

and these low values have been partly attributed by these authors to the pithing of the animals.

The peripheral release of catecholamines (epinephrine and norepinephrine) does not seem to play any significant role in the hypertensive response of physostigmine (5, 9). This observation is based on the fact that these authors did not find any difference in the pressor response of physostigmine in normal as well as in the adrenalectomized animals. They further suggested (9) that although physostigmine is known to increase the output of epinephrine from the adrenals of the cat (6) it is unlikely that this effect occurs in the rat. These results contradict their statement that physostigmine will not increase the peripheral release of catecholamines from the adrenals of the rat. The results presented in this paper clearly demonstrate such an effect in the rat.

The authors have shown that catecholamines are being released following injection of physostigmine and if these were contributing to the hypertensive response then one would have seen a bigger rise of blood pressure following physostigmine injection in normal animal with intact adrenals than in adrenalectomized animals. In fact the experiments showed an increase in the pressor response of physostigmine in adrenalectomized animals. Therefore these results show that release of catecholamines from the adrenal glands does not contribute to the hypertensive response of physostigmine.

Some doubt also exists in the literature as to whether the adrenal medullary secretion plays any significant role in the cardiovascular regulations (10). Since most of the catecholamine present in the adrenal medulla is adrenaline (85%) (11), it is possible that its effect on β -adrenergic receptors in certain vascular beds leads to vasodilation and masks the vasoconstriction effect. This could probably explain why a significant increase in the

pressor response of physostigmine in the adrenalectomized animals as compared to controls is seen. This could not be attributed to the change of sensitivity of the cardiovascular system, after adrenalectomy, since pressor responses of epinephrine and norepinephrine were the same in both normal and adrenalectomized animals. This is further supported by experiment with propranolol where an increase in the pressor response of physostigmine occurred. The slight but significant ($p < 0.05$) increase in the pressor response in the adrenalectomized animals is difficult to explain with the data available.

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Keyphrases

Physostigmine—catecholamine output
Adrenal gland catecholamine output—physostigmine effect
Central mediation—physostigmine activity

In Vitro Utilization of Diethylstilbestrol by Rat Liver

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A technique is described for the study of the utilization of diethylstilbestrol (DES) by rat liver *in vitro*. The disappearance of DES and the seasonal variations were determined on liver slices and homogenates. A chromatographic identification of diethylstilbestrol monoglucuronide (DESGA) as a reaction product in the assays of slices was carried out. The effect of liver preincubation with saline phosphate buffer, of phenylmercuric acetate, and of uridine diphosphate glucuronic acid on DES utilization by liver was studied. The possibility of a DES utilization pathway other than monoglucuronide formation is discussed. A thin-layer chromatographic study was carried out with DES and DESGA.

DIEETHYLSTILBESTROL (α, α' -diethylstilbene-diol) is an orally active synthetic estrogen

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that has been utilized in therapeutics to produce prostatic tumor regression; it inhibits glutamate dehydrogenase by breaking the enzyme into inactive subunits (1), uncouples oxidative phosphorylation with mitochondrial volume expansion (2), and inhibits electron transport between cytochromes *b* and *c* (3).

In spite of its important activity at physiological and enzymatic levels, and of its use in medicine and veterinarian practice, very little is known about its metabolism and mechanism of action. Reports by Zondek (4), Engel (5), Riegel (6), and Hopwood (7) showed that liver *in vitro* is capable of utilizing DES; however, in no case is the nature of the reaction involved nor the type of product formed mentioned, only the loss of estrogenic activity of DES after the incubation period with liver being ascertained by means of biological assay.

Other studies point to the role of DES as a tyrosinase (8) and horseradish peroxidase (9) substrate but no mention is made of the reaction products, except to the appearance of water soluble metabolites.

Experiments with ^{14}C -labeled DES have demonstrated that this compound, like the natural estrogens, enters the enterohepatic circulation and is eliminated to a high degree through feces (10). Knoche (11) using ^{14}C - and ^3H -labeled DES in rats studied the *in vivo* oxidation with production of labeled H_2O and CO_2 and four radioactive metabolites.

The excretion of DES in the urine as a monoglucuronide (DESGA) is well known (12), but the enzymatic mechanism of glucuronide formation has not been subjected to detailed investigation. Zimmerberg (13) studied the quantitative DES utilization by rat liver slices and attributed substrate disappearance to conjugation processes with glucuronic acid and the sulfate but the reaction products were not identified. Hartiala (14) employing chromatography, indirectly pointed out the formation of DESGA in rat duodenal mucosa slices. Dutton and Storey (15) showed the formation of a small amount of glucuronide in rat liver homogenates.

The present report presents a systematic study of DES disappearance following incubation period with rat liver slices and homogenates. Starting from the incubation mixture, the technique employed allows for the determination of DES without DESGA interference or any other phenolic compounds. The amount of DES consumed was calculated by difference with a blank incubated without substrate and added after protein precipitation.

A thin-layer chromatographic study of DES and DESGA was performed for qualitative as well as semiquantitative identification of reaction products.

EXPERIMENTAL

Chemicals—DES and uridine diphosphate glucuronic acid (UDPGA) were provided by Sigma

Chemical Co. and tris(hydroxymethyl)aminomethane (tris) and phenylmercuric acetate, by Schuchardt. All solvents were redistilled and diethyl ether was maintained free from peroxides by storage over ferrous sulfate and was freshly distilled before use.

Animals—Wistar male rats of 240–280 g. were sacrificed by cervical dislocation and the livers immediately removed and washed with $0.15\text{ M KCl}-3.2 \times 10^{-4}\text{ M KHCO}_3$ previously cooled to 0° . Slices of 250 mg. each were obtained with a Stadler-Riggs hand microtome and 15% homogenates were prepared with $0.15\text{ M KCl}-3.2 \times 10^{-4}\text{ M KHCO}_3$ in a Potter-Elvehjem homogenizer cooled to 0° .

Preparation of DESGA—DESGA was obtained from the urine of rabbits fed DES at a daily rate of 500 mg. for 5 days. Following the procedures given by Dodgson *et al.* (12), a chromatographically pure product of m.p. 176° was obtained after three crystallizations from benzene-acetone. The presence of glucuronic acid in the product was demonstrated by hydrolysis with concentrated HCl vapors and elution of the hydrolyzed product on Silica Gel FH₂₅₄ plate with ethyl acetate-2-propanol-water (6:2.5:1.5). Two spots were found: a red spot on the site of application similar to the one yielded by DES when given the same treatment, and another spot with the same R_f as glucuronic acid.

Thin-layer Chromatography—The plates used in the thin-layer chromatography were Silica Gel G and Merck precoated Silica Gel FH₂₅₄. They were heated for 30 min. at 110° before use. The Merck precoated plates were observed at $254\text{ m}\mu$.

For the detection of glucuronic acid, 2% aniline phthalate in water-saturated *n*-butanol was used; for the detection of DES the following methods were applied: (a) direct observation after 2 days of exposure to daylight; (b) 1% I_2 in methanol; (c) 50% H_2SO_4 v/v followed by heating for 3 min. at 100° ; (d) equal volumes of 1% FeCl_3 and 1% potassium ferricyanide mixed before use followed by application of 10% HCl, and (e) 0.5 *N* Folin-Ciocalteu's reagent and exposure to ammonia vapor. The last four detecting agents can be also used for the detection of DESGA.

Solubility Determination—Aliquots of a DES solution containing 2 mg./ml. in acetone were placed in tubes and evaporated to dryness. Five-milliliter portions of the solutions in which DES solubility was to be determined were added to the residue. The tubes were hermetically sealed and shaken at 25° for 24 hr. The contents were filtered only where an excess of DES occurred and the latter ascertained in triplicate by the method outlined below.

Calibration Curve for DES—A modified Tubis technique (16) has been employed for the determination of DES. A standard curve was obtained by treatment of 1 ml. DES solution containing from 0 to 65 mcg./ml. in 30% ethanol with 4 ml. of 4% $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ in 0.1 *N* NaOH and 0.25 ml. of 1 *N* Folin-Ciocalteu's reagent. The contents were shaken and readings taken at $540\text{ m}\mu$ after 45 min. at 20° . A linear function was obtained over the range studied.

DES Determination in the Incubation Mixture—One milliliter of 20% trichloroacetic acid and 4 ml. of ethanol were added to 5 ml. of the incubation

mixture, over a 10-min. period and the contents filtered. Five milliliters of a freshly prepared 2% NaHCO₃ was added to an aliquot of 5 ml. from the filtrate, toward formation of the sodium salts of DESGA and of the other acids so as to prevent their removal by diethyl ether. The mixture was extracted twice with 10-ml. portions of diethyl ether. The ether was removed on the water bath and the aqueous alcoholic residue evaporated under vacuum at a temperature below 45°. The solid residue was lacking in any substance which could interfere with subsequent DES determination.

Two milliliters of 30% ethanol, 8 ml. of 4% Na₂CO₃·10H₂O in *N* NaOH, and 0.50 ml. of *N* Folin-Ciocalteu's reagent were added to the solid residue. The contents were shaken and readings taken at 540 m μ after 45 min. at 20°. Reagent volumes double those described for the calibration curve were used so that readings should be within the limits of the standard curve. In order that the amount of DES determined could be related to total DES the former was multiplied by 2 (correction factor for using an aliquot of 5 ml. from the filtrate) and by 2 again (correction factor for using double the amount of reagents as in the calibration curve). The amount of DES utilized by the liver was calculated as the difference between the blanks incubated without DES and with DES after precipitation with trichloroacetic acid and the samples incubated with DES for 90 min. at 37°. Assays were done in triplicate.

Isolation of DESGA from the Incubation Mixture—An incubation volume five times greater than in the assays of slices was used in conjunction with the DESGA analysis. The reaction was stopped with 5 ml. of 12 *N* H₃PO₄ and 20 min. later, 20 ml. of ethanol was added and following a period of 20 min. the mixture was centrifugated and concentrated to a volume of 10 ml. Four extractions were carried out with 10-ml. portions of diethyl ether and the pooled ethereal layers were washed with 2 ml. of 0.01 *N* H₃PO₄ and then concentrated to 5 ml. They were extracted with three 5-ml. portions of a saturated NaHCO₃ solution. The aqueous portion was washed with 3 ml. of ether, acidified with 2.7 ml. of 12 *N* H₃PO₄, and then extracted with four 20-ml. portions of ether. The pooled ethereal layers were washed with 4 ml. of 0.01 *N* H₃PO₄ and evaporated to dryness. The residue was dissolved in 80% ethanol and an aliquot applied to the Silica Gel FH₂₅₄ plate. The plate was completely developed with benzene-ethanol (6:4) in order to separate contaminating substances with less polarity; once dry it was developed with ethyl acetate-2-propanol-water (6:2.5:1.5) or *n*-butanol-acetic acid-water (6:2:2). A spot of the same *R_f* as the standard DESGA, which is absent in the blank, was detected.

TABLE I—*R_f* VALUES OF DES AND DESGA

	<i>R_f</i> in Solvent Systems ^a					
	1	2	3	4	5	6
DES	0.39	1.00	1.00	0.72	0.90	0.95
DESGA	0.00	0.82	0.30	0.00	0.00	0.90

^a Solvent systems employed were: 1, benzene-ethanol (9:1); 2, 1-butanol-acetic acid-water (6:2:2); 3, ethyl acetate-2-propanol-water (6:2.5:1.5); 4, chloroform-ethanol (8:2); 5, benzene-ethanol (6:4); 6, methanol-acetic acid-water (8:1:1).

TABLE II—DES SOLUBILITY IN AQUEOUS ALCOHOLIC SOLUTIONS^a

Solvent	Solubility, mg./ml.
Water	0.012
10% Ethanol	0.030
20% Ethanol	0.083
30% Ethanol	0.496
30% Propylene glycol	0.036
40% Propylene glycol	0.135
50% Propylene glycol	0.420
60% Propylene glycol	1.212
70% Propylene glycol	3.572

^a Percentages refer to v/v.

RESULTS

Thin-layer Chromatography—Table I presents *R_f* values for DES and DESGA with the different solvents used. The detection limits for DES with the methods applied were: for 1, 4, and 5, 0.60–0.90 mcg.; for 2, 0.20 mcg., and for 3, 1.00–1.30 mcg. (see *Experimental*).

Solubility Determinations—Table II presents DES solubilities in water solutions of ethanol and propylene glycol.

Utilization by Liver—Table III shows DES utilization by rat liver slices in the presence and absence of phenylmercuric acetate, and inhibitor of UDP-glucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17 (17) and the seasonal variations observed. Table IV depicts DES utilization by rat liver homogenates in the presence and absence of UDPGA, and the seasonal variations observed.

DISCUSSION

The amount of DES consumed by rat liver slices cannot be exclusively attributed to monoglucuronide formation since the actual amount of the latter detected was far lower than the theoretically expected one, should all DES have been transformed into DESGA. The utilization of DES in the presence of inhibitor and by liver previously inactivated by preincubation further supports the same assumption. The difference between the amount of DES utilized in the absence and presence

TABLE III—DES UTILIZATION BY RAT LIVER SLICES^a

Conditions	DES Consumed, mcg. Mean \pm SD	<i>p</i> ^d
Spring		
Without inhibitor (10) ^b	44.40 \pm 7.85	
With inhibitor (10)	35.12 \pm 7.30	<0.025
Without inhibitor (8) ^c	17.32 \pm 10.10	<0.01
Autumn		
Without inhibitor (7)	44.24 \pm 8.50	
With inhibitor (7)	40.64 \pm 7.96	>0.2

^a The composition of the incubation mixture was as follows: NaCl, 96.3 mM; KCl, 4 mM; NaHCO₃, 6.3 mM; MgCl₂·6H₂O, 9.9 mM; Na₂HPO₄·2H₂O, 19.8 mM; HCl, 4 mM; DES, 0.149 mM; phenylmercuric acetate, 0.5 mM; 3% v/v propylene glycol; liver slice, 250 mg.; final volume, 5 ml.; pH, 7.4; incubation with shaking at 37° for 90 min.

^b Numbers in parentheses indicate number of animals used. ^c Assay done after slice preincubation with saline phosphate buffer for 24 hr. at 37°. ^d Level of significance, Student *t* test; *p* value compared with the assay performed in the absence of inhibitor.

TABLE IV—DES UTILIZATION BY RAT LIVER HOMOGENATES^a

Conditions	DES Consumed, mcg.		<i>p</i> ^c
	Mean	± SD	
	Spring		
With UDPGA (7) ^b	21.40	± 8.17	
Without UDPGA (7)	7.36	± 5.56	<0.01
	Autumn		
With UDPGA (7)	0.00		
Without UDPGA (7)	0.00		

^a The composition of the incubation mixture was as follows: Tris, 35 mM; MgCl₂·6H₂O, 10.5 mM; UDPGA, 0.67 mM; DES, 0.149 mM; 3% v/v propylene glycol; 15% homogenate, 1 ml.; final volume, 5 ml.; pH, 7.4; incubation with shaking at 37° for 90 min. ^b Numbers in parentheses indicate number of animals used. ^c Level of significance, Student *t* test; *p* value compared with the assay performed in the presence of UDPGA.

of inhibitor is statistically significant ($p < 0.025$) in the experiments carried out in spring and relates to the amount of DESGA detected. The same experiments carried out in autumn did not yield statistically significant differences ($p > 0.2$).

Jellinck (8) using ¹⁴C-labeled DES and tyrosinase pointed out that after the incubation of the mixture production of water-soluble metabolites ensued, a phenomenon that was accentuated by the presence of proteins, not affected by thiol reagents and totally inhibited by potassium cyanide. The latter finding is of interest considering that Zimmerberg (13) obtained complete DES recoveries from incubation mixtures of rat liver slices and cyanide. It is possible that in the present report the DES disappearance in the presence of phenylmercuric acetate could be due to an oxidative process that would transform DES into a water-soluble metabolite and which might explain the incomplete recovery of DES by ether extraction.

The DES disappearance in the liver system inactivated by preincubation at 37° for 24 hr. can be interpreted as an adsorption phenomenon on proteins. Similar retention phenomena upon inactive proteins have been described for DES by Riegel *et al.* (6) with boiled liver and in the case of estrone by Jellinck (18, 19) with boiled liver, albumin, and hemin.

As far as the rat liver homogenates is concerned, the difference between the amount of DES consumed in the presence and absence of UDPGA is statistically significant ($p < 0.01$) and it seems logical to attribute this difference exclusively to the formation of glucuronide. The disappearance of DES in the absence of UDPGA, which is very variable, can be due to residual levels of UDPGA or other unknown pathways of utilization. Non-

enzymatic phenomena do not seem to play any role at all, as there has not been any case of DES disappearance in experiments undertaken in autumn.

Seasonal variations, although previously described, are not too well known. Dutton (20) and Hartiala (21) have pointed out greater glucuronyl-transferase activity in winter than in spring with *o*-aminophenol as substrate. Periodic variations have been observed in relation to synthetic hypoglycemic drugs (22) the toxicity being greater from December to March. No explanation for the phenomenon observed in this study can be advanced at the present time.

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Keyphrases

Diethylstilbestrol—rat liver utilization *in vitro*
 Metabolite—diethylstilbestrol monoglucuronide
 Solubility—diethylstilbestrol
 TLC—analysis
 Colorimetric analysis—spectrophotometer