formed might be excreted unconjugated as in the case of benzoic acid arising from toluene. The above urines also gave positive results with iodoform tests, suggesting the possible formation of $Ph.CH(CH_3).CH(OH).CH_3$ and $Ph.CH(CH_3).CH_2.CH(OH).CH_3$.

Finally, in the fourth group, the two tertiary compounds give relatively high glucuronic acid conjugations. In *tert.*-butylbenzene, the only expected reaction is the oxidation of one or more of the methyl groups. The urine obtained after feeding with this compound was only feebly reducing, suggesting that the ester glucuronide, $PhC:(CH_3)_2.CO_2G$, is produced only in small amounts. The high glucuronic acid conjugation, however, could be accounted for by the formation of conjugated $\beta\beta$-dimethyl-β-phenylethanol ($Ph.C(CH_3)_2.CH_2OH$). With *tert.*-pentylbenzene, the reducing urine suggests the excretion of an ester glucuronide either of $Ph.C(CH_3)(C_2H_5).CO_2H$ or of $Ph.C(CH_3)_2.CH_2.CO_2H$.

SUMMARY

1. The glucuronic acid conjugation of nine alkylbenzenes has been studied in rabbits.

2. These alkylbenzenes can be divided into four groups according to the amount and nature of the conjugated glucuronic acid excreted after their administration.

3. These groups are (*a*) toluene, (*b*) ethyl-, *n*-propyl-, and *n*-butyl-benzene, (*c*) *iso*propyl-, *sec.*-butyl-, and *sec.*-pentyl-benzene, and (*d*) *tert.*-butyl- and *tert.*-pentyl-benzene.

4. About 18% of an oral dose of 350 mg./kg. body weight of toluene is eliminated unchanged in the expired air.

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Studies in Detoxication

56. THE METABOLISM OF ALKYLBENZENES. STEREOCHEMICAL ASPECTS OF THE BIOLOGICAL HYDROXYLATION OF ETHYLBENZENE TO METHYLPHENYLCARBINOL

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The oxidation of ethylbenzene to methylphenylcarbinol in rabbits was first observed by Neubauer (1901). This observation was confirmed by Thierfelder & Daiber (1923), who also showed that acetophenone was reduced in the rabbit to the same carbinol. Since the formation of methylphenylcarbinol involves the production of an asymmetric carbon atom, the stereochemical implications of the oxidation of ethylbenzene become important. Attempts to deal with this aspect were made by Thierfelder & Daiber (1923) and Thierfelder & Klenk (1924*a*), and they suggested that ethylbenzene and acetophenone were metabolized to the same stereoisomer of methylphenylcarbinol. We shall show, however, that ethylbenzene is hydroxylated in the rabbit to both stereoisomers of methylphenylcarbinol, whereas acetophenone is reduced to only one form, namely (−)-methylphenylcarbinol. A preliminary account of this work has been published (Smith, Smithies & Williams, 1953).

EXPERIMENTAL

Materials and Methods

Materials. The compounds used, except styrene epoxide, were commercial products which were purified before use. The ethylbenzene had b.p. 134–136°, acetophenone, 202°; (±)-methylphenylcarbinol, 204–206°; β-phenylethanol, 220°; and styrene, 146°. Styrene epoxide, b.p. 188–192°, was prepared according to Hickinbottom (1948). (−)-Methylphenylcarbinol, b.p. 204°, $[\alpha]_D^{20} - 47°$ in ethanol (*c*, 1), was prepared according to Downer & Kenyon (1939) by resolution from the (±) carbinol.

Analytical methods. The chinchilla rabbits (3 kg. wt.) used were maintained on an unvarying diet of 70 g. rat cubes (diet 41; Associated London Flour Millers) and 100 ml. water per day, and their urine was analysed daily. The compounds studied were mixed with water and administered by stomach tube. All the compounds administered except phenylethanol and styrene were well tolerated. β-Phenylethanol and styrene seemed to affect the appetite of the animals. Glucuronic acid was estimated by the modification of the naphthoresorcinol method described by Paul (1951), and ethereal sulphate turbidimetrically according to Sperber (1948) but using standard curves based on the recovery of potassium *p*-nitrophenylsulphate from rabbit urine.

Isolation of metabolites

(Melting points are corrected, and all optical rotations are for 1% solns. in $CHCl_3$, unless otherwise stated, and are accurate to ±1°.)

After administration of (−)-methylphenylcarbinol. This carbinol (2 g.) was fed to a black rabbit. The animal became drowsy after 10 min. but soon recovered. The 24 hr. urine (155 ml.) contained about 17% of the dose of carbinol as glucuronide (otherwise known as glucosiduronic acid, see previous paper, p. 317). The glucuronide gum (0·4 g.) was isolated via the basic lead salt (Kamil, Smith & Williams, 1951). This was methylated with ethereal diazomethane, and the product acetylated with pyridine and acetic anhydride. The mixture was poured into water (10 ml.) and after several hours the solid (0·16 g., $[\alpha]_D^{15} - 82°$) was collected. After recrystallization from 90% ethanol, the *methyl* ((−)-*methylphenylcarbinyl-tri*-O-*acetyl*-β-D-*glucosid*)*uronate* formed colourless needles, m.p. 117°; $[\alpha]_D^{15} - 82·5°$ in $CHCl_3$ (*c*, 1). (Found: C, 57·9; H, 6·1. $C_{21}H_{26}O_{10}$ requires C, 57·5; H, 6·0%.)

The conjugation of the carbinol in this particular animal seemed unexpectedly low. In another rabbit which received 1 g. of the (−)-carbinol, the glucuronic conjugation was 47% of the dose.

After administration of (±)-methylphenylcarbinol. Thierfelder & Klenk (1924*a*) injected (±)-methylphenylcarbinol into rabbits and found it to yield mainly hippuric acid and methylphenylcarbinyl glucuronide, and a small amount of mandelic acid. The glucuronide (25% of the dose) was isolated as a monohydrated potassium salt with $[\alpha]_D - 117·3°$ (in water). The large rotation of this compound suggests that Thierfelder & Klenk isolated the (−)-form of methylphenylcarbinyl glucuronide only. However, both isomers of this glucuronide are formed on feeding with the (±)-carbinol, and these were isolated as follows.

Quantitative measurements showed that about 50% of the administered carbinol is excreted as glucuronides (see Table 1). A total of 24 g. of the (±)-carbinol were fed to twelve rabbits and their urine was collected for 24 hr. The glucuronide gum (27 g., ethanol soluble), isolated in the usual way by the lead acetate procedure, was methylated and acetylated to yield the triacetyl methyl ester. This ester (18·84 g. equivalent to 22% of the dose of carbinol) had $[\alpha]_D^{20} - 51°$ and m.p. 90–95° and appeared to contain almost equal amounts of the (+)- and (−)-glucuronides. Two recrystallizations of this material from methanol yielded 8·4 g. of ester (fraction *A*) with $[\alpha]_D^{20} - 72°$, and two further recrystallizations of *A*, from 90% ethanol and 90% methanol, respectively, yielded 6·2 g. of the pure methyl ((−)-methylphenylcarbinyl-tri-*O*-acetyl-β-D-glucosid)uronate, m.p. and mixed m.p. 117° and $[\alpha]_D^{20} - 83°$. (Found: C, 57·3; H, 5·6%.) The mother liquors from fraction *A* were evaporated and the residue was repeatedly recrystallized from methanol until the m.p. and $[\alpha]_D$ became constant. After ten crystallizations, 1·54 g. of the pure *methyl* ((+)-*methylphenylcarbinyl-tri*-O-*acetyl*-β-D-*glucosid*)*uronate* were obtained as colourless needles, m.p. 129° and $[\alpha]_D^{20} - 15°$ in $CHCl_3$ (*c*, 1). (Found: C, 57·5; H, 5·8; $C_{21}H_{26}O_{10}$ requires C, 57·5; H, 6·0%.)

In another experiment in which 12 g. of the carbinol were fed, the unresolved triacetyl methyl ester had $[\alpha]_D^{18} - 50·2°$. Thus, in both experiments, the unresolved ester contained roughly equal parts of the (+)- and (−)-forms, since the specific rotation of the (±)-form calculated from the rotations of the pure (+)- and (−)-forms is −49°. It is concluded from these experiments that both forms of methylphenylcarbinol are equally well conjugated.

After administration of acetophenone. Thierfelder & Klenk (1924*b*) concluded that about 50% of a dose of acetophenone is excreted by rabbits as a glucuronide. This figure agrees with our finding of 47% (see Table 1). The glucuronide of acetophenone urine was isolated by Thierfelder & Daiber (1923) as the monohydrated potassium salt of methylphenylcarbinyl glucuronide with $[\alpha]_D - 124·4°$ (in water). This high rotation suggests that it is the glucuronide of (−)-methylphenylcarbinol. It is likely that Thierfelder & Daiber (1923) did not appreciate this, because when they hydrolysed the glucuronide they obtained a racemized methylphenylcarbinol of $[\alpha]_D + 0·7°$, whereas the pure isomers of this carbinol show $[\alpha]_D$ about ±43°. It is now shown that acetophenone yields only (−)-methylphenylcarbinyl glucuronide, identical with that obtained by feeding with (−)-methylphenylcarbinol.

The 24 hr. urine of four rabbits, which had, between them, received 8 ml. of acetophenone, yielded 7·5 g. of glucuronide gum by the method of isolation by basic lead acetate. This was converted into the triacetyl methyl ester. The crude ester (5 g.) had $[\alpha]_D^{20} - 79·2°$, and one recrystallization from ethanol yielded the pure methyl ((−)-methylphenylcarbinyl-tri-*O*-acetylglucosid)uronate, m.p. and mixed m.p. 117°; $[\alpha]_D^{20} - 83·5°$. (Found: C, 57·6; H, 6·1.) This experiment was repeated twice, with similar results. No evidence was obtained for the presence of the (+)-isomer in the mother liquors of the (−)-isomer.

After administration of ethylbenzene. Six rabbits between them received 15 g. of ethylbenzene by mouth, and the ether-soluble glucuronide gum (15·6 g.) of the urine, collected during the next 36 hr., was prepared via the basic lead salt. The gum yielded 12·7 g. of crude triacetyl methyl ester of $[\alpha]_D - 42°$. Four recrystallizations of this ester from methanol yielded 2·5 g. of the pure methyl ((−)-methylphenylcarbinyl-tri-*O*-acetylglucosid)uronate, m.p. and mixed m.p. 117°; $[\alpha]_D^{21} - 82°$. (Found: C, 57·8; H, 6·3.)

The mother liquors from the (−)-ester were evaporated and the residue was fractionally crystallized from methanol. This yielded 2·7 g. of material, $[\alpha]_D$ −27°. This was recrystallized 7 times from 90% aqueous methanol yielding 0·15 g. of the methyl ((+)-methylphenylcarbinyl-tri-*O*-acetylglucosid)uronate, m.p. 127°, which did not depress the m.p. of the authentic compound prepared by feeding with (±)-methylphenylcarbinol. By working up mother liquors, a total of 0·7 g. of the (+)-isomer was eventually obtained; $[\alpha]_D^{20}$ −13·5°. (Found: C, 57·1; H, 5·9%.)

In two other experiments, 19·5 g. and 20 g. of ethylbenzene yielded 10·6 and 10 g. of the crude triacetyl methyl ester, $[\alpha]_D^{20}$ −50·3° and $[\alpha]_D^{20}$ −47°, respectively. In both these experiments the pure (−)-isomer was isolated, but complete purification of the (+)-isomer was not successful, although material with $[\alpha]_D$ −20 to −30° was obtained.

After administration of β-phenylethanol. The conjugation of phenylethanol with glucuronic acid was so low that no attempt was made to isolate the glucuronide, which might have been 2-phenylethyl glucuronide. The excretion of ether-soluble acid, determined according to Bray, Neale & Thorpe (1946), was high (61 and 76% of the dose of 0·46 g./kg. body weight in two experiments). As expected, this alcohol was oxidized rapidly to phenylacetic acid, as shown by the isolation of phenaceturic acid by ether extraction of the urine. The conjugate with glycine was recrystallized from 80% aqueous ethanol and had m.p. and mixed m.p. 143–144°.

After administration of styrene. According to Spencer, Irish, Adams & Rowe (1942), some 50–90% of styrene are oxidized in the animal to benzoic acid. In our experiments, we found also small amounts of glucuronide (see Table 1). Attempts were made to isolate the glucuronide, but crystalline material could not be obtained. After the glucuronide gum had been methylated and acetylated, a triacetyl methyl ester which was not a derivative of phenylmethylcarbinol was obtained as a non-crystalline solid with a high positive rotation ($[\alpha]_D^{19}$ +41·5°).

After administration of styrene epoxide. The conjugation of this compound with glucuronic acid was similar to that of styrene. The urine of a rabbit which had received 1·5 g. of styrene epoxide had a strongly positive rotation. In a 2 dm. tube, the urine, which had been diluted to 250 ml., acidified, treated with charcoal and filtered, had α_D +0·28°.

RESULTS

The glucuronic acid and ethereal sulphate excretions for the six compounds mentioned above are given in Table 1. In all cases the ethereal sulphate excretion suggests that none of these compounds is hydroxylated in the aromatic ring to any significant extent. Only acetophenone shows an ethereal sulphate output above 1%. All compounds, however, give rise to glucuronides, particularly ethylbenzene (32%), acetophenone (47%) and (±)-methylphenylcarbinol (50%). The excretion of these glucuronides is practically complete one day after dosing. The glucuronic acid conjugation of β-phenylethanol is low (7%), as was expected and is similar to that found for primary aliphatic alcohols (Kamil, Smith & Williams, 1953). This alcohol appears to be mainly oxidized to phenylacetic acid, since the excretion of ether-soluble acids is high, and phenaceturic acid can be readily isolated from the urine. Styrene and styrene epoxide both show low, but significant, glucuronic acid conjugations of about 15% and form a glucuronide of unknown constitution.

DISCUSSION

Thierfelder & Daiber (1923) and Thierfelder & Klenk (1924*a*) found that, when (±)-methylphenylcarbinol, acetophenone, or ethylbenzene, was administered to rabbits, the same methylphenylcarbinylglucuronide could be isolated from the urine as a potassium salt. On hydrolysis with 2% sulphuric acid, the glucuronide obtained from the urine of animals which had received acetophenone yielded a methylphenylcarbinol with $[\alpha]_D$ +0·7°, whereas the (+)-isomer has $[\alpha]_D$ about +43° (undiluted). Thierfelder and his co-workers thought that acetophenone and ethylbenzene were converted *in vivo* into both isomers of methylphenylcarbinol, because acetophenone and ethylbenzene yielded the same glucuronide as (±)-methylphenyl-

Table 1. *The glucuronic acid and ethereal sulphate conjugation of rabbits receiving ethylbenzene and related compounds*

Compound administered	Dose (mg./kg.)	Glucuronic acid (% of dose)		Ethereal sulphate (average % of dose)
		Individual experiments	Average	
Ethylbenzene	433	—	32*	0*
Acetophenone	450	37, 52, 53	47	3
(±)-Methylphenylcarbinol	460	40, 54, 55	50	0
β-Phenylethanol	460	8, 8, 6	7	0
Styrene†	391	(16)‡	—	(1)‡
Styrene epoxide	421	17, 14	15	0

* See preceding paper (Smith, Smithies & Williams, 1954).

† Ether-soluble acid (mainly hippuric acid) excreted was 40% of the dose.

‡ One animal only; two other animals fed with styrene lost their appetite, and therefore estimations were not continued. Styrene at this dose appeared to have a toxic effect.

carbinol, and because hydrolysis of the glucuronide from acetophenone urine yielded a methylphenylcarbinol which was almost optically inactive. Thierfelder and his co-workers must have believed also that the potassium salt of methylphenylcarbinyl glucuronide, which they isolated and which had $[\alpha]_D - 124°$ (in water), was a derivative of (±)-methylphenylcarbinol. However, in view of its high rotation, this salt must be the potassium salt of (−)-methylphenylcarbinyl glucuronide. This is supported by our observation that the methyl ((−)-methylphenylcarbinyl-tri-*O*-acetylglucosid)-uronate has $[\alpha]_D - 83°$. It seems probable that when Thierfelder & Daiber (1923) hydrolysed their glucuronide, the liberated carbinol was racemized by the acid (cf. McKenzie & Clough, 1913). In the present work, the possibility of racemization has been avoided by working entirely with glucuronides.

This investigation shows that ethylbenzene is hydroxylated in rabbits to both isomers of methylphenylcarbinol, since the glucuronides of both (+)- and (−)-methylphenylcarbinols can be isolated from the urine as the triacetyl methyl esters. In fact, ethylbenzene appears to yield approximately the same mixture of glucuronides as when (±)-methylphenylcarbinol is fed. In both cases the triacetyl methyl ester isolated had $[\alpha]_D - 42$ to $-50°$. An equal mixture of the methyl esters of triacetyl (+)- and (−)-methylphenylcarbinyl glucuronides should have $[\alpha]_D - 49°$, since the derivative of the (+)-isomer has $[\alpha]_D - 15°$ and that of the (−)-isomer $[\alpha]_D - 83°$. It thus appears that ethylbenzene yields (+)- and (−)-methylphenylcarbinols in equal amounts and that both carbinols are equally well conjugated *in vivo*. Acetophenone, on the other hand, yields only one methylphenylcarbinyl glucuronide, which is identical with that obtained on feeding (−)-methylphenylcarbinol. It is interesting to note that (−)-methylphenylcarbinol is related to L(+)-alanine and hence to L(−)-glyceraldehyde and L(+)-lactic acid (cf. Brewster, Hughes, Ingold & Rao, 1950; Brewster, Hiron, Hughes, Ingold & Rao, 1950). L(+)-Lactic acid is the form produced biologically from pyruvic acid in animal tissues, and it appears that the carbonyl groups of acetophenone and pyruvic acid are asymmetrically reduced *in vivo* to alcohols of the same configuration.

The mechanism of the biological hydroxylation of ethylbenzene to methylphenylcarbinol is not known. One possible path via styrene and acetophenone,

$$\mathrm{Ph.CH_2.CH_3} \xrightarrow{-2H} \mathrm{Ph.CH{:}CH_2} \xrightarrow{+O} \mathrm{Ph.CO.CH_3} \xrightarrow{+2H} \mathrm{Ph.CH(OH).CH_3},$$

is not likely to be of significance, since styrene does not yield methylphenylcarbinol (Spencer *et al.* (1942) state that 50–90% of styrene is metabolized to benzoic acid) and acetophenone yields only(−)-methylphenylcarbinol. Another path involving styrene epoxide,

$$\mathrm{Ph.CH_2.CH_3} \xrightarrow{-2H} \mathrm{Ph.CH{:}CH_2} \xrightarrow{+O} \mathrm{Ph.CH(O)CH_2} \xrightarrow{+2H} \mathrm{Ph.CH(OH).CH_3},$$

seemed possible because an epoxide had been isolated as a metabolite of the insecticide Heptachlor (1:4:5:6:7:10:10-heptachloro-4:7-*endo*methylene-4:7:8:9-tetrahydroindane; Radomski & Davidow, 1953*a*, *b*) and because styrene epoxide can be reduced to methylphenylcarbinol by lithium aluminium hydride (Trevoy & Brown, 1949). However, neither styrene nor its epoxide yields this carbinol in the rabbit and it seems unlikely therefore that styrene epoxide is an intermediate in the hydroxylation of ethylbenzene.

Ethylbenzene might be expected to undergo ω-oxidation yielding eventually phenylacetic acid,

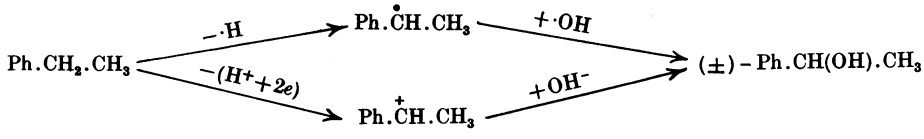
$$\mathrm{Ph.CH_2.CH_3} \longrightarrow \mathrm{Ph.CH_2.CH_2.OH} \longrightarrow \mathrm{Ph.CH_2.CO_2H}.$$

However, neither phenylacetic nor phenaceturic acid has yet been found in ethylbenzene urine, although β-phenylethanol is largely converted into phenaceturic acid. Thierfelder & Daiber (1923) and Thierfelder & Klenk (1924*a*) found both isomers of mandelic acid to be formed in small amounts from ethylbenzene, (±)-methylphenylcarbinol and acetophenone. The stereochemistry of the formation of mandelic acid, however, has not yet been satisfactorily worked out, since the extent of its formation appears to be small (about 1–2%). Benzoic acid (about 25% of the dose) is also a metabolite of ethylbenzene (Thierfelder & Daiber, 1923) and a possible scheme for its formation, which is suggested by the evidence available, is as follows:

$$\mathrm{Ph.CH_2.CH_3} \longrightarrow \mathrm{Ph.CH(OH).CH_3} \longrightarrow \mathrm{Ph.CH(OH).CO_2H} \longrightarrow \mathrm{Ph.CO_2H}.$$

According to this scheme, the initial oxidation of ethylbenzene occurs entirely at the α-methylene group, ω-oxidation being a secondary reaction resulting in mandelic acid. This position of hydroxylation of ethylbenzene is, however, not unexpected, since the methylene group vicinal to the benzene ring is known to be specially reactive (cf. Waters, 1945). Most alkylbenzenes are autoxidized at this carbon atom (Waters, 1945) and uncatalysed chlorination also takes place at this point (cf. Fieser & Fieser, 1950). It is to be expected, therefore, that a number of alkylbenzenes will, in general, be hydroxylated *in vivo* at the carbon atom vicinal to the benzene ring if it carries a replaceable hydrogen atom. This point is being investigated.

Since it appears that neither acetophenone, nor styrene, nor styrene epoxide, are intermediates in the formation of (±)-methylphenylcarbinol from ethylbenzene *in vivo*, the only suggestion we can make concerning the mechanism of the reaction is that hydrogen is abstracted from the reactive α-methylene group by an enzyme, leaving a free radical or a carbonium ion. This could then combine with ·OH or OH^-, respectively, to form methylphenylcarbinol, which would be expected to be racemic, since tervalent-carbon free radicals are planar, and when a three-covalent carbon atom carries a positive charge the valencies are coplanar (cf. Barnett, 1950), as shown above.



When ethylbenzene is oxidized chemically by processes which proceed by a free radical mechanism, methylphenylcarbinol, acetophenone, and 3:3-diphenylbutane, may be produced (cf. Emerson *et al.* 1948).

SUMMARY

1. (±)-Methylphenylcarbinol is partly excreted by rabbits as (±)-methylphenylcarbinyl glucuronide, which can be resolved into the (+)- and (−)-diastereoisomers by fractional crystallization of the triacetyl methyl esters.

2. Acetophenone is reduced in rabbits to (−)-methylphenylcarbinol, which is excreted as a glucuronide which is identical with the glucuronide isolated after feeding with the (−)-carbinol.

3. Ethylbenzene gives rise to both (+)- and (−)-methylphenylcarbinol in rabbits. The carbinols were characterized as the triacetyl methyl esters of the corresponding glucuronides.

4. Ethylbenzene does not undergo oxidation in the benzene ring *in vivo*.

5. Styrene and styrene epoxide do not appear to yield methylphenylcarbinol in rabbits.

6. Phenylacetic and phenaceturic acids were not detected as metabolites of ethylbenzene, but phenaceturic acid was isolated as the main metabolite of β-phenylethanol.

7. The mechanism of the biological oxidation of ethylbenzene is discussed and it is suggested that this hydrocarbon is initially oxidized at the active methylene group to yield (±)-methylphenylcarbinol, which could then yield mandelic and benzoic acids.

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