose was recovered unconjugated after 2 hr, and one third was glucuronidated, while the remaining third consisted of other, as yet unidentified conjugates. In the older neonates, the amount of these unknown conjugates increased at the expense of unconjugated and glucuronidated radioactivity.

The glucuronide fraction from neonatal mice was further analyzed after enzymic hydrolysis by radio-TLC and GLC-mass fragmentography. In addition to DES, the three oxidative metabolites Z,Z-DIES, 1-hydroxy-DES, and 1-hydroxy-$\psi$-DES (for formulas see Figure 5) were identified. This clearly indicated the capability of the neonatal mouse for the oxidative metabolism of DES in addition to conjugation reactions. Similar oxidative metabolites were also observed by autoradiography in the glucuronide fraction from near-term mouse fetuses after administration of $^{14}$C-DES to the mother.[115]

In the rat, maternal-fetal transfer of DES and fetal metabolism were investigated by Fischer et al.[46] and Miller et al.[124,125] In Fischer's study, $^{14}$C-DES (0.2 mg/kg) was injected i.v. into pregnant rats on the 13th or 20th day of gestation and the radioactivity determined after 10, 30, 60, and 90 min in the maternal liver, brain, plasma and also in the placenta and whole fetus. In addition, radioactivity was extracted from these tissues and separated into unconjugated and conjugated (probably glucuronidated) material. The maximum concentration of total radioactivity was always found at the first time point (10 min), and no difference in the distribution and decline of

unconjugated radioactivity in maternal tissues in middle and late gestation was observed. The rate of decline of total radioactivity was similar in all tissues except for the fetus, where the decline was slower. Since the radioactivity in the fetus was only about one fourth to one half of the concentration found in the maternal plasma or placenta, it was concluded that rats had a partial placental barrier for DES similar to that observed in the mouse. Interestingly, the 13-day fetus, in contrast to the maternal plasma and placenta, contained only unconjugated material, whereas the 20-day fetus also contained conjugated radioactivity, the relative amount of which increased with time. This indicated that the placenta in middle gestation did not permit conjugates of DES to penetrate, and the 13-day fetus was not able to conjugate DES, whereas the near-term fetus had conjugating capability (see below) or/and the placenta became permeable to maternal DES conjugates in late gestation. Analysis of the unconjugated radioactivity of day-13 fetuses by reverse-isotope-dilution with DES showed that compounds other than DES were also present, the amount of which increased with time (from 44% at 10 min to 72% at 90 min). Whether these oxidative in vivo metabolites, which were not further identified were formed in the fetus or transferred from the mother remains to be clarified.

The placental transfer of $^{14}$C-labeled DES after chronic infusion into near-term pregnant rats or direct injection into 20-day fetuses was studied by Miller et al.[124] After 3 hr of infusion, the maternal plasma levels of radioactivity were constant. Surprisingly, the concentration of radioactivity in the fetal plasma at that time was 2.7 times that of maternal plasma. An even higher concentration of radioactivity was observed in the fetal ovary, testes, and uterus (by a factor of 15 to 20 as compared to maternal plasma) and in the chorioallantoic placenta and the visceral yolk sac (by a factor of 4 to 5). When DES was injected into the fetuses, the radioactivity rapidly appeared in the maternal plasma, indicating that the fetal-maternal transfer was also operative. Analysis by TLC of maternal plasma, fetal plasma, fetal reproductive tissue, placenta, and yolk sac showed that both DES and glucuronides were present. In the fetal reproductive tissue, only 30% of the radioactivity was associated with DES, 25% was glucuronides, and 45% was unidentified products, considered to be oxidative DES metabolites.

A few in vitro studies demonstrated the capability of fetal rats to oxidize and glucuronidate DES. Using a slice technique, the metabolism of $^{14}$C-DES by fetal liver, chorioplacenta and visceral yolk sac was investigated and compared to that in maternal liver.[125] After 2 hr of incubation at 37°C, all tissues concentrated the radioactivity when compared to the medium. According to TLC and high-pressure liquid chromatography (HPLC) analysis, less than 4% of the $^{14}$C in the maternal and fetal liver was unchanged DES, and more than 85% were unconjugated metabolites. About 10% of the radioactivity was bound nonextractably to macromolecular material precipitated with trichloroacetic acid. The chorioplacenta and visceral yolk sac obviously had a lower capacity to oxidize DES, since more than 90 and 54%, respectively, of the $^{14}$C in these tissues after incubation was associated with unchanged DES.

In an in vitro study carried out by Lucier and Shah,[97] it was found that hepatic glucuronidation of DES in the rat fetus took place on the 16th day of gestation and around parturition, but could not be detected on the 5th or 10th day of gestation. The perinatal development of rat liver glucuronyl transferase activity was the same for DES and the natural estrogen, estradiol-17$\beta$, but differed from the transferase activity for testosterone and the xenobiotics p-nitrophenol and 1-naphthol.

The ontogenic development of glucuronide conjugation and oxidative metabolism of DES in the neonatal rat was investigated by Fischer and Weissinger.[45] As a measure for the conjugative capability, the decline of unconjugated material was determined in the whole body of rats of different age after injection of a 17$\mu$g/kg dose of $^{14}$C-

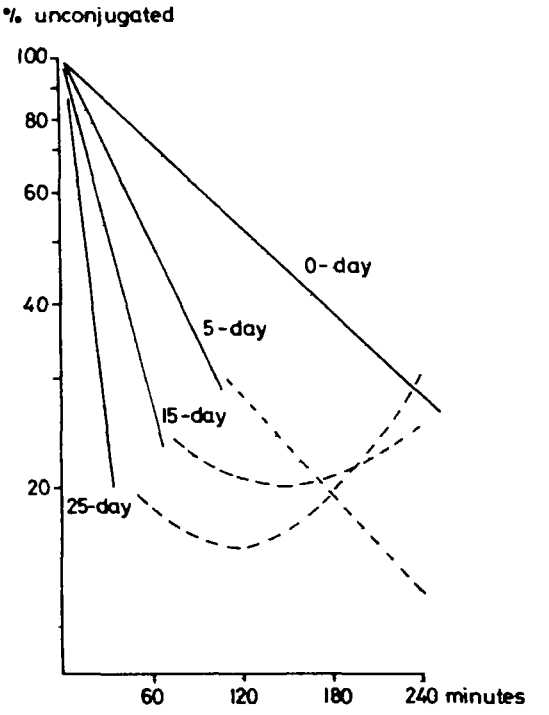



FIGURE 11. Age-dependent conjugation of DES in the rat. Solid lines represent unconjugated material found in whole-body homogenates of rats of different age (newborn, 5, 15, 25 days old) at various times after injection of a certain dose of $^{14}$C-DES. The steeper decline in older rats reflects the increased capacity for conjugation of DES. Broken lines indicate the reappearance of unconjugated material due to intestinal hydrolysis of conjugates. (Adapted from Fischer, L. J. and Weissinger, J. L., *Xenobiotica*, 2, 399, 1972.)

DES (Figure 11). The unconjugated radioactivity declined with a half-life of 131, 55, 53, and 14 min in animals on the day of birth and at age 5, 15, and 25 days, respectively. No difference was observed between 25- and 50-day-old rats. In the older animals (from the 15th day on) the unconjugated radioactive material in the body extract increased again after about 60 min (dashed lines in Figure 11), and it was found that this was caused by the intestinal hydrolysis of biliary excreted glucuronide conjugates. The deconjugative activity in the rat reached maturity at the same time as glucuronidation, i.e., between the 15th and 25th day after birth.

Evidence for an age-related increase in the capacity for oxidative metabolism of DES was also obtained in this study. Reverse-isotope-dilution analysis of the unconjugated radioactive material revealed that in newborn rats, after 4 hr, 88% was unchanged DES, whereas in 25-day-old animals, this value decreased to 38%. This suggests that oxidative metabolism of DES is low at birth and develops with increasing age. It is not known at what age the adult levels are reached.

The fate of DES was also studied in the fetus of the Syrian golden hamster.[56] Six hours after oral and i.p. administration of $^{14}$C-DES to pregnant near-term hamsters, 0.07% and 0.5 to 3% of the dose, respectively, were found in the fetuses; only 30 to 40% of the fetal radioactivity was unconjugated. In order to minimize the influence of maternal metabolism and to recover amounts of fetal metabolites sufficient for

identification, $^{14}$C-DES was also injected directly into the fetuses through the uterine wall after opening the peritoneal cavity. Six hours after intrafetal injection, 88% of the administered radioactivity was still present in the fetuses, while 5% was found in the placentae and 6% in the maternal liver. The metabolites were extracted from whole-body homogenates of the fetuses and separated by HPLC.[54] Z,Z-DIES indenestrol A, 1-hydroxy-DES, 1-hydroxy-$\psi$-DES, 1-hydroxy-Z,Z-DIES, 3'-methoxy-DES and 3'-methoxy-DIES (for formulas see Figure 5) were identified through GLC/mass fragmentography of the fetal extracts. Since most of these metabolites appeared in larger amounts after intrafetal injection of DES than after i.p. injection of the same dose into the mother, it was likely that they were formed in the fetal rather than in the maternal organism.

The only data available on the placental transfer and perinatal metabolism of DES in primates appear to stem from the rhesus monkey. In a study reported in an abstract, Slikker et al.[150] determined the placental passage of radio-labeled DES, DES-glucuronide, and estradiol-17$\beta$ from the mother to the fetus. After simultaneous i.v. injection of $^{14}$C-DES and $^{3}$H-estradiol-17$\beta$, the $^{3}$H was eliminated from maternal plasma and accumulated in the urine more rapidly than the $^{14}$C. The fetal plasma levels of both radioisotopes reached a maximum after 10 to 20 min, and thereafter remained higher than maternal plasma levels. Water-soluble metabolites, probably conjugates, of DES, which could not be extracted with ether, were found in fetal and maternal plasma. When DES-glucuronide was administered i.v. to the mother, it was rapidly eliminated from maternal plasma, and no significant transfer into the fetus could be observed. From these results it was concluded that DES readily crossed the placenta and was converted to water-soluble metabolites in both the fetal and maternal organism.

Indications for fetal conjugation and oxidative metabolism of DES in the rhesus monkey was also obtained in experiments of Metzler et al.[121] Radio-labeled DES was injected into the fetoplacental unit *in situ*, on the 80th and 115th day of gestation, and the extracts from placenta and fetal liver and plasma were separated into unconjugated radioactivity and glucuronides. After 15 min, unconjugated radioactivity predominated in the placenta. On the other hand, considerable amounts of glucuronides, sometimes exceeding the unconjugated material, were found in fetal liver and plasma, especially in the older fetus. The finding that the ratio of glucuronide to unconjugated radioactivity was highest in the fetal liver and lowest in the placenta suggested that the fetal liver was the site of glucuronide formation.

The unconjugated and glucuronidated material was further analyzed by radio-GLC, and DES was found to be the major compound present in all samples. However, several small peaks were seen in the radio gas chromatogram, indicating the presence of oxidative metabolites, but none of them could be unambiguously identified.

When $^{14}$C-DES was administered orally to a 1-day-old female rhesus monkey, 4% of the dose was recovered from the 5 hr urine, and 78% of the urinary radioactivity was found to be associated with the glucuronide fraction.[121]

## III. BIOLOGICAL ACTIVITY OF DES AND ITS METABOLITES

The biotransformation of DES to a variety of metabolites raises the question as to whether all of the biological effects of DES are caused by the parent compound or may be due to different metabolites. Although this question has already been asked as early as 1962 by Gawienowski et al.,[51] data are only recently becoming available on the affinity of DES metabolites to the estrogen receptor and other estrogen binding proteins, and on the chemical reactivity of metabolic intermediates, which is of particular interest for the toxicity of DES.

### A. Estrogenic Activity

DES has repeatedly been shown to have an intrinsic estrogenicity about equal to that of estradiol-17$\beta$, i.e., the affinity to the estrogen receptor of target tissues is about the same for both compounds (for review see Reference 106). The estrogenicity of DES in vivo, determined by the immature mouse uterine weight assay, is at least two times higher for the trans-isomer (E-DES, see Figure 5) than for the cis-form (Z-DES), and the estrogenic activity retained in the cis-DES in vivo probably has to be attributed for the most part to the trans-isomer arising through facile isomerization.[176]

It was recognized quite early that metabolization of DES led to a decrease or loss of estrogenic activity. For a long period of time, conjugation with glucuronic acid or sulfate was considered to be the only pathways operative in DES metabolism since these two conjugates were the first tested for estrogenicity. Simpson and Wilder-Smith,[147] by using the cornified vaginal smear assay, found that DES was about 17 times more active than its monoglucuronide after subcutaneous injection into ovariectomized rats. Similarly, Umberger[165] reported that DESG, upon s.c. administration to weanling rats and mice, had about one seventh and one fourth, respectively, of the estrogenicity of DES in these two species, as measured by the uterine weight response. After oral administration, however, the estrogenicity of DESG was about two thirds that of DES in the rat and one half in the mouse. The higher estrogenicity of DESG after oral as compared to parenteral administration was probably due to the increased release of DES from the conjugate through intestinal hydrolysis (see II.A.2.). Bishop et al.[13] reported that DES-disulfate is about one half as active as an estrogen as DES in man and in the rat after oral administration. Again, this high residual activity was most likely due to DES liberated through hydrolysis of the sulfate in vivo. So far, no in vitro data on the receptor affinity of DES glucuronides and sulfates appear to exist.

Following the identification of oxidative DES metabolites (see II.B.1.) those metabolites available as synthetic substances were tested for estrogenicity in vivo and in vitro by using the immature mouse uterine weight bioassay and the mouse uterine cytoplasmic receptor binding assay, respectively.[82] There was a good correlation in the ranking of compounds from in vivo experiments and in vitro receptor binding studies. The important DES metabolite Z,Z-DIES had no significant estrogenicity, in contrast to its isomer, E,E-DIES, which is, however, not formed metabolically (see II.B.1.). Also, the metabolites 1-hydroxy-Z,Z-DIES and 4′-hydroxypropiophenone were void of estrogenic activity.[183] On the other hand, the suspected metabolic intermediate E-DES-3,4-oxide (see Figure 5) has retained a considerable estrogenic activity, which is about one half that of E-DES in vivo and one tenth in vitro.[82] 1-Hydroxy-DES and the ring-hydroxylated and methoxylated metabolites have not yet been tested. In the ring-derivatized compounds, estrogenic activity should be low or absent, since positioning of methyl groups ortho to the phenolic hydroxyls of DES abolishes the uterotropic activity.[19]

### B. Affinity to Other Estrogen-Binding Proteins

The affinity of DES and its metabolites to proteins other than the estrogen receptor is of interest because this determines the distribution of these substances and their availability to the different compartments, including the fetus.[107] Two such proteins are the specific estrogen-carrying protein in serum ("sex steroid-binding globulin", SSBG, in humans, and "estrogen-binding protein", EBP, in rats), and the alpha-fetoprotein (AFP). AFP is a glycoprotein mainly occurring in the amniotic fluid, but is also present in the serum and uterine cytoplasm of immature rats, whereas its concentration is low or undetectable in adult rat serum and uterus, respectively.

DES, unlike estradiol-17$\beta$, appears to have no appreciable affinity to either SSBG or EBP.[130] Thus far, no data exist on the binding of DES metabolites to these proteins.

In a recent study, the affinity of DES and a few of its metabolites to AFP from mouse amniotic fluid was investigated by McLachlan et al.[107] Neither DES nor DES-3,4-oxide, Z,Z-dienestrol, and indenestrol A were able to compete with radio-labeled estradiol-17$\beta$ for binding, thus indicating that DES and the tested metabolites lacked affinity to mouse amniotic AFP. Differences in binding to AFP between DES and estradiol-17$\beta$ were previously reported.[135]

### C. Chemical Reactivity

Metabolism of radio-labeled DES both in vivo and in vitro has frequently been observed to lead to nonextractable binding of the radioactivity to macromolecules, which indicates the formation of chemically reactive metabolites of DES. For example, Krishna et al.[83] reported in an abstract that DES in vivo became covalently bound to hepatic proteins in the rat after i.p. injection. In female hamsters, 13 to 15% of the total radioactivity present in the liver 3 hr after i.p. injection of $^{14}$C-DES was bound to ethanol-precipitable material, and in female mice 13 hr after the oral administration of $^{14}$C-DES, the nonextractable label accounted for up to 57% of the total liver radioactivity.[181] Nonextractable binding of radio-labeled DES was also observed for plasma proteins of rats in vivo,[132] and for macromolecules precipitated from the whole-body homogenate of neonatal mice.[115] The amount of tissue-bound $^{14}$C 2 hr after i.p. injection of $^{14}$C-DES into mice aged 1, 4, and 8 days was 1.2, 3.8, and 5.8% of the dose, respectively. The age-related increase in binding may indicate an enhanced capability for oxidative metabolism.

After in vitro incubation of $^{14}$C-DES with chicken liver homogenate, Hopwood et al.[73] noted that only 82% of the dose was extracted with ethanol while 95% was recovered in control experiments. When $^{14}$C-DES was incubated with rat liver microsomes at a concentration of $10^{-4}$M, covalent binding of radioactivity to the microsomes was found to occur at a rate of 160 pmol/mg/min.[84] The formation of the reactive metabolites responsible for the observed binding was thought to be catalyzed by cytochrome P-450 because a NADPH-generating system was required and the binding could be blocked by carbon monoxide or SKF-525. Moreover, covalent binding was increased after pretreating rats with phenobarbital. Engel et al.,[35] by studying the in vitro metabolism of $^{14}$C-DES with rat liver homogenates and microsomes, also noted that an appreciable amount of radioactivity was bound to the washed microsomal pellet when the incubations were carried out under aerobic conditions in the presence of NADPH. In the absence of NADPH or under anaerobic conditions, only negligible amounts of radioactivity were bound to microsomes.

Binding of $^{3}$H-DES to DNA in vitro was observed after metabolic activation through microsomes from rat liver and on incubation with primary mouse fetal cells in culture.[15] The labeled DNA obtained from the microsomal incubations upon enzymic hydrolysis and chromatography on Sephadex® LH 20 gave at least eight radioactive deoxyribonucle osides.

Although these findings clearly establish the formation of reactive DES metabolites, the question remains as to which metabolites account for the binding, since thus far, the structures of the adducts have not been elucidated. From the pathways operative in the oxidative biotransformation of DES (see II.B.) several reactive intermediates are likely. These include epoxides of the aromatic ring and olefinic double bond as well as the 4′,4″-quinone and -semiquinone (Figure 5). In addition, the ortho-quinone and -semiquinone arising through further oxidation of the catechol 3′-hydroxy-DES, and the ester conjugates of the allylic alcohols 1-hydroxy-DIES and 1-hydroxy-$\psi$-DES must be taken into consideration. To date, only a few data are available on the reactivity of these metabolites, primarily because the compounds have not yet been synthesized in pure form.

As a model compound for an ester conjugate of 1-hydroxy-DIES, its acetate has been tested for alkylating potential in the 4-(*p*-nitrobenzyl)pyridine test.[114] A positive reaction has been obtained and is shown to be due to the allylic ester structure. Synthetic E-DES-3,4-oxide has failed to give a positive result in this test, but this is most likely due to the high tendency of the epoxide to rearrange to 3,3-di(*p*-hydroxyphenyl)-hexan-4-one. Other stilbene oxides which do not rearrange that easily, such as 4-acetaminostilbene oxide, are clearly positive in this test.[133]

A way to rather selectively produce the 4′,4″-semiquinone and -quinone of DES is through oxidation of DES with peroxidase/hydrogen peroxide (see II.B.2.). When $^{14}$C-DES is incubated in a system containing peroxidase from horseradish or mouse uterus and albumin or DNA, nonextractable binding of radioactivity to these macromolecules is found.[117,120] Binding does not occur when the enzyme or hydrogen peroxide is omitted. These findings imply that the 4′,4″-quinone and/or semiquinone are reactive compounds. The DES-quinone may also be the binding species in the studies of Blackburn et al.,[14] who have observed binding to DNA when DES is treated with iodine. It has been concluded from kinetic data that the binding is the result of a net 2-electron oxidation of DES, rather than of its iodination. Oxidation of DES with iodine, on the other hand, most likely leads to the 4′,4″-quinone as an intermediate, since its tautomerization product, Z,Z-DIES, (see II.B.2.) is found as a major compound under the conditions used by Blackburn et al.[14,181]

It may be of interest in this context to mention that the catechols 2-hydroxy-estradiol-17$\beta$ and 2-hydroxy-17$\alpha$-ethinyl-estradiol, which are major metabolites of these steroid estrogens, are also activated by mouse uterus peroxidase to DNA-binding forms.[119]

## IV. IMPLICATION OF METABOLISM FOR THE TOXICITY OF DES

The formation of reactive metabolites through oxidative biotransformation of DES raises the question as to whether metabolic activation of DES plays a role in the toxicity of this compound. The covalent binding of DES metabolites to hepatic macromolecules has been suggested as the biochemical mechanism of the centrolobular necrosis caused by high doses (200 mg/kg) of DES in rat liver.[83,84] More recently, Metzler[111,112] and Engel et al.[35] have pointed out that reactive metabolites may also be involved in the carcinogenicity of DES, i.e., DES, after metabolic activation, might be able to transform cells to malignancy and thus initiate cancer. This quality, according to current understanding, would require an interaction with the genetic material of somatic cells leading to mutations. Although the capability of DES metabolites to bind to DNA has been clearly established (see III.C.), the genotoxic activity of DES has remained questionable until recently, because DES has been inactive in several short-term tests for mutagenicity. For example, several laboratories have tested DES employing the Salmonella/microsome test (Ames test), but did not find any mutagenicity. Moreover, eleven derivatives of DES including five metabolites have been tested under various metabolic conditions in four tester strains, but again, all tests were negative.[53] In addition, DES did not transform cells in culture.[160]

These results seemed to indicate that DES has no genotoxicity and cannot be an initiator of tumors. Recently, however, this situation has changed. DES has been found to be an inducer of unscheduled DNA synthesis in HeLa cells in the presence of liver microsomes from phenobarbital treated rats.[103] This test measures the removal of covalently bound DNA adducts through repair mechanisms and is considered a useful short-term test of chemicals for carcinogenic activity. DES is also active in a skin test system in hairless mice, behaving "as a classical carcinogen" in this test in contrast to different steroidal estrogens (estradiol, ethinylestradiol, mestranol) and $\alpha$-

dienestrol, which are all negative.[47] Very recently, DES has also been found to be a good inducer of sister chromatid exchanges (SCE), another assay correlating well with carcinogenicity.[140] The potency of DES to cause SCE is even stronger than that of the carcinogen, benzo(*a*)pyrene, but can be suppressed with α-naphthoflavone, suggesting that metabolic activation of DES is necessary for the induction of SCE. Indeed, the metabolites E-DES-3,4-oxide and Z,Z-DIES are more active than DES in this test by 10 and 70 times, respectively.

These recent studies clearly indicate that DES gains genotoxicity through metabolic activation, in analogy to those chemical carcinogens that have to be activated to electrophiles in order to be mutagenic and carcinogenic.[123] If reactive metabolites are involved in the mechanism of DES carcinogenicity, the question arises as to why DES exerts its carcinogenic effect preferentially in estrogen target organs. Three reasons could possibly account for this organotropism.[120] First, the estrogenic nature of DES may promote the formation of tumors through an increase in cell proliferation. It has been well-established that tissues are most susceptible to carcinogens when their rate of proliferation is high. Thus two qualities of DES, namely its activation to genotoxic metabolites (leading to tumor initiation) and its stimulation of cell growth (leading to tumor promotion) may be necessary for the manifestation of tumors.

The second cause for the organotropism of DES carcinogenicity may be that estrogen receptors are involved in the accumulation of reactive metabolites in the estrogen target organs, and may even facilitate the access of genotoxic metabolites to the nuclear DNA. This possibility is exemplified by E-DES-3,4-oxide, which exhibits a considerable estrogenicity both in vitro and in vivo (see III.A.) and has genotoxic potential (see III.C.).

Thirdly, it is conceivable that reactive DES metabolites are preferentially formed in estrogen target organs due to the presence of a particular enzyme. In this context, the enzyme peroxidase deserves special attention, since it has been shown that peroxidase is a marker enzyme for those tissues depending on estrogens for growth.[3,98,99] Moreover, it can be easily induced in the estrogen target organs by estrogens including DES.[17] Since peroxidase can metabolize DES to reactive forms, probably the 4′,4″-semiquinone and -quinone, capable of binding to DNA (III.C.), this enzyme may cause an organ specific bioactivation of DES.[116] Moreover, it may play a role in the fetotoxicity of DES. The target tissue for the carcinogenic and teratogenic effect of DES in the fetus is the Müllerian duct, which represents the embryonic precursor of the female upper genital tract.[106] In the mouse fetus, which is exposed to DES transplacentally, it has been demonstrated that DES reaches the Müllerian duct.[146] Presently, however, it is not known whether peroxidase is active prenatally or can be induced by DES in the Müllerian duct.

Other known reasons for the organotropism of carcinogens, such as insufficient inactivation of reactive metabolites or delayed repair of the DNA-bound adducts in the susceptible organs may also contribute to the organotropism of DES carcinogenicity, but no data are available thus far to evaluate their role.

At this time, the different mechanisms possibly contributing to the organotropic tumorigenicity of DES cannot be distinguished, even for the male hamster kidney, a nongenital-tract target organ of DES carcinogenicity. Estrogen receptors have been demonstrated in the renal adenocarcinoma of hamsters treated with DES,[93] and the male hamster kidney has been reported to contain considerable levels of peroxidase.[108]

## V. FATE OF DES AND ITS METABOLITES IN THE ENVIRONMENT

Due to its use as a growth promotant in beef cattle (for review see McMartin et al.[109]), considerable amounts of DES are released into the environment.[107] Most of this

material enters the environment in the form of excreta from the treated animals. Metabolic studies have shown that the major portion of the DES administered to cattle is excreted in the feces as unconjugated DES, and a smaller proportion in the urine, mostly as DES-glucuronide.[5] In the presence of bacteria, this conjugate can be easily hydrolyzed. Therefore, the waste from cattle must be expected to contain predominantly unchanged DES. The further fate of this compound in the environment appears to depend upon the treatment of the waste. Under aerobic conditions at ambient temperature, an extensive degradation of DES in the ruminant feces occurs, leaving less than 5% unchanged DES after 7 days.[6] On the other hand, feedlot waste stored in a concrete pit for 3 months still contained about 75% of the excreted DES.[141]

When DES-containing waste is used agriculturally as fertilizer, degradation in the soil and uptake into plants are of importance. Early studies by Gregers-Hansen[57] with $^{14}$C-DES (labeled in the methylene group) showed that some decomposition of DES occurred in the soil, as indicated by the release of $^{14}CO_2$. Over a period of 6 months, 12 to 28% of the radioactivity added to the soil as $^{14}$C-DES was converted to $^{14}CO_2$, the amount depending on the soil type and the presence of organic matter such as straw or compost. In addition, a considerable proportion (some 20 to 50%) of the radioactivity could not be extracted with benzene but only with water, and was thought to result from incorporation of radioactivity by microorganisms or from oxidation of DES. These water-soluble products were not further identified.

Gregers-Hansen was also the first to study uptake of DES into plants.[58] Pot experiments, in which $^{14}$C-DES was added to the soil, were performed on rye grass, red clover, and mushrooms. Radioactivity was found in the plants harvested during a period of 2 to 8 months, but the amounts were too small for identification. The concentration factor, i.e., the ratio of ppm DES-related material in plant dry matter to ppm in soil, was smaller than 0.2 in all cases, indicating that no concentration of DES occurred in the plants. When, however, the uptake of the DES-glucuronide by maize in a water culture was studied, the concentration of extractable radioactivity in the plants became constant after 4 days and was about 5 times higher in the green parts of the plants than in the nutrient solution. It was concluded from this study that the concentrations of DES taken up by plants from soil under practical conditions, i.e., less than 10 μg DES/kg soil, did not exceed the amount of estrogens naturally occurring in plants.

Similar findings and conclusions were reached in the study of Hacker et al.[60] Uptake of radioactivity into corn, cucumbers, and peas grown in solutions containing $^{14}$C-DES or $^{14}$C-DES-glucuronide was higher for the conjugate. For pot experiments, the excreta of a steer injected with $^{14}$C-DES was mixed with two soil types with a pH of 6.5 and 7.5, and wheat, pinto beans, lettuce, radishes, onions, and tomatoes were grown in the soil-manure mixture under greenhouse conditions. Independent from the soil type, all plant parts were found to be radioactive, the radioactivity being highest in the roots. However, in most plant parts no estrogenic activity could be found by the mouse uterine weight assay, although the amounts of material present were sufficient for detection if it had been unchanged DES. Therefore, the radioactivity in the plants must have been associated with products other than DES. When the soil was extracted with ethanol and the extract analyzed by chromatography, 3 to 6 radioactive compounds were found. However, 80 to 90% of the radioactivity was not extractable from the soil. Repeated use of the soil-manure mixture showed that after 5 weeks the remaining radioactivity in the soil was no longer taken up by the plants. In addition, migration of radioactivity in the soil appeared to be very small.

Uptake of radioactivity from $^{14}$C-DES into alfalfa has also been described by Ferrando and Valette.[40]

Finally, the combined influence of plants, animals and microorganisms on the fate of DES was studied in model ecosystems.[20] In an aquatic system containing algae, water fleas, mosquito larvae, snails and fish, DES and some nine unidentified metabolites were found after 3 days. DES concentrated to a considerable degree in the alga (100 times the concentration of that in water) and snail (484 times), and to a lesser extent in fish (14 times). A terrestrial-aquatic system was also employed in order to better simulate the feedlot situation. $^{14}$C-DES was introduced into the model feedlot ecosystem by administration to either mice or chickens, the excreta of which were incorporated by the system. After 33 days, unchanged DES constituted 17 to 25% of the extractable radioactivity. Seven radioactive compounds other than DES were also found by TLC, none of which was identified. DES and some of its metabolites were detected in all organisms in the system, and the snails and fish even accumulated DES and the more lipophilic compounds. The model ecosystem studies clearly showed that DES was degraded in the environment, but a significant portion of the compound persisted in the water and organisms as the parent molecule over a period of at least several weeks.

## VI. CONCLUSION

After 40 years of extensive use in human medicine and in agriculture, and despite continuous interest in this synthetic estrogen both from theoretical and practical points of view, our present understanding of the interactions of DES with biological systems, including the environment, cannot be considered satisfactory. Although there is a large quantity of information on the nature of metabolites, the rates and routes of excretion, and on species differences, important gaps in the pharmacokinetics of DES remain to be filled in order to fully understand the fate of this compound. Even larger deficiencies exist in its pharmacodynamics, i.e., in our understanding of the mechanisms through which DES exerts its biological effects, particularly its toxicity. Answers to questions concerning the mechanism of teratogenicity and transplacental carcinogenicity of DES, as well as the common features and the differences in the disposition and toxicity of DES and other estrogenic compounds, including the natural estrogens, are only beginning to emerge. It may take quite some time and effort to clarify the mechanism of DES toxicity, of which metabolism is only one aspect. An understanding of DES toxicity, however, may contribute to our general concepts of hormonal toxicity and, eventually, provide a scientific basis for the development of safer estrogens.

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