Metabolic Hydroxylations of *trans*-Stilbene

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1. A study was made of the hydroxylation of *trans*-stilbene in rabbits, guinea pigs and mice, as well as by rabbit liver microsomes. 2. In the rabbit *in vivo*, *trans*-stilbene is converted into 4-hydroxy-, 4,4'-dihydroxy-, 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene, and hydroxylation plays a more significant role in the metabolism of *trans*-stilbene than has previously been reported. 3. Investigation of the hydroxylation of 4-hydroxystilbene in the rabbit *in vivo* demonstrated its ready conversion into 4,4'-dihydroxystilbene and established its intermediacy in the formation of this compound and the methylated analogues of 3,4-dihydroxystilbene. 4. Hydroxylation of *trans*-stilbene in the guinea pig was found to follow a pattern similar, both qualitatively and quantitatively, to that in the rabbit. 5. Studies in the mouse revealed only limited yields of 4,4'-dihydroxy-stilbene. 6. Studies of the hydroxylation of *trans*-stilbene and 4-hydroxystilbene by rabbit liver microsomes located two of the reactions that occur with these compounds *in vivo*. 7. Work with a solubilized liver-microsomal preparation provided evidence that 'stilbene hydroxylase' activity is not completely lost on solubilization, thus allowing for future microsomal enzyme-isolation studies.

Numerous stilbene analogues have been prepared as potential therapeutic agents. Among these are hypocholesterolaemic agents (Hughes, Moore & Stebbins, 1964), oestrogens (Grundy, 1957) and sympathomimetic amines (Drefahl & Fischer, 1956). However, little is known about the metabolism of stilbenes, particularly about the role of hydroxylations. A study of hydroxylations of *trans*-stilbene *in vivo* and *in vitro* was considered as an initial step toward the understanding of stilbene metabolism.

Stroud (1939, 1940) reported that *trans*-stilbene is hydroxylated in the rabbit to yield 4,4'-dihydroxystilbene. Newer procedures for the assay of a number of hydroxystilbenes were developed (Sinsheimer & Smith, 1967) and applied to a study of the hydroxylation of *trans*-stilbene *in vivo* in the rabbit, guinea pig and mouse. Hydroxylation of *trans*-stilbene by rabbit liver microsomes was also studied to locate the site of hydroxylation and correlate the activity found *in vivo* and *in vitro*.

MATERIALS

All melting points were determined on a Kofler block and are corrected. Ultraviolet spectra were obtained with a Beckman DK-2A ratio-recording spectrophotometer. Infrared spectra were determined in KBr on a Perkin-Elmer model 337 infrared spectrophotometer.

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trans-Stilbene and 2-hydroxy-, 4-hydroxy-, 4,4'-dihydroxy- and 4-hydroxy-3-methoxy-stilbene were purchased or prepared and purified as described by Sinsheimer & Smith (1967). All stilbenes used were of the *trans* configuration. The sodium salt of NADP⁺ and the dipotassium salt of glucose 6-phosphate (both from Sigma Chemical Co., St Louis, Mo., U.S.A.) and glucose 6-phosphate dehydrogenase (Calbiochem, Los Angeles, Calif., U.S.A.) were used as purchased.

3-Hydroxy-4-methoxystilbene. A mixture of phenylacetic acid (1.36 g.), 3-hydroxy-4-methoxybenzaldehyde (1.52 g.), piperidine (0.2 ml.) and dimethylformamide (5 ml.) was refluxed for 4 hr. and then poured over 20 g. of ice containing 1 ml. of conc. HCl. The resulting suspension was exhaustively extracted with ether. The ether layer was washed with water and saturated NaCl soln., dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo* to give a brown oil (2.02 g.). Chromatography over silica gel (75 g., 100 mesh) with benzene-methanol (24:1, v/v) and recrystallization from cyclohexane gave 3-hydroxy-4-methoxystilbene as fine white needles (97 mg.). 3-Hydroxy-4-methoxystilbene had m.p. 159°, $\nu_{\max}$ 3450 (OH), 2983 and 2883 (CH₃) and 1607 cm.⁻¹ (C=C), $\lambda_{\max}$ in ethanol 231, 302 and 323 m μ (ϵ 13 200, 20 900 and 25 100 respectively) (Found: C, 79.6; H, 6.3; C₁₅H₁₄O₂ requires C, 79.6; H, 6.2%).

3,4-Dihydroxystilbene. A mixture of 4-hydroxy-3-methoxystilbene (100 mg.) and dry pyridine hydrochloride (1.5 g.) was heated at 205–210° for 40 min. in an atmosphere of N₂. The molten mixture was then poured over ice. The resulting suspension was exhaustively extracted with methylene chloride. The methylene chloride layer was washed with water and saturated NaCl soln., dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo* to give a pink crystalline solid (75 mg.). Sublimation of this

solid at 90–100° and 0.005 mm. Hg for 56 hr. gave 3,4-dihydroxystilbene as a white crystalline powder (24 mg.). 3,4-Dihydroxystilbene had m.p. 168–170°, $\nu_{\max}$. 3377 (OH, broad) and 1589 cm⁻¹ (C=C), $\lambda_{\max}$. in ethanol 232, 302 and 325 m μ (ϵ 14500, 20400 and 26300 respectively) (Found: C, 79.0; H, 5.7; C₁₄H₁₂O₂ requires C, 79.2; H, 5.6%).

4-Trimethylsiloxystilbene. A mixture containing 4-hydroxystilbene (54 mg.), pyridine (dried over KOH pellets) (0.7 ml.), hexamethyldisilazane (0.2 ml.) and trimethylchlorosilane (0.1 ml.) was allowed to react at room temperature for 10 min. Two portions (10 μ l.) of this mixture were then chromatographed at 175° with an F & M 720 gas chromatograph (Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with a thermal conductivity detector and two 6 ft. $\times$ $\frac{1}{8}$ in. copper columns packed with 3% SE-52 on Chromosorb W (60–80 mesh; hexamethyldisilazane-treated) (Applied Science Laboratories, State College, Pa., U.S.A.). Collection of the compound (R_f from solvent front 7.1 min.) in a U-shaped open capillary tube gave 4-trimethylsiloxy-stilbene (1 mg.) as a white crystalline solid. 4-Trimethylsiloxystilbene had $\nu_{\max}$. 2956 and 2851 (CH₃), 1590 (C=C) and 925 cm⁻¹ (C–O–Si) (Nelson & Smith, 1966), $\lambda_{\max}$. in cyclohexane 228, 301 and 316 m μ (ϵ 15100, 29500 and 28200 respectively).

4,4'-Di(trimethylsiloxy)stilbene. A mixture of 4,4'-dihydroxystilbene (5.3 mg.), dry pyridine (0.10 ml.), hexamethyldisilazane (0.05 ml.) and trimethylchlorosilane (0.05 ml.) was allowed to react at room temperature for 10 min. Vapour-phase chromatography of one portion (20 μ l.) of this mixture under the same conditions as described for 4-trimethylsiloxystilbene and collection of the material (R_f from solvent front 28.9 min.) gave 4,4'-di(trimethylsiloxy)stilbene (0.9 mg.) as a white crystalline solid. 4,4'-Di(trimethylsiloxy)stilbene had $\nu_{\max}$. 2958 and 2854 (CH₃), 1603 (C=C) and 922 cm⁻¹ (C–O–Si), $\lambda_{\max}$. in cyclohexane 230, 305 and 322 m μ (ϵ 11000, 27500 and 26300 respectively).

METHODS

Analytical procedures. The separation and quantitative analysis of 2-hydroxy-, 4-hydroxy-, 4,4'-dihydroxy- and 4-hydroxy-3-methoxy-stilbene were performed with the paper-chromatographic systems (1–3), thin-layer-chromatographic systems (1–6), thin-layer-chromatographic fluorimetry (system 6) and gas-liquid-chromatographic method (with trimethylsilyl ethers) described by Sinsheimer & Smith (1967). Two additional thin-layer-chromatographic (silica gel) systems [7, *n*-hexane-chloroform-methanol (10:9:1, by vol.); 8, toluene-piperidine (5:2, v/v)] were developed and used for the analysis of the above hydroxy-stilbenes plus 3-hydroxy-4-methoxy- and 3,4-dihydroxy-stilbene (Table 1). Detection after paper chromatography and thin-layer chromatography was by fluorescence in u.v. radiation (320–400 m μ) after overspraying of the chromatograms with 10% NaOH in aq. 65% ethanol. After development with system 8, thin-layer-chromatographic fluorimetry of 4-hydroxy- and 4,4'-dihydroxy-stilbene (0.1–0.3 μ g. of each) was performed with λ_{fl} . 450 m μ and λ_{exc} . 365 m μ and of 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene (0.1–0.3 μ g. of each) with λ_{fl} . 465 m μ and λ_{exc} . 375 m μ .

Preparation of injections. *trans*-Stilbene (15–30 mg./ml.)

or 4-hydroxystilbene (15 mg./ml.) was dissolved in peanut oil (U.S.P.) by gentle heating. Sterilization of control or stilbene-containing oil was accomplished by passage over a Millipore filter (0.45 μ pore size) (with a Stainless Pressure Filter Holder supplied by Millipore Corp., Bedford, Mass., U.S.A.).

Experiments with animals. Young adult male New Zealand rabbits (2.20–2.85 kg.), adult male albino guinea pigs (Hartley strain, 725–745 g.) and young adult male albino mice (IRC strain, 18–20 g.) (obtained from the Animal-Care Center of the University of Michigan, Ann Arbor, Mich., U.S.A.) were housed in stainless-steel metabolism cages that permitted separation of urine from faeces. During all investigations, animals were allowed to feed on Rockland laboratory-animal diets (Teklad Co., Monmouth, Ill., U.S.A.) and tap water *ad libitum*.

Two young adult male rabbits were injected intramuscularly once daily for 5 days with 2 ml. of peanut oil. Control urine was collected during this time and in subsequent investigations once or twice daily, according to the yield of urine. Collected urine was maintained at –19° to –23° until it was extracted. After the control collection, the same rabbits were injected once daily for 10 days with 60 mg. of *trans*-stilbene (21–26 mg./kg.) in 2 ml. of peanut-oil solution. During this time and for 4 days after the last injection urine was collected.

The above rabbits, after a rest of 4 months, were injected intramuscularly once daily for 5 days with 30 mg. of 4-hydroxystilbene (7 mg./kg.) in 2 ml. of peanut-oil solution. Urine was collected during the days of injections and for 4 days after the last injection.

Three adult male guinea pigs were injected intramuscularly with 1 ml. of peanut oil daily for 3 days. During the injection days and for 36 hr. after the last injection, control urine was collected. After a rest of 4 days, the same animals were each injected for 5 days with 30 mg. of *trans*-stilbene (36–39 mg./kg.) in 1 ml. of peanut-oil solution. During this time, and for 4 days after the last injection, urine was collected.

In the mouse study, control urine was collected for 12 days from 25 albino mice (18–20 g. each). After a 3-month period, the mice were each subcutaneously injected daily with 0.75 mg. of *trans*-stilbene (20 mg./kg., average) in 0.05 ml. of peanut-oil solution, for 10 days. Urine was collected during the injection days and for 48 hr. after the last injection.

Urine extraction and fractionation. Urine, pooled into fractions (1–2.5 l.), was first centrifuged at 600g for 1 hr. The precipitate recovered was dried *in vacuo* and was then continuously extracted with ether in a Soxhlet extractor for 48 hr. Evaporation of this ether extract to dryness *in vacuo* gave fraction A.

The supernatant urine was adjusted to pH 7.0 with conc. HCl and extracted continuously with ether for 48 hr. The ether extract was back-extracted with 5% (w/v) HCl and 5% (w/v) NaHCO₃ (this step was omitted in the mouse study), washed with water and saturated NaCl soln., dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo* to give fraction B.

After ether extraction at pH 7.0, urine was heated on a steam plate to remove dissolved ether. The treated urine was then mixed with conc. HCl (50 ml./l.) and refluxed for 2 hr. The hydrolysed urine was adjusted to pH 7.0 with 30% NaOH and extracted continuously with ether for

48 hr. This extract was treated in the same way as the one from the unhydrolysed urine and gave fraction *C*.

Chromatographic analysis of urine fractions. Fractions *A*, *B* and *C* were examined directly by paper and thin-layer chromatography. In both qualitative and quantitative (thin-layer-chromatographic fluorimetry) analyses, metabolite fractions were compared with controls and reference compounds spotted and developed on the same chromatograms.

Vapour-phase chromatography of metabolite and control fractions as their trimethylsilyl derivatives was performed with reaction mixtures prepared with fraction *A*, *B* or *C* (15–100 mg.), dry pyridine (0.7 ml.), hexamethyldisilazane (0.2 ml.) and trimethylchlorosilane (0.1 ml.). Control fractions were compared with metabolite fractions at equivalent concentrations.

Isolation of metabolically formed 4-hydroxy- and 4,4'-dihydroxy-stilbene as their trimethylsilyl ethers. After trimethylsilylation of portions of metabolite fraction *B* (21–26 mg.) derived from rabbits injected with *trans*-stilbene, the trimethylsilyl derivatives were submitted to preparative vapour-phase chromatography under the conditions described in the Materials section for reference 4-trimethylsiloxystilbene. The compounds (R_f from solvent front 6.5 and 25 min.) were collected in U-shaped open capillary tubes, and i.r. and u.v. analyses were performed on these derivatives as described in the Materials section for the reference trimethylsilyl ethers of 4-hydroxy- and 4,4'-dihydroxy-stilbene.

Microsomal preparations and incubations. Rabbit liver microsomes in 0.25 M-sucrose (1 ml. $\equiv$ 1.25 g. of liver or 15 mg. of protein) and a solubilized liver microsomal preparation (1 ml. $\equiv$ 1.08 g. of liver or 12 mg. of protein) were prepared as described by Lu & Coon (1968).

In the standard procedure, a solution of *trans*-stilbene or 4-hydroxystilbene (0.42–0.50 μ mole) in ethanol (0.02 ml.) was mixed with one of the above-mentioned microsomal preparations (0.5 ml.), 1 M-tris-HCl buffer, pH 7.6 (0.2 ml.), NADP⁺ (1 μ mole), dipotassium glucose 6-phosphate (5 μ moles) and glucose 6-phosphate dehydrogenase (0.2 unit) in a 15 ml. open glass centrifuge tube. Control mixtures were prepared in the same way but with a microsomal preparation that had previously been boiled for 5 min. A

blank mixture was also prepared in the same way but without any substrate. The microsomal, control and blank mixtures (0.97 ml. each) were incubated at 37° for 1.5 hr.

Extraction and analysis of incubation mixtures. Each incubation mixture was treated with 1 N-HCl (1 ml.) to stop the reaction, and exhaustively extracted with ether. The ether layer was washed with water and saturated NaCl soln., dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*.

Thin-layer chromatography on silica-gel-G plates with solvent systems 2, 6, 7 and 8 was used for the qualitative determination of phenolic stilbene metabolites. Quantitative determination was by thin-layer-chromatographic fluorimetry after development with solvent system 8. Extracts of control and blank preparations were compared with extracts of metabolite-incubation mixtures.

RESULTS

***trans*-Stilbene hydroxylations in rabbits.** Fractions *A*, *B* and *C*, obtained from urine of rabbits injected with *trans*-stilbene, were submitted to paper (systems 1–3) and thin-layer (systems 1–7) chromatography. All chromatograms revealed the presence of 4-hydroxy-, 4,4'-dihydroxy- and monomethylated 3,4-dihydroxy-stilbene by fluorescence detection. Both 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene were observed after development in system 7 followed by system 8 (Table 1). Neither 2-hydroxy- nor 3,4-dihydroxy-stilbene was present in the urine fractions.

Identification of the metabolites 4-hydroxy- and 4,4'-dihydroxy-stilbene was also based on isolation of these compounds as their trimethylsilyl ethers (preparative vapour-phase chromatography) and comparison, with excellent agreement, of their i.r. and u.v. spectra with those of reference derivatives.

Quantitative analysis of 4-hydroxy- and 4,4'-dihydroxy-stilbene in fractions *A*, *B* and *C* was through trimethylsilylation and vapour-phase

Table 1. R_F values and fluorescence of some hydroxystilbenes

The hydroxystilbenes were chromatographed on thin-layer-chromatography plates of silica gel G (Warner-Chilcott Laboratories, Morris Plains, N.J., U.S.A.) in the following solvent systems: 7, *n*-hexane-chloroform-methanol (10:9:1, by vol.); 8, toluene-piperidine (5:2, v/v). [These systems were developed in addition to those described as systems 1–6 by Sinzheimer & Smith (1967).] Compounds were detected by fluorescence in u.v. radiation (320–400 μ) after chromatograms were oversprayed with 10% NaOH in aq. 65% ethanol.

Compound	Solvent	...	R_F		Colour reactions fluorescence
			7	8	
2-Hydroxystilbene			0.49	0.47	Yellow
4-Hydroxystilbene			0.39	0.31	Blue
3,4-Dihydroxystilbene*			0.10	0.04	Blue-tan
4,4'-Dihydroxystilbene			0.07	0.21	Bright blue
3-Hydroxy-4-methoxystilbene			0.60	0.37	Orange
4-Hydroxy-3-methoxystilbene			0.60	0.24	Blue-green

* This compound rapidly decomposed when chromatographed.

Table 2. *Hydroxylated metabolites of trans-stilbene and 4-hydroxystilbene*

Male rabbits, guinea pigs and mice were injected with *trans*-stilbene or 4-hydroxystilbene as described in the Methods section. Urine collected during and 4 days after the injections was extracted with ether at pH 7.0, before and after hydrolysis. After back-extraction with 5% HCl and 5% NaHCO₃, the ether extracts were examined for qualitative and quantitative determination of metabolites. Faeces were not examined.

Species	Compound injected	Metabolite	Conversion (%)	
			Free	Conjugated
Rabbit	<i>trans</i> -Stilbene	4-Hydroxystilbene	1.5	1.3
		4,4'-Dihydroxystilbene	2.5	4.6
		3-Hydroxy-4-methoxystilbene	0.1	0.5
		4-Hydroxy-4-methoxystilbene	0.3	0.3
Rabbit	4-Hydroxystilbene	4-Hydroxystilbene*	4.5	2.4
		4,4'-Dihydroxystilbene	9.4	9.3
		3-Hydroxy-4-methoxystilbene	0.3	1.0
		4-Hydroxy-3-methoxystilbene	0.7	0.5
Guinea pig	<i>trans</i> -Stilbene	4-Hydroxystilbene	0.3	1.5
		4,4'-Dihydroxystilbene	3.4	4.1
		Monomethylated stilbene catechol	1.0†	
Mouse	<i>trans</i> -Stilbene	4,4'-Dihydroxystilbene	0.5	0.8

* Material recovered unchanged or conjugated.

† Total conversion for both free and conjugated monomethylated catechol (see the text).

chromatography of these fractions. Assay of 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene was by sequential thin-layer chromatography in systems 7 and 8 followed by thin-layer-chromatographic fluorimetry. The percentage conversions of *trans*-stilbene into 4-hydroxy-, 4,4'-dihydroxy-, 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene in the rabbit, as detected in the urine, are shown in Table 2.

Hydroxylation of 4-hydroxystilbene in rabbits. Fractions A, B and C, obtained from urine of rabbits injected with 4-hydroxystilbene, were submitted to thin-layer chromatography (systems 1, 2, 3, 7 and 8). 4-Hydroxy-, 4,4'-dihydroxy-, 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene were observed in these fractions. Quantitative analyses as described in the above section gave the results shown in Table 2. No 3,4-dihydroxy-stilbene was detected in the above-mentioned fractions.

trans-Stilbene hydroxylations in guinea pigs. Urine fractions A, B and C from guinea pigs injected with *trans*-stilbene were analysed by thin-layer chromatography (systems 1, 2, 3 and 7). Fluorescence detection revealed the presence of 4-hydroxy-, 4,4'-dihydroxy- and monomethylated 3,4-dihydroxy-stilbene in fractions B and C. These fractions were then subjected to sequential thin-layer chromatography with system 7 followed by system 8. However, unlike the rabbit studies, this procedure gave no conclusive indication of 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-stilbene in fractions B and C because of interfering native materials and the apparent low conversion

into these compounds (Table 2). No 2-hydroxy- or 3,4-dihydroxy-stilbene was observed in either fraction B or fraction C.

4-Hydroxy- and 4,4'-dihydroxy-stilbene were assayed by vapour-phase chromatography of trimethylsilyl ethers (Table 2). There was an indication of the formation of monomethylated stilbene catechol in less than 1% yield as estimated by fluorescence detection in thin-layer-chromatographic systems 1 and 3.

trans-Stilbene metabolism in mice. Urinary fractions A, B and C, obtained from mice injected with *trans*-stilbene, were qualitatively analysed by paper (systems 1, 2 and 3) and thin-layer (systems 1, 2, 5 and 6) chromatography. Fractions B and C revealed the presence of 4,4'-dihydroxystilbene in all the solvent systems, but none of the other metabolites found in rabbits and guinea pigs were present.

These results were confirmed by electron-capture vapour-phase-chromatographic analysis of a chloromethyltrimethylsilylated portion of fraction B. The detection limits of such ethers have been shown to be low (Sinsheimer & Smith, 1967). A unique peak (*R_t* 4.75 min. compared with *R_t* 10.1 for the ether of 4,4'-dihydroxystilbene) that did not correspond to the reference stilbenes was detected by this procedure. However, low yields prevented its isolation.

Assay of the conversion into 4,4'-dihydroxy-stilbene in mice in fractions B and C was by thin-layer-chromatographic fluorimetry after development in system 6. The percentage conversion of *trans*-stilbene was 0.5% into free and

0.8% into conjugated 4,4'-dihydroxystilbene (Table 2).

Hydroxylation of trans-stilbene and 4-hydroxystilbene in vitro by rabbit liver microsomes. Ether extracts of rabbit liver microsomes incubated with *trans*-stilbene and 4-hydroxystilbene were submitted to thin-layer chromatography in systems 2, 6, 7 and 8. Extracts of *trans*-stilbene incubation mixtures showed both 4-hydroxy- and 4,4'-dihydroxy-stilbene and no intact *trans*-stilbene, whereas an extract of a 4-hydroxystilbene incubation mixture revealed 4,4'-dihydroxystilbene and substrate, 4-hydroxystilbene. In no instance was 2-hydroxy-, 3,4-dihydroxy-, 3-hydroxy-4-methoxy- or 4-hydroxy-3-methoxy-stilbene observed.

After thin-layer chromatography in system 8, the extracts were subjected to thin-layer-chromatographic fluorimetry with the results shown in Table 3.

Hydroxylation of trans-stilbene and 4-hydroxystilbene in vitro by solubilized rabbit liver microsomes. Ether extracts of incubation mixtures consisting of *trans*-stilbene and 4-hydroxystilbene treated with solubilized rabbit liver microsomes were subjected to thin-layer chromatography in systems 2, 7 and 8.

trans-Stilbene was shown to be converted into 4-hydroxystilbene, whereas 4-hydroxystilbene was converted into 4,4'-dihydroxystilbene. Thin-layer-

chromatographic fluorimetry, after development in system 8, gave the results shown in Table 4.

DISCUSSION

In general, the nature and role of hydroxylations in the metabolism of stilbenes have not been fully established. Elson, Goulden & Warren (1958) examined the excretion of glucuronides after the administration of 4-aminostilbene and 4-acetylaminostilbene to rats. They suggested from their results that 20–40% of the injected dose was converted into hydroxylated compounds, 'probably' 4-amino- or 4-acetamido-4'-hydroxystilbene. Anderson, Enomoto, Miller & Miller (1964) investigated the metabolism of 4-amino- and 4-acetamido-stilbene in rats and found up to 2.1% conversion of each into 4-acetamido-3-hydroxystilbene and an undetermined percentage conversion into a product that they believed was 4-acetamido-4'-hydroxystilbene. Similar studies (Baldwin & Smith, 1961) in the rat led to the qualitative determination of 4-amino-4'-hydroxystilbene as a metabolite of 4-dimethylaminostilbene.

Hydroxylation of trans-stilbene in rabbits. *trans*-Stilbene was reported (Stroud, 1939, 1940) to be excreted (to the extent of 2.5%) in the urine of rabbits as 4,4'-dihydroxystilbene. Our findings

Table 3. *Hydroxylation of trans-stilbene and 4-hydroxystilbene by rabbit liver microsomes*

Rabbit liver microsomes (in 0.25 M-sucrose; 0.5 ml. $\equiv$ 7.5 mg. of protein) from livers of adult New Zealand rabbits were incubated with 0.50 μ mole of *trans*-stilbene or 0.45 μ mole of 4-hydroxystilbene and 0.02 ml. of ethanol, 0.2 ml. of 1 M-tris-HCl buffer, pH 7.6, 1 μ mole of NADP⁺, 5 μ moles of glucose 6-phosphate and 0.2 unit of glucose 6-phosphate dehydrogenase (total volume 0.97 ml.) for 1.5 hr. in air at 37°.

Product ... Compound incubated	Conversion (%)		Yield (m μ moles/mg. of protein/hr.)	
	4-Hydroxystilbene	4,4'-Dihydroxystilbene	4-Hydroxystilbene	4,4'-Dihydroxystilbene
<i>trans</i> -Stilbene	14.6	0.20	6.53	0.08
4-Hydroxystilbene	—*	5.20	—	2.09

* 56.2% of starting 4-hydroxystilbene was recovered.

Table 4. *Hydroxylation of trans-stilbene and 4-hydroxystilbene by solubilized rabbit liver microsomes*

Solubilized rabbit liver microsomes (0.5 ml. $\equiv$ 6.5 mg. of protein) were incubated with 0.42 μ mole of *trans*-stilbene or 4-hydroxystilbene dissolved in 0.02 ml. of ethanol with the buffer-cofactor solution described in Table 3. Incubations were carried out in air for 1.5 hr. at 37°.

Product ... Compound incubated	Conversion (%)		Yield (m μ mole/mg. of protein/hr.)	
	4-Hydroxystilbene	4,4'-Dihydroxystilbene	4-Hydroxystilbene	4,4'-Dihydroxystilbene
<i>trans</i> -Stilbene	0.10	0	0.04	0
4-Hydroxystilbene	—*	0.23	—	0.11

* 95% of starting 4-hydroxystilbene was recovered.

A greater amount of the monomethylated catechols was formed in this experiment than when *trans*-stilbene was administered. Therefore extensive attempts were made with thin-layer chromatography to find 3,4-dihydroxystilbene, the intermediate proposed in Scheme 1. These experiments gave no conclusive evidence for the presence of this compound in any of the fractions chromatographed. However, hydroxylation of phenols to catechols is a well-established reaction in mammals (Williams, 1964), and the apparent absence of 3,4-dihydroxystilbene in these studies is likely to

be a function of its instability; 3,4-dihydroxystilbene, synthesized as reference material, was very unstable in air and readily decomposed when chromatographed. Thus fluorescence was of limited utility in detecting 3,4-dihydroxystilbene, since this compound was rapidly oxidized to non-fluorescent materials that would be indistinguishable from the native urinary products co-chromatographed in the area of this material.

When either *trans*-stilbene or 4-hydroxystilbene was administered to rabbits, the ratio of 3-hydroxy-4-methoxy- to 4-hydroxy-3-methoxy-stilbene was about 1:1. These results differ from those obtained with other catechol compounds in the rabbit. For instance, with protocatechuic acid or 3,4-dihydroxyphenylalanine (DeEds, Booth & Jones, 1957) and homoprotocatechuic acid (Scheline, Williams & Wit, 1960) only *m*-methylated products were formed.

As with *trans*-stilbene, a small amount of 4-hydroxystilbene (1.7%) was converted into 4,4'-dihydroxybiphenyl in the rabbit, as shown by vapour-phase chromatography.

Hydroxylation of trans-stilbene in guinea pigs. The hydroxylated compounds formed from *trans*-stilbene and excreted in the urine of guinea pigs were found to be similar, both qualitatively and quantitatively, to those observed in rabbits (Table 2).

Again, the unstable 3,4-dihydroxystilbene was not found in this study. It is noteworthy that 2-hydroxystilbene was absent both from this investigation and from that of *trans*-stilbene hydroxylations in rabbits. 2-Hydroxystilbene, after thin-layer chromatography and treatment with alkali, shows brilliant yellow fluorescence that makes it readily apparent at concentrations estimated at one-tenth that of any hydroxystilbenes actually found as metabolites.

Hydroxylation of trans-stilbene in mice. The only metabolite identified in the mouse studies was 4,4'-dihydroxystilbene. The low yield of metabolites and the necessity to change the mode of administration from intramuscular to subcutaneous injection limited a comparative study of the metabolism of *trans*-stilbene in this species.

Hydroxylation of trans-stilbene and 4-hydroxystilbene in vitro. The hydroxylations of *trans*-stilbene and 4-hydroxystilbene by rabbit liver microsomes confirmed the major hydroxylations observed in the studies *in vivo*. The conversion of *trans*-stilbene into 4,4'-dihydroxystilbene *in vitro* is noteworthy, since studies with compounds such as biphenyl (Creaven, Parke & Williams, 1962, 1965) and naphthalene (Booth & Boyland, 1958; Posner, Mitoma & Udenfriend, 1961) did not reveal a similar dihydroxylation. The lack of 2-hydroxystilbene *in vitro* is in agreement with the results

obtained *in vivo*. Absence of the monomethylated catechols probably indicates a need for added cofactor or the localization of catechol *O*-methyltransferase in the soluble portion of liver homogenates reported by Axelrod (1965).

The lowered hydroxylase activity observed with the solubilized microsomal preparation may reflect inhibitory activity of the solubilizing reagents. The retention of some 'stilbene hydroxylase' activity by this preparation, however, is encouraging in terms of any future isolation of microsomal enzyme systems.

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REFERENCES

- Anderson, R. A., Enomoto, M., Miller, E. C. & Miller, J. A. (1964). *Cancer Res.* **24**, 128.
- Axelrod, J. (1965). In *Transmethylation and Methionine Biosynthesis*, p. 74. Ed. by Shapiro, S. K. & Schlenk, F. Chicago: University of Chicago Press.
- Baldwin, R. W. & Smith, W. R. D. (1961). *Rep. Brit. Emp. Cancer Campgn.* **39**, 422.
- Booth, J. & Boyland, E. (1958). *Biochem. J.* **70**, 681.
- Creaven, P. J., Parke, D. V. & Williams, R. T. (1962). *Biochem. J.* **85**, 5.
- Creaven, P. J., Parke, D. V. & Williams, R. T. (1965). *Biochem. J.* **96**, 879.
- DeEds, F., Booth, A. N. & Jones, F. T. (1957). *J. biol. Chem.* **225**, 615.
- Drefahl, G. & Fischer, F. (1956). *Liebigs Ann.* **598**, 167.
- Elson, L. A., Goulden, F. & Warren, F. L. (1958). *Brit. J. Cancer*, **12**, 108.
- Grundy, J. (1957). *Chem. Rev.* **57**, 281.
- Hughes, G. M. K., Moore, P. F. & Stebbins, R. B. (1964). *J. med. Chem.* **7**, 511.
- Lu, A. Y. H. & Coon, M. J. (1968). *J. biol. Chem.* **243**, 1331.
- Nelson, R. F. & Smith, J. G. (1966). *TAPPI*, **49**, 215.
- Posner, H. S., Mitoma, C. & Udenfriend, S. (1961). *Arch. Biochem. Biophys.* **94**, 269.
- Scheline, R. R., Williams, R. T. & Wit, J. G. (1960). *Nature, Lond.*, **188**, 849.
- Sinsheimer, J. E. & Smith, R. V. (1967). *J. pharm. Sci.* **56**, 1280.
- Sinsheimer, J. E. & Smith, R. V. (1968). *J. pharm. Sci.* **57**, 713.
- Smith, R. V. (1968). Ph.D. Dissertation: University of Michigan, Ann Arbor. Ann Arbor, Mich.: University Microfilms, no. 68-13405.
- Stroud, S. W. (1939). *Nature, Lond.*, **144**, 245.
- Stroud, S. W. (1940). *J. Endocrin.* **2**, 55.
- Williams, R. T. (1964). In *Biochemistry of Phenolic Compounds*, p. 216. Ed. by Harborne, J. B. London: Academic Press (Inc.) Ltd.