Paschkis, 1929) can be explained by the high protein reserves present in the livers of these animals (Kosterlitz, 1947). It would seem that insulin has a specific effect in preventing protein breakdown, for it is known that it can prevent the increase of amino-acids in the blood of eviscerated dogs, an effect which is independent of the blood-sugar level (Mirsky, 1938; Frame & Russell, 1946; Ingle, Prestrud & Nezamis, 1947).

This investigation has given evidence that during insulin hypoglycaemia part of the free glutamic acid and glutamine present in the liver is metabolized for energy production.

SUMMARY

1. The amounts of glutamine and glutamic acid present in the rat liver are markedly reduced by the injection of insulin.

2. The level of free ammonia found in the liver is also reduced after insulin administration, but this is probably a post-mortem effect and due to the reduced glutamine content.

3. The decreases appear to be linked with the associated hypoglycaemia, for they are largely prevented by glucose ingestion.

4. The decreases are not related to the adrenaline release caused by the hypoglycaemia, as they occur in rats after adrenal demedullation.

5. No change could be detected in the concentration of the other free amino-acids or of the proteins of the liver during insulin hypoglycaemia.

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Metabolism of Derivatives of Toluene

5. THE FATE OF THE XYLENOLS IN THE RABBIT, WITH FURTHER OBSERVATIONS ON THE METABOLISM OF THE XYLENES

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In an investigation of the fate of the xylenes in the rabbit (Bray, Humphris & Thorpe, 1949), evidence was obtained that these compounds give rise to varying amounts of phenolic excretion products, but their nature was not determined. It is theoretically possible that they may be either xylanols or hydroxytoluic acids. Since toluic acids do not appear to be hydroxylated in the rabbit (Bray, Thorpe & Wood, 1949), it seemed likely that xylanols might be found as the primary hydroxylation products of the xylenes. It was hoped that an investigation of

the fate of the xylanols in the rabbit might help to elucidate the nature of the minor metabolites of the xylenes.

The metabolic fate of the xylanols does not appear to have been investigated previously. These compounds possess two similar 'potential centres for conjugation' (cf. Bray, Ryman & Thorpe, 1948), and in the light of the results obtained from our earlier investigations (summarized by Thorpe, 1950), it was expected that they would follow similar metabolic pathways to those taken by the cresols

(Bray, Thorpe & White, 1950*b*) since, by analogy with the xylenes (Bray, Humphris & Thorpe, 1949), it seemed unlikely that oxidation of both methyl groups in individual molecules would occur to any considerable extent. Consequently, the principal metabolites of the xyenols expected were xylenyl sulphates and glucuronides, whilst dihydroxyxylenes and hydroxytoluic acids might occur as minor metabolites.

In the investigation reported here, the increases in unconjugated non-acidic phenol, ether-soluble acid, ethereal sulphate and ester and ether glucuronides caused by administration of 2:3-, 2:4-, 2:5-, 2:6-, 3:4- and 3:5-xylen-1-ols have been determined. Crystalline glucuronides have been isolated as metabolites of all six xyenols. Small amounts of the xyenols were found to be excreted unconjugated. Examination of ether extracts of xyenol urines by paper chromatography has shown the presence of small quantities of both phenolic and reducing substances in addition to those present in normal urine.

The excretion of non-acidic phenols by rabbits which had received the xylenes has also been estimated, and the xyenol excreted as a metabolite of *p*-xylene has been isolated and characterized.

MATERIALS AND METHODS

Diet and dosage. The rabbits used were does weighing 2–3 kg. The diet used throughout was that of rabbit pellets and water previously described (Bray, Ryman & Thorpe, 1947). The xylenols (Light and Co.) were administered by stomach tube as suspensions in water. In doses of 1 g. they had, in general, no appreciable toxic effect, though occasionally 2:3- and 2:6-xylen-1-ols caused narcosis lasting for periods up to 1 hr. As with the cresols, the toxic effects could be minimized by allowing the rabbits their daily food ration before administration of the dose.

2:5-Dihydroxy-1:3-dimethylbenzene was prepared from 5-amino-1:3-dimethylbenzene (British Drug Houses Ltd.) by the method of Nölting & Forel (1885).

Estimation of metabolites of xylenols. The excretion of ester and ether glucuronides, ether-soluble acid, ethereal sulphate and non-acidic phenol was estimated as previously described for the cresols (Bray *et al.* 1950*b*). Unconjugated phenol only was estimated in xyenol urines, but both free and conjugated in xylene urines. The appropriate xyenol was used as standard in each case.

RESULTS

Quantitative studies

Normal excretion of metabolites. The average daily excretion for individual rabbits was: free non-acidic phenol, 12–18 mg. (calculated as xyenol); total non-acidic phenol, 90–99 mg. (calculated as xyenol); ethereal sulphate, 32–49 mg. SO₃; ether-soluble acid, 441–1055 mg. (calculated as hippuric acid) and

reducing material, 159–236 mg. (calculated as glucuronic acid). The average differences between individual values and corresponding weekly averages were $\pm 18\%$ (3 mg.), $\pm 7\%$, $\pm 10\%$ (4 mg.), $\pm 5\%$ and $\pm 7\%$, respectively.

Table 1. *Excretion of metabolites of xylenols in the rabbit*

(Results are expressed as percentage of dose which was 1 g. in all cases except for 2:4-xylen-1-ol, where it was 850 mg.)

300 mg./

	No. of experiments	Excretion	
		Range (%)	Average (%)
2:3-Xylen-1-ol:			
Free non-acidic phenol	4	0.4-1.6	1
Ethereal sulphate	3	11-14	13
Ester glucuronide	6	0-3	1
Ether glucuronide	6	37-69	50
Ether-soluble acid	6	55-86	69
2:4-Xylen-1-ol:			
Free non-acidic phenol	4	0-5	2
Ethereal sulphate	3	12-14	13
Ester glucuronide	6	0-4	1
Ether glucuronide	7	35-56	46
Ether-soluble acid	6	49-75	64
2:5-Xylen-1-ol:			
Free non-acidic phenol	4	1-4	3
Ethereal sulphate	5	11-21	14
Ester glucuronide	13	0-5	1
Ether glucuronide	9	45-75	58
Ether-soluble acid	10	47-87	69
2:6-Xylen-1-ol:			
Free non-acidic phenol	4	0-2	1
Ethereal sulphate	3	16-17	16
Ester glucuronide	6	0-5	1
Ether glucuronide	6	53-74	65
Ether-soluble acid	6	68-84	77
3:4-Xylen-1-ol:			
Free non-acidic phenol	4	2-5	3
Ethereal sulphate	3	8-9	8
Ester glucuronide	13	0-8	2
Ether glucuronide	8	50-73	65
Ether-soluble acid	8	58-76	69
3:5-Xylen-1-ol:			
Free non-acidic phenol	4	0-3	1
Ethereal sulphate	3	9-12	10
Ester glucuronide	15	0-15	2
Ether glucuronide	6	58-76	72
Ether-soluble acid	6	73-91	83

Metabolites of xylenols. The results obtained are summarized in Table 1. There is an overall similarity between the metabolism of the xylenols and that of the cresols (Bray *et al.* 1950*b*), the major portion of the dose (77–93 %) being excreted as *O*-conjugates. Since xylenylglucuronides are readily soluble in ether, it would be expected that the values for both ether-soluble acid and ether glucuronide would be due mainly to xylenylglucuronide. It is seen from Table 1 that the values for ether glucuronide are somewhat lower than those for the ether-soluble acid.

This difference probably arises from decomposition occurring during the preliminary hydrolysis which is carried out in 5N-sulphuric acid. This was found to give maximum liberation of reducing material from xylanol urines, but control experiments, using pure xylenylglucuronides, gave results corresponding to recoveries of glucuronic acid of 58–93 %, as shown in Table 2, presumably due to decomposition under

Table 2. *Percentage recovery of glucuronic acid liberated by hydrolysis of xylenylglucuronides*

(Hydrolysis with 5N-H₂SO₄ for 1.5 hr. in boiling-water bath; glucuronic acid estimated by reducing value.)

	Recovery (%)
2:3-Dimethylphenylglucuronide	84
2:4-Dimethylphenylglucuronide	64
2:5-Dimethylphenylglucuronide	58
2:6-Dimethylphenylglucuronide	93
3:4-Dimethylphenylglucuronide	77
3:5-Dimethylphenylglucuronide	89

the rather drastic conditions of hydrolysis. Thus the ether glucuronide values are probably low, and if corrections based on the control experiments are applied, values in most cases in better accord with the ether-soluble acid results are obtained. The extra excretion of ester glucuronides is very small with all the isomers, and while this may be due to traces of glucuronides of hydroxytoluic acids, it is difficult to distinguish it with certainty from the normal fluctuations in the base-line values for ester glucuronide excretion. With regard to the excretion of free non-acidic phenol (xylenols), it will be shown later that these compounds were isolated from xylanol urines as collected. Control experiments showed that the amount of free xylanol did not increase appreciably as the extraction time was increased from 6 to 24 hr., so that it seems unlikely that the material isolated is formed during extraction owing to decomposition of conjugates.

Qualitative studies

Isolation of unconjugated xylanols. Continuous ether extraction for 24 hr. of 24 hr. xylanol urines as collected (pH 7.8–8.0) gave in all cases dark-brown, partially crystalline syrups, having the characteristic smell of xylanol. Attempts at purification of these products by recrystallization were unsuccessful, and the free xylanols were finally isolated as carbanilates. The syrup was heated for 10 min. in a boiling-water bath with phenyl isocyanate (1 ml. for the syrup from the urine of four rabbits, corresponding to a total dose of 4 g. xylanol). After cooling, the carbanilate, together with unreacted isocyanate and some carbanilide, was extracted with light petroleum (b.p. 60–80°) and the solvent removed under reduced pressure. The solid residue

was recrystallized from light petroleum and in every case the crystals obtained were shown by comparison with authentic specimens to be the carbanilate of the xylanol administered. The observed melting points agreed with those quoted by Johnson, Shennan & Reed (1944), which are given in parentheses, and were: 2:3-xylan-1-ol, 176° (176°); 2:4-xylan-1-ol, 99° (102°); 2:5-xylan-1-ol, 161° (162°); 2:6-xylan-1-ol, 132° (133°); 3:4-xylan-1-ol, 118° (120°); 3:5-xylan-1-ol, 150° (150°). Yields, 10–20 mg. from urine corresponding to doses of 2–3 g. of the xylanol.

Isolation of xylenylglucuronides. In the isolation of these conjugates advantage was taken of their solubility in ether. 3:5-Dimethylphenylglucuronide could be obtained crystalline by direct extraction of the acidified urine with ether, but the following procedure was found more suitable and applicable to all the xylanols: The crude lead salt of the glucuronide was isolated from the urine by the usual procedure (cf. Bray *et al.* 1947), suspended in water and the free acid liberated by treatment with hydrogen sulphide. After removal of lead sulphide the filtrate was extracted continuously with ether. Evaporation of solvent from the extract left a light-brown syrup which crystallized on standing. The crystals were washed by repeated centrifugation with ether and finally recrystallized by dissolving in a minimum volume of absolute ethanol or acetone, and adding light petroleum (b.p. 60–80°) until a slight cloudiness was procured. Each glucuronide formed fine silky colourless needles. Yield about 2 g. from 8 g. xylanol. They had the following properties:

2:3-Dimethylphenylglucuronide hemihydrate, m.p. 156°, $[\alpha]_D^{20} - 74.2^\circ$ in water (c, 0.4). (Found: C, 55.2; H, 6.4; equiv. by titration, 326. C₁₄H₁₈O₇·½H₂O requires C, 54.8; H, 6.2%; equiv. 307.) 2:4-Dimethylphenylglucuronide dihydrate, m.p. 78°, $[\alpha]_D^{21} - 138^\circ$ in water (c, 0.25). (Found: C, 50.3; H, 6.7; equiv. 330. C₁₄H₁₈O₇·2H₂O requires C, 50.2; H, 6.6%; equiv. 334.) 2:5-Dimethylphenylglucuronide hemihydrate, m.p. 154°, $[\alpha]_D^{21} - 97^\circ$ in water (c, 0.25). (Found: C, 55.2, 55.3; H, 6.2, 6.4; equiv. 304. C₁₄H₁₈O₇·½H₂O requires C, 54.8; H, 6.2%; equiv. 307.) 2:6-Dimethylphenylglucuronide, m.p. 185°, $[\alpha]_D^{21} - 50^\circ$ in water (c, 0.25). (Found: C, 56.6; H, 6.2; equiv. 315. C₁₄H₁₈O₇ requires C, 56.2; H, 6.0%; equiv. 298.) 3:4-Dimethylphenylglucuronide hemihydrate, m.p. 142°, $[\alpha]_D^{21} - 100^\circ$ in water (c, 0.25). (Found: C, 54.8, 55.0; H, 6.4, 6.2; equiv. 315. C₁₄H₁₈O₇·½H₂O requires C, 54.8; H, 6.2%; equiv. 307.) 3:5-Dimethylphenylglucuronide monohydrate, m.p. 165–170°, $[\alpha]_D^{20} - 78^\circ$ in water (c, 1). (Found: C, 53.0; H, 6.5; equiv. 317. C₁₄H₁₈O₇·H₂O requires C, 53.2; H, 6.3%; equiv. 316.)

Paper chromatography of ether extracts of xylanol urines

Using the paper chromatographic technique previously described (Bray, Thorpe & White, 1950a), but with benzene saturated with formic

acid as the developing solvent, it was shown that ether extracts of all the xylénol urines contain phenolic compounds in addition to those found in normal rabbit urine. The identification of these compounds is hindered by the lack of the appropriate reference compounds. One compound, obtained as a metabolite of both 2:6- and 3:5-xylén-1-ol, has been identified as 2:5-dihydroxy-1:3-dimethylbenzene by comparison with an authentic specimen. It seems probable that both dihydroxyxylenes and hydroxytoluic acids are formed as metabolites since some spots are obtained on the chromatograms which react with ammoniacal silver nitrate, but only feebly (pale brown) with diazotized *p*-nitraniline, whereas other spots react with diazotized *p*-nitraniline giving bright colours (usually purple), but not with ammoniacal silver nitrate. The former behaviour would be expected of dihydric phenols and the latter of hydroxytoluic acids. Further investigation of the nature of these minor metabolites has been postponed pending the synthesis of the necessary reference compounds.

*Further experiments on the metabolism
of the xylenes*

In our previous investigation (Bray, Humphris & Thorpe, 1949) evidence was obtained that all three xylenes were hydroxylated to a small extent in the rabbit. We have now determined the amounts of 'extra' unconjugated and total non-acidic phenol excreted by rabbits receiving the xylenes, using the colorimetric method described by Bray *et al.* (1950*b*). The results are given in Table 3. The standards used were 3:4-, 2:4- and 2:5-xylén-1-ols for *o*-, *m*- and *p*-xylenes, respectively. These are likely hydroxylation products, although *o*- and *m*-xylenes may theoretically give rise to more than one xylénol.

Table 3. *Excretion of non-acidic phenols by rabbits after administration of xylenes*

(Results expressed as percentage of dose. Four experiments with each compound.)

	Dose (g.)	Unhydrolysed urine		Hydrolysed urine	
		Range (%)	Average (%)	Range (%)	Average (%)
<i>o</i> -Xylene	1.35	0.0-1	0.1	1-3	2
<i>m</i> -Xylene	1.7	0.0-1	0.1	3-6	4
<i>p</i> -Xylene	1.74	0.0-4	0.3	0.5-3	2

Whilst there is some difference in the intensities of colour given by different xylenols, this is not great enough to affect the results obtained to any appreciable degree. The values for *m*- and *p*-xylenes are in general accord with those suggested by the earlier investigation, but in the case of the *o*-isomer there

is a considerable difference. Whereas previously we found that about 6% of the dose was excreted as ethereal sulphate and there was evidence that 10-15% might be excreted as ether-type glucuronide, Table 3 indicates that only 2% of *o*-xylene is excreted in the form of non-acidic phenol. A possible explanation of this discrepancy would be the presence in the urine of an acidic phenol, e.g. hydroxytoluic acid, which could form ethereal sulphate and ether glucuronide. This is not, however, supported by examination of ether extracts of *o*-, *m*- and *p*-xylene urines by paper chromatography, which has so far failed to reveal phenolic metabolites such as those found in xylénol urines. Xylenols were not detected on the chromatograms since we have not yet found a solvent mixture suitable for chromatography of these phenols which in all solvents used so far move along with the solvent front.

Attempts were made to isolate the hydroxylation products of the xylenes from metabolic urines. Continuous ether extraction of *p*-xylene urine as collected (pH 7.8-8.0) gave a small amount of syrupy material which smelt of xylénol. Treatment with phenyl isocyanate gave the carbanilate of 2:5-xylén-1-ol (m.p. 161°) identical with an authentic specimen. Yield, 15 mg. from 6.8 g. *p*-xylene. Similar treatment of extracts of *o*- and *m*-xylene urines gave only products with indefinite melting points, suggesting that mixtures of xylenols are formed.

DISCUSSION

As already noted, the metabolism of xylenols is very similar to that of the cresols, the major part of the dose being excreted conjugated through the hydroxyl group with sulphuric and glucuronic acids. A small amount is excreted unchanged and another small portion of the dose is oxidized and hydroxylated. Little difference was observed in the metabolism of the six isomers. These findings are fully in accord with the generalizations suggested by our earlier investigations (see Thorpe, 1950).

In the light of the results obtained from this investigation, it seems most likely that the phenolic material excreted after the administration of xylenes consists mainly of xylenols, both free and conjugated. Since the administration of the xylenols themselves gives rise to only very small amounts of further oxidation or hydroxylation products, it seems reasonable that the amounts of such compounds arising from the xylenes are very small indeed.

SUMMARY

1. The metabolism of 2:3-, 2:4-, 2:5-, 2:6-, 3:4- and 3:5-xylén-1-ols in the rabbit has been studied.
2. All the isomers follow essentially similar metabolic paths, 8-16% being excreted as ethereal

sulphate and 50–72 % as ether glucuronide. 1–3 % is excreted unconjugated.

3. Small amounts of the xlenols have been isolated by direct extraction of the metabolic urines and characterized as carbanilates.

4. Crystalline glucuronides have been isolated as metabolites of all six isomers.

5. Evidence has been obtained by means of paper chromatography of the excretion of small amounts of phenolic and reducing metabolites of the xlenols.

2:5-Dihydroxy-1:3-dimethylbenzene has been identified as a metabolite of both 2:6- and 3:5-xylene-1-ols.

6. The xylenes have been shown to give rise to non-acidic phenol, 0.1–0.3 % being excreted free and 2–4 % conjugated. 2:5-Xylene-1-ol was isolated from *p*-xylene urine and characterized as its carbanilate.

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A Study of Enzymes that can Break Down Tobacco-Leaf Components

1. DIGESTIVE JUICE OF *HELIX* ON LEAF FIBRE

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Knowledge of the detailed composition of the leaf, and more especially of its enzymes and other macromolecular components, has lagged behind the corresponding knowledge of animal tissues and bacteria. There are many reasons for this, but an important one is the mechanical strength of parts of the leaf structure. Furthermore, polysaccharides are important structural elements and leaves have little polysaccharidase other than amylase; they are not therefore weakened by autolytic processes as many other tissues are. Treatments that have been found effective in making extracts from seeds and from most animal tissues are not generally applicable to leaves, and satisfactory alternatives have not yet been developed.

Much work has been done on constituents extracted from leaves that have been dried or frozen before grinding, but many of the materials in which we are interested are not stable in the dry or frozen state. For many years the standard technique with fresh leaves has been to grind by hand in a mortar, or by passage through a domestic meat mincer. This was improved by Osborne & Wakeman (1920),

Chibnall (1939) and Lugg (1939), who used mechanical mortars and domestic grinders in which rubbing is to some extent substituted for the cutting action of the meat mincer. This development was extended by Bawden & Pirie (1944) and Crook (1946), who used a power-driven triple-roller mill, and achieved even finer subdivision, and by Wildman & Bonner (1947), who used a colloid mill. These processes increased the extraction of protein, but Bawden & Pirie (1944) pointed out that fine grinding cannot be looked on simply as a subdivision of the leaf with concomitant release of imprisoned substances; the process brings about both combination between substances originally separate and dissociation of complexes originally present. They suggested that the intense local heating, which is a probable result of any grinding process, is responsible for the observed formation of artifacts. Crook (1946) was able to disperse 95 % of the tobacco-leaf protein, but he confirmed that an alteration in the properties of some of the protein is brought about by the grinding. Grinding, by any method involving rubbing, is not therefore a wholly satisfactory pre-