Biochemical Pharmacology, Vol. 36, No. 19, pp. 3135–3140, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

METABOLISM OF DIETHYLSTILBESTROL IN HAMSTER HEPATOCYTES

GÜNTER BLAICH,*† ERICH PFAFF‡ and MANFRED METZLER*

*Institute of Pharmacology and Toxicology, University of Würzburg, Federal Republic of Germany and ‡Institute of Toxicology, University of Tübingen, Federal Republic of Germany

(*Received* 18 *November* 1986; *accepted* 9 *March* 1987)

Abstract—Under certain modulating conditions the liver of the male Syrian golden hamster is a target organ for the carcinogenic effect of the synthetic estrogen diethylstilbestrol (DES). As a basis for mechanistic studies aimed at elucidating the role of metabolic activation in the process of DES-induced neoplasia, the metabolism of ^{14}C-DES was investigated in freshly isolated hamster hepatocytes. Three oxidative metabolites of DES, *viz.* *Z,Z*-dienestrol, 3′-hydroxy-DES and 1-hydroxy-*E*-DES, were formed in 14.2, 9.1, and 0.3% yield, respectively, when hepatocytes were incubated with 50 nmol DES/mg cellular protein for 60 min. Glucuronides (4.0%) and sulfates (2.8%) of DES and of the oxidative metabolites were also found, and non-extractable binding of radioactivity to cellular protein was observed indicating the formation of reactive intermediates. The capability of hamster hepatocytes to oxidize and conjugate DES should allow the investigation of the effects of modulators on the metabolic activation of DES in this cellular system in order to help clarify the mechanisms of DES-induced hepatocarcinogenesis.

The male Syrian golden hamster is uniquely susceptible to the carcinogenic activity of the synthetic estrogen diethylstilbestrol (DES).§ Whereas renal tumors but no hepatic tumors occur in a high incidence after six to eight months of continuous DES-treatment [2], hepatic tumors can be induced at similarly high incidence by simultaneous treatment with DES and 7,8-benzoflavone (7,8-BF), but not with DES nor 7,8-BF alone [3].

The mechanisms underlying tumor formation are unknown in both cases. In addition to being a potent estrogen, DES is extensively biotransformed in numerous mammalian species to oxidative metabolites, some of which are capable of damaging cellular macromolecules [4]. Both hormonal effects and metabolic activation are thought to act in concert in the process of DES-induced renal neoplasia [5, 6]. A similar mechanism may apply for the hepatic tumors evoked by DES and 7,8-BF. It is conceivable that 7,8-BF, a known inducer of drug-metabolizing enzymes, modulates the metabolism of DES in the hamster liver and thereby contributes to organ specific tumorigenesis.

In order to test this hypothesis, we have started to investigate the organ-specific biotransformation of DES. In this communication we report on the metabolism of DES in hamster hepatocytes.

† Correspondence to: Dr. G. Blaich, Institute of Toxicology, University of Würzburg, Versbacher Strasse 9, D-8700 Würzburg, Federal Republic of Germany.

§ Abbreviations used: DES, diethylstilbestrol, 3,4-bis-(*p*-hydroxyphenyl)hex-3-ene; DIES, dienestrol, 3,4-bis-(*p*-hydroxyphenyl)hexa-2,4-diene; *E* and *Z* are configurational designations, the nomenclature of DES metabolites follows the recommendation of Metzler and McLachlan [1]; 7,8-BF, 7,8-benzoflavone; GC/MS, gas chromatography/mass spectrometry; HPLC, high-pressure liquid chromatography; LDH, lactate dehydrogenase; TCA, trichloroacetic acid; TMS, trimethylsilyl.

MATERIALS AND METHODS

Chemicals and reagents. (Monoethyl-2-^{14}C)-diethylstilbestrol (spec. radioact. 53 mCi/mmole, the Radiochemical Centre, Amersham, U.K.) was shown by HPLC to be of 95% radiochemical purity and to consist of 77% E- and 18% Z-isomer. Unlabelled E-DES and 4′-hydroxy-propiophenone were purchased from Merck (Darmstadt, F.R.G.). *Z,Z*-Dienestrol (DIES) and 1-hydroxy-*Z,Z*-DIES were prepared in our laboratory as previously described [7]. 1-Hydroxy-E-DES and 3′-hydroxy-E-DES were kindly provided by Dr. John A. McLachlan (National Institute of Environmental Health Sciences, Research Triangle Park, U.S.A.) and Z-DES by Dr. P. Murphy (Lilly Research Laboratories, Indianapolis, IN).

Collagenase was from Boehringer (Mannheim, F.R.G.) and from Knoll AG (Ludwigshafen, F.R.G.). Silicone oils were purchased from Wacker Chemie (München, F.R.G.), lumagel from Baker Chemical B.V. (Deventer, Netherlands), digitonin and succinate from Merck (Darmstadt, F.R.G.), β-glucuronidase/arylsulfatase and trypan blue from Serva GmbH (Heidelberg, F.R.G.). The following radiochemicals were bought from NEN Chemicals GmbH (Dreieich, F.R.G.): $^{3}H_2O$ (5 mCi/ml), (carboxy-^{14}C)-dextran (MT 70000; 1 mCi/g) and ^{36}chloride (as $Na^{36}Cl$ solution, 3 mCi/g). All other chemicals and solvents used were of analytical grade.

Animals. Male Syrian golden hamsters (90–100 g body weight) were obtained from the Zentralinstitut für Versuchstiere (Hannover, F.R.G.). The animals had access to standard lab chow (Altromin 1324, Altrogge, Lage/Lippe, F.R.G.) and tap water *ad libitum*. They were kept under controlled conditions of temperature and humidity on a 12-hr light, 12-hr dark cycle and acclimated at least 4 weeks prior to use.

Isolation of liver cells. Liver cells were isolated according to the procedure of Berry and Friend [8] with modifications by Seglen [9] and Baur *et al.* [10]. During preparation the liver was perfused with Ca^{2+}-free Hank's medium at 37° via the vena portae. To disintegrate the liver, perfusion was carried out via the vena portae with a recirculating medium containing 1.25 mM calcium, 2 mM pyruvate and 0.05% collagenase. After about 10–15 min, the perfusion was continued via the vena cava for 15–20 min when the liver disintegrated. Subsequently, the tissue was transferred into a round-bottomed flask and enzymatic treatment was continued for another 15 min under slow rotation. After washing, the cells were stored in suspension (30–40 mg protein/ml) at 0° in a medium containing 137 mM NaCl, 5 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 5 mM glucose and 3 mM phosphate pH 7.2.

Cellular protein was determined by a modified biuret method [11]. Stainability by Trypan blue was tested by mixing equal volumes of cell suspension and 0.4% Trypan blue solution in incubation medium (see below) at room temperature. Only cell preparations in which less than 5% of the cells were stained were used for metabolic studies. The efflux of lactate dehydrogenase (LDH) was measured according to the procedure of Bergmeyer and Bernt [12]: The enzyme which leaked out of the cells was determined in the supernatant of centrifuged samples. Total LDH activity was measured after lysis of the cells by 0.1% Triton X-100. The aqueous cell volume was measured using ^3H_2O (2 μCi/ml) and (carboxy-^{14}C)-dextran (0.5 μCi/ml) as described by Baur *et al.* [10]. Membrane potential was determined with the aid of $Na^{36}Cl$ (0.5 μCi/ml) as described elsewhere [10]. The concentration of intra- and extracellular Na^+ and K^+ was determined by flame photometry [10].

Incubations. Incubation of cells was carried out at 37° in a standard medium containing 137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 3 mM phosphate, 10 mM succinate and 20 mM morpholino-3-propanesulfonic acid adjusted with Tris base to pH 7.3. Incubations contained 1 mg cellular protein/ml corresponding to about 1.5×10^6 cells/ml. For the determination of energetic parameters, cells were separated from incubation medium by centrifugal filtration as described by Klingenberg and Pfaff [13]. Radioactivity of the cells was measured by liquid scintillation counting after dissolving the pellet in lumagel.

Metabolic studies were carried out in a total volume of 2 ml of the standard medium with cells at a concentration of 1 mg cellular protein/ml in the presence of 50 nmol DES/mg cellular protein. At the end of the incubation time, 100 μl of an aqueous 1M citrate/1M ascorbate solution was added and the aqueous phase extracted four times with a 2-fold volume of diethyl ether/ethyl acetate (1:1, v/v). The combined organic extracts were concentrated to dryness under reduced pressure and the residue dissolved in 200 μl methanol for HPLC analysis. The cellular protein in the extracted aqueous phase was precipitated by adding 1 ml of a 20% aqueous solution of trichloroacetic acid (TCA). After centrifugation and washing with 2 ml 5% TCA solution, the precipitate was dissolved in 1N aqueous sodium hydroxide and an aliquot was analysed for radioactivity. Heat-inactivated cells (96° for 10 min) were used in control incubations. In some experiments (see Results), the extraction was carried out with diethyl ether/ethanol (3:1, v/v) after saturation of the incubation mixture with ammonium sulfate. Chromatography of the extract on alumina for the separation of unconjugated and conjugated metabolites and hydrolysis of the conjugates by glucuronidase/aryl sulfatase at 37° for 20 hr were performed as described previously [14].

High performance liquid chromatography (HPLC) was carried out using a Waters instrument. A 25 cm × 4.6 mm Zorbax ODS column packed with RP-18 phase (Du Pont, Wilmington, U.S.A.) was operated at 42° with a flow rate of 1 ml/min and a linear solvent gradient (solvent A: water–methanol 8:2 v/v; solvent B: methanol) changing from 40 to 100% B in 24 min. The eluate was monitored with a u.v. detector at 254 nm and collected in 0.3 min fractions. Radioactivity was measured in a model 3390 liquid scintillation counter with automatic external standardization (Packard Instruments, Frankfurt, F.R.G.). HPLC fractions used for analysis by gas chromatography/mass spectrometry (GC/MS) were evaporated to dryness under reduced pressure and derivatized with O,N-bis(trimethylsilyl)acetamide. GC/MS was performed on a Finnigan 4510 GC/MS as previously described [15].

RESULTS

Viability of hamster hepatocytes

In order to ensure that viable cells were used for the metabolic studies, hepatocytes of male hamsters were incubated in standard medium (see Materials and Methods). At the time points indicated samples were taken from the incubation and analyzed for Na^+/K^+ distribution (Fig. 1A) and membrane potential across the plasma membrane (Fig. 1B). When hepatocytes taken directly from the stock solution were incubated, a rapid K^+-uptake and a decrease in intracellular Na^+ were noted (Fig. 1A). After approximately 20 min, a steady state was reached with intracellular concentrations of about 30 mM for Na^+ and 105 mM for K^+. Addition of 0.02 mg/ml digitonin, which is known to make the plasma membrane permeable and to disturb the Na^+/K^+ distribution, resulted in extensive K^+ release and Na^+ uptake (Fig. 1A). A membrane potential of about 20–25 mV, calculated from the ^{36}Cl distribution across the membrane, was rapidly built up and maintained during the incubation time of 60 min (Fig. 1B). Addition of digitonin resulted in a collapse of the membrane potential, demonstrating that the hepatocytes were in a physiologically excellent state.

As a further viability criterion, the efflux of lactate dehydrogenase (LDH) from the cells was determined in blank incubations run parallel to incubations with DES. LDH in the medium increased only very slowly during the incubation time of 60 min but was strongly enhanced when digitonin was added (Fig. 1B), increasing to about 50% of the LDH activity of cells lysed with Triton X-100.

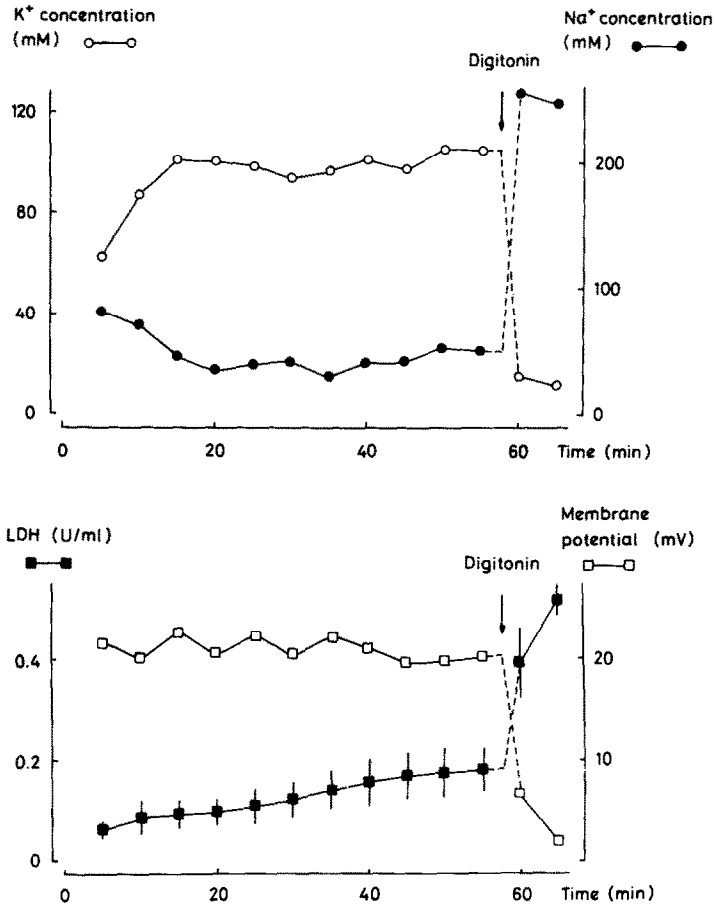

Fig. 1. Viability criteria of isolated hamster hepatocytes. A (upper chart): intracellular Na^+ and K^+ concentrations. B (lower chart): membrane potential and LDH efflux. Na^+ and K^+ concentrations and membrane potential represent the mean value of two different preparations, LDH is the mean ± SD of five cell preparations.

Identification of DES metabolites

Freshly-prepared hepatocytes were incubated with ^{14}C-DES (see Materials and Methods) for various lengths of time (see below). Following exhaustive extraction of the incubation mixture with ethyl acetate/diethyl ether, protein was precipitated from the aqueous phase. The amount of radioactivity was determined in the organic extract, the precipitated protein and the aqueous supernatant. After the maximum incubation time of 1 hr, the organic phase contained about 92% of the total radioactivity, the precipitated protein 3% and the aqueous phase 5%.

The radioactivity in the organic extract was analyzed by HPLC. An efficient separation of DES metabolites by HPLC was achieved by using a modification of a previously published method [15]. Figure 2A shows the chromatogram of eight synthetic reference compounds. When the extract of the 60 min incubation was chromatographed under the same conditions, six radioactive peaks were observed in the chromatogram (Fig. 2B). The major peak cochromatographed with *E*-DES, whereas three other peaks cochromatographed with *Z*-DES, *Z,Z*-dienestrol (*Z,Z*-DIES) and 3'-hydroxy-*E*-DES. For the unequivocal identification of these metabolites, the respective fractions of the HPLC run were collected and analyzed by gas chromatography/mass spectrometry after formation of the trimethylsilyl derivatives. The mass spectra (Fig. 3) were identical to those of the synthetic reference compounds. Attempts to identify the small peak eluting at 24 min (Fig. 2B) have so far been unsuccessful.

In addition to these metabolites, a very polar peak of radiolabeled material, representing 3–4% of the total radioactivity of the extract, eluted very early in the HPLC chromatrogram (Fig. 2B). By changing the extraction solvent from ethyl acetate/ether to ethanol/ether and saturating the aqueous phase with ammonium sulfate (see Materials and Methods), this peak could be enhanced to about 7–8% with a simultaneous decrease of the radioactivity remaining in the aqueous phase. When this extract was analyzed by chromatography on an alumina column to separate unconjugated material, sulfates and glucuronides [14], it was found that the extract contained 93.3 ± 1.1% unconjugated material, 2.8 ± 1.3% sulfates and 4.0 ± 0.7% glucuronides (mean of three 60 min-incubations ± standard deviation). These conjugates were then enzymatically hydrolyzed and identified by HPLC (Table 1). In addition to *Z,Z*-DIES, 3'-hydroxy-*E*-DES and *Z*-DES, 1-hydroxy-*E*-DES was found as a metabolite in the sulfate and glucur-

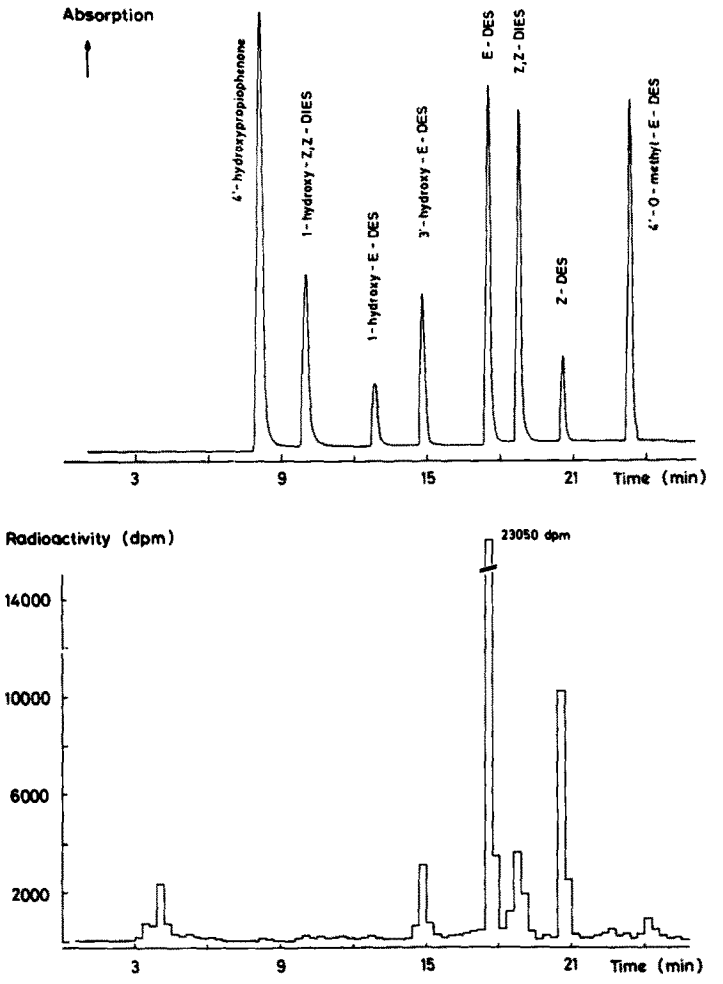

Fig. 2. Separation of DES metabolites by HPLC. A (upper chart): reference compounds. B (lower chart): radioactivity profile of metabolites of ^{14}C-DES from a 60 min-incubation with hamster hepatocytes

onide fraction. The identity of this metabolite was confirmed by cochromatography with the synthetic reference compounds in HPLC; the amount was too small for gas chromatography/mass spectrometry.

A time-course study of the DES metabolism in hepatocytes (Fig. 4) revealed a continuous consumption of *E*-DES with a concomitant formation of the metabolites *Z,Z*-DIES and 3'-hydroxy-*E*-DES; in

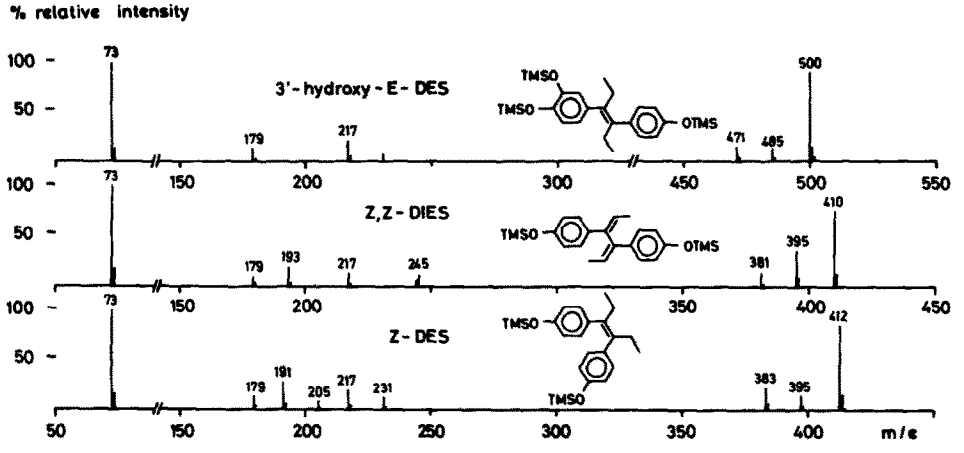

Fig. 3. Mass spectra of DES metabolites from isolated hamster hepatocytes.

Table 1. Oxidative metabolites of DES in the sulfate and glucuronide fraction

Metabolite	Sulfate fraction	Glucuronide fraction
1-Hydroxy-*E*-DES	1.4 ± 0.2	5.3 ± 0.2
3'-Hydroxy-*E*-DES	1.5 ± 0.2	1.8 ± 0.2
E-DES	32.4 ± 0.8	20.6 ± 0.9
Z,Z-DIES	16.5 ± 2.9	20.6 ± 1.7
Z-DES	27.4 ± 1.7	24.4 ± 0.8

The conjugates were treated with β-glucuronidase and aryl sulfatase and the deconjugated metabolites were analyzed by HPLC. Data are presented as % of radioactivity recovered after HPLC (mean ± SD of 3 incubations)

contrast, *Z*-DES remained nearly constant (Fig. 4A). The amounts of radioactivity bound to the precipitated protein and remaining in the supernatant also increased with time in the incubations but not in the controls (Fig. 4B).

DISCUSSION

The purpose of this study was to elucidate the capability of isolated hamster hepatocytes for oxidative biotransformation and metabolic activation of the carcinogen, diethylstilbestrol (DES). For the use of isolated hepatocytes in metabolic studies, the viability of the cells is of crucial importance. During the isolation procedure liver cells can be damaged by the removal of calcium, by mechanical stress and by the action of lytic enzymes. Many investigators confine themselves to the Trypan blue exclusion test. This test has proved to be inadequate for detecting small alterations of the plasma membrane and early events in ageing of the cells. On the other hand, the membrane potential and intracellular K^+ and Na^+ concentrations are very sensitive criteria of viability [10]. When the hamster hepatocytes used for the metabolitic study were examined according to these criteria, the cells proved to be of high viability (Fig. 1).

To our knowledge, the metabolism of DES has not previously been studied in isolated hepatocytes of

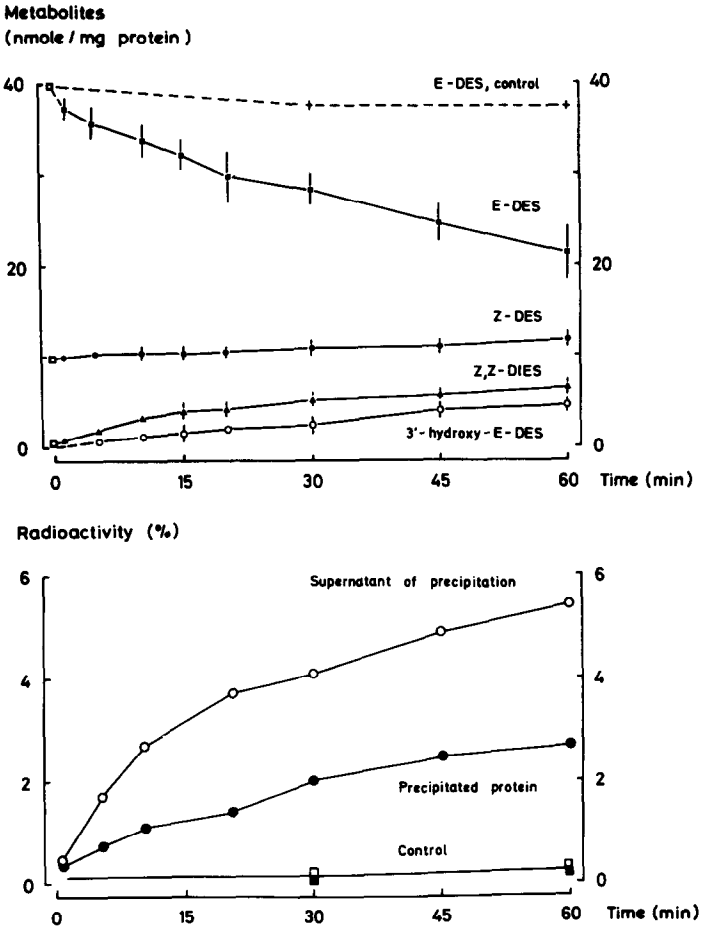

Fig. 4. Time course of the metabolism of DES in hamster hepatocytes. A (upper chart): individual metabolites. The data were obtained by HPLC analysis and represent the mean value ± SD of 4 incubations. Controls were obtained with boiled hepatocytes (10 min at 96°). B (lower chart): radioactivity precipitated with protein (●) and radioactivity remaining in the supernatant (○) are expressed as % of the total recovered radioactivity (mean of 2 incubations). Controls of protein-bound (■) and non-precipitated radioactivity (□) were determined with boiled hepatocytes.

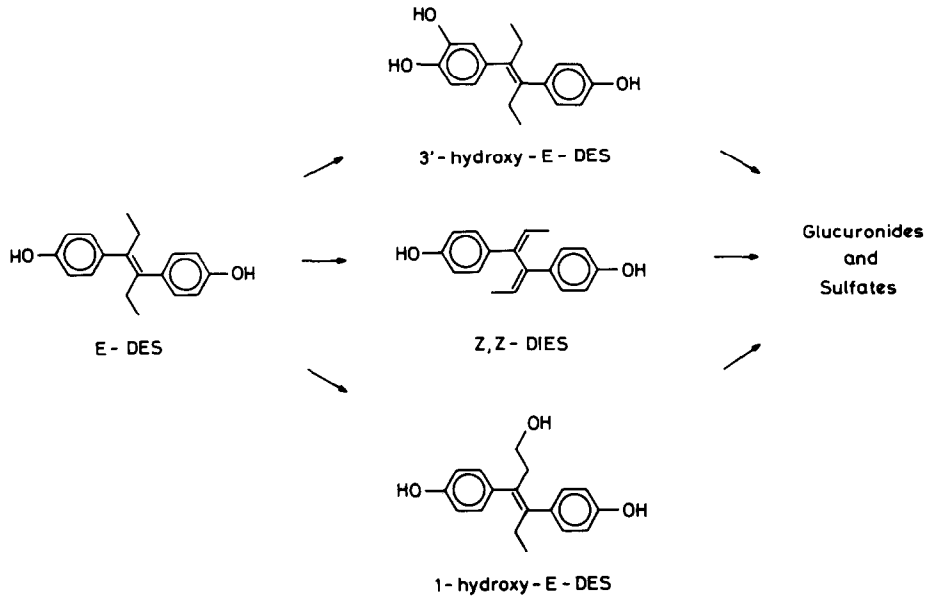

Fig. 5. Metabolic pathways of DES in hamster hepatocytes.

any species. The results of the present study clearly show that DES is metabolized by male hamster hepatocytes to a number of oxidative metabolites and also to conjugates. The oxidative metabolites comprise *Z,Z*-dienestrol (Z,Z-DIES), 3'-hydroxy-DES and 1-hydroxy-DES; this indicates that at least three oxidative pathways are operative in the hepatocytes in addition to the formation of glucuronides and sulfates (Fig. 5). The metabolism involves reactive intermediates, as demonstrated by non-extractable binding of radioactivity to cellular protein (Fig. 4B).

The metabolic pattern of DES found in isolated hamster hepatocytes is, in general, in good agreement with metabolites identified in the bile and urine of intact hamsters [14, 16] and also with metabolites formed from DES upon incubation with hamster liver microsomes *in vitro* [15]. However, an interesting difference from the microsomal metabolism is the apparent failure of hamster hepatocytes to form the *Z*-isomers of the oxidative metabolites shown in Fig. 5: neither 3'-hydroxy-*Z*-DES nor 1-hydroxy-*Z*-DES were found among the cellular metabolites although they would have been detected under the HPLC conditions used. *Z*-DES was present in the radioactive DES used for the metabolic study and its amount did not change during incubation (Fig. 4A). This suggests that neither hydroxylation of the *Z*-DES nor isomerization of the oxidative metabolites of *E*-DES took place in hepatocytes. This is in contrast to the microsomal metabolism of DES, where large amounts of *Z*-DES and *Z*-configurated oxidative DES metabolites were observed [15].

The ability to metabolize DES along oxidative and conjugative routes, including reactive intermediates, should make the isolated hamster hepatocytes a suitable cell model system to study the effect of 7,8-benzoflavone pretreatment on DES metabolism, in order to help clarify the role of metabolic activation in the neoplastic effects of DES.

Acknowledgements—This study has been supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 172.) We thank Mrs H. Raabe and Mrs S. Kasperek for technical assistance and Mrs J. Colberg for the recording of the mass spectra. We are also grateful to Knoll AG, Ludwigshafen, for providing collagenase and to Doktor Robert Pfleger-Stiftung (Bamberg, F.R.G.) for mass spectrometric equipment.

REFERENCES

1. M. Metzler and J. A. McLachlan, *J. Envir. Path. Tox.* **2**, 579 (1978).
2. H. Kirkman and R. L. Bacon, *J. natn. Cancer Inst.* **13**, 745 (1952).
3. J. J. Li and S. A. Li, *J. natn. Cancer Inst.* **73**, 543 (1984).
4. M. Metzler, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot) Vol. 6, p. 191. Elsevier, New York (1984).
5. M. Metzler, *Archs Toxic.* **55**, 104 (1984).
6. J. J. Li and S. A. Li, *Archs Toxicol.* **55**, 110 (1984).
7. M. Metzler, *Tetrahedron* **34**, 3113 (1978).
8. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
9. P. O. Seglen, *Exp. Cell Res.* **74**, 450 (1972).
10. H. Baur, S. Kasperek and E. Pfaff, *Hoppe-Seyler's Z. physiol. Chem.* **356**, 827 (1975).
11. L. Szarkowska and M. Klingenberg, *Biochem. Z.* **338**, 674 (1963).
12. H. U. Bergmeyer and E. Bernt, in *Methoden der enzymatischen Analyse,* (Ed. H. U. Bergmeyer), 2nd Edn Vol. 1, p. 533. Verlag Chemie, Weinheim (1970).
13. M. Klingenberg and E. Pfaff, *Meth. Enzym.* **10**, 680 (1967).
14. R. Gottschlich and M. Metzler, *Xenobiotica* **10**, 317 (1980).
15. H. Haaf and M. Metzler, *Biochem. Pharmac.* **34**, 3107 (1985).
16. M. Metzler, *Biochem. Pharmac.* **24**, 1449 (1975).