known acid phosphatase and acid pyrophosphatase, a potent alkaline pyrophosphatase, active only in the presence of added Mg^{2+}, was found to be present in all vegetable tissues tested. Fresh plant products showed more alkaline pyrophosphatase activity than the stored products. The richest sources are green leaves, which also contain large amounts of acid pyrophosphatase. However, some leaves like plantain leaves and grass blades, even though possessing large amounts of alkaline pyrophosphatase, were found to contain only negligible amounts of acid pyrophosphatase. Perhaps the alkaline pyrophosphatase is more essential for plant tissues where photosynthesis is taking place. Plant tissues, unlike animal tissues, do not contain any alkaline phosphatase.

REFERENCES

Naganna, B. (1951). *Curr. Sci.* **20**, 101.
Naganna, B., Raman, A., Venugopal, B. & Sripathi, C. E. (1955). *Biochem. J.* **60**, 215.

Metabolism of Ethers in the Rabbit

2. NUCLEAR-SUBSTITUTED ANISOLES

By H. G. BRAY, VALDA M. CRADDOCK and W. V. THORPE
Department of Physiology, The Medical School, University of Birmingham

(*Received* 9 *November* 1954)

In a previous paper (Bray, James, Thorpe & Wasdell, 1953) it was shown that when anisole was given to a rabbit, the main product excreted in urine was *p*-methoxyphenol; no phenol could be detected, so that it seemed improbable that demethylation was a significant metabolic pathway. Kossel (1880, 1883) and Lehmann (1889) made similar observations for phenetole in the dog, *p*-ethoxyphenol being the chief metabolite. Smith & Williams (1949*a*, *b*), however, isolated *p*-acetamidophenylglucuronide from the urine of rabbits which had received either phenacetin or *p*-phenetidine, showing that in the presence of an amino group in the *para* position, the ethoxy group could be hydrolysed. Huggins, Jensen & Cleveland (1948) also observed cleavage of *p*-nitrophenyl ethers in the rat. It was, therefore, of interest to investigate the effect of other substituents on the stability of alkoxy groups *in vivo*. The present study has been confined to methoxy compounds, which were *m*- and *p*-nitro-, *p*-chloro-, *p*-methyl-, *p*-methoxy-, *p*-hydroxy-, *p*-carboxy- and *p*-cyano-anisole. The effect of each of these compounds on the excretion by the rabbit of ethereal sulphates, ether glucuronides (glucosiduronic acids), free phenols and of other appropriate metabolites has been determined and certain metabolites have been isolated and characterized. The kinetics of some of the reactions have also been investigated. Some preliminary experiments showing that substituted aromatic ethers can be demethylated by rabbit tissue slices have also been carried out. Huggins *et al.* (1948) have shown that some *p*-nitrophenyl ethers can be demethylated by homogenates of rat tissues.

MATERIALS AND METHODS

Materials. *m*- and *p*-Nitroanisoles, *p*-chloroanisole, *p*-bromoanisole, *p*-methoxyanisole (*p*-dimethoxybenzene), *p*-methoxyphenol (*p*-hydroxyanisole) and anisic acid (*p*-carboxyanisole) were purchased. *p*-Methylanisole (*p*-methoxytoluene), b.p. 176°, was prepared by methylation of *p*-cresol with dimethyl sulphate and NaOH. *p*-Cyanoanisole was obtained by heating *p*-methoxybenzamide (anisamide) with PCl_5 (cf. Henry, 1869). 3- and 4-Nitrocatechols were prepared according to Weselsky & Benedikt (1882) and 4-chlorocatechol by the method of Frejka, Šefránek & Zika (1937). For the preparation of 4-cyanocatechol (cf. Hoesch & Zarzecki, 1917), 3:4-dihydroxybenzaldoxime was heated in boiling acetic anhydride for 1 hr. After removal of excess acetic anhydride by distillation *in vacuo*, the product was deacetylated by keeping overnight in 2·5 N-NaOH. Cyanocatechol was then extracted with ether from the acidified solution. Recrystallization from water of the residue left after evaporation of the ether gave colourless plates of 4-cyanocatechol dihydrate, m.p. 83°. (Found: loss at 105°, 19·9. Calc. for $C_7H_5O_2N, 2H_2O$: water, 21·0%.) On heating at 105° the dihydrate gave 4-cyanocatechol, m.p. 152° (Hoesch & Zarzecki give 156°). The nitrile was unchanged by boiling in 5 N-H_2SO_4 for 1 hr.

All melting points agreed with those given in the literature. Melting points recorded in this paper are uncorrected.

Preparation of derivatives. For characterization or isolation of the phenols formed as metabolites, derivatives were prepared as described by Johnson, Shennan & Reed (1950). Quinol ditoluene-*p*-sulphonate was, however, obtained by the method of Porteous & Williams (1949). p-*Cyanophenyl* p-*nitrobenzyl ether* forms pale yellow elongated platelets, m.p. 164°. (Found: C, 66·1; H, 3·8; N, 11·4. $C_{14}H_{10}O_3N_2$ requires C, 66·1; H, 3·9; N, 11·0%.) Since the isolation of derivatives of the phenols provided the main evidence for demethylation, some indication of the quantitative efficiency of the preparation of these derivatives was required.

The yields of pure derivatives, recrystallized to constant m.p., obtained using authentic specimens of the phenols on the scale used for isolation experiments are given in Table 1. These yields were used in calculating the percentage of the dose of an anisole excreted (estimated excretion) in the form of a phenol isolated as the particular derivative.

Animals, diet and dosage. The rabbits used were does of body weight 2·5–3·5 kg. and were maintained on a diet of rabbit pellets and water (Bray, Ryman & Thorpe, 1947). The compounds studied were administered by stomach tube in doses of 0·7 g. as suspensions or emulsions in water. No toxic effects were observed.

Table 1. *Yields of derivatives of various phenols*

Derivatives were prepared from pure phenols as described by Johnson *et al.* (1950) except that from quinol which was prepared according to Porteous & Williams (1949).

Phenol	Derivative	M.p. (°)	Yield (%)
p-Chlorophenol	*p*-Chlorophenoxyacetic acid	156	56
p-Cresol	*p*-Cresyl carbanilate	106	61
	p-Methylphenoxyacetic acid	138	38
	p-Cresyl toluene-*p*-sulphonate	68	80
p-Cyanophenol	*p*-Cyanophenyl *p*-nitrobenzyl ether	164	39
p-Methoxyphenol	*p*-Methoxyphenyl benzoate	86	60
	p-Methoxyphenyl *p*-nitrobenzyl ether	84	52
m-Nitrophenol	*m*-Nitrophenyl toluene-*p*-sulphonate	112	73
p-Nitrophenol	*p*-Nitrophenyl *p*-nitrobenzyl ether	187	54
Quinol	Quinol ditoluene-*p*-sulphonate	157	54

Plan of experiments in vivo. The 24 hr. experiments were performed in the usual way, the normal excretion of metabolites being determined on the days preceding and following the experiment. All administered compounds were excreted within 24 hr. The plan of the kinetic experiments has been described (Bray, Thorpe & White, 1951).

Paper chromatography. The apparatus and general procedure used were as previously described (Bray, Thorpe & White, 1950*a*). Table 2 summarizes the conditions used for the separation and identification of the metabolites investigated. All identifications were made by comparison with authentic reference compounds run on the paper alongside the unknown.

Analytical methods. Ethereal sulphate was determined in 24 hr. urines by Folin's (1905–6) gravimetric method and in urine collected during kinetic experiments by a turbidimetric method (Bray, Humphris, Thorpe, White & Wood, 1952*b*). Ester glucuronide was determined by a copper reducing method (Bray, Neale & Thorpe, 1946) and total glucuronide by a naphthoresorcinol method (Bray *et al.* 1952*b*). A ninhydrin method (Bray, Clowes, Thorpe, White & Wood, 1952*a*) was used for determination of conjugated glycine. Phenols were determined by means of the Folin–Ciocalteu reagent (Bray, Thorpe & White, 1950*b*; Bray *et al.* 1952*b*) with the exception of *m*- and *p*-nitrophenols, which give a feeble colour with this reagent.

Unconjugated nitrophenols were determined after reduction to the corresponding aminophenol. Urine (25 ml.) was extracted exhaustively with ether. The residue left after removal of solvent from the extract was dissolved in water (5 ml.) and this solution was heated with H_2SO_4 (5 ml. 2N) and zinc dust (0·5 g.) for 1 hr. in a boiling-water bath, using a 'cold finger' to reduce evaporation. The mixture was brought to pH 7 with NaOH and extracted continuously for 6 hr. with peroxide-free ether. Water (10 ml.) was added to the ether extract and the ether carefully removed by evaporation. Aminophenol was determined in the aqueous residue using the Folin–Ciocalteu reagent. Linear calibration curves with the Spekker photoelectric absorptiometer using a Chance OR2 red filter were obtained for each aminophenol, a drum reading of 0·1 corresponding to 9 μg. of *m*-nitrophenol and 15 μg. of *p*-nitrophenol. Recovery of the phenols added to urine was 93 ± 4 and 85 ± 3% respectively.

An attempt was made to determine methoxy compounds in the residue obtained after removal of solvent from light petroleum (b.p. 40–60°) extracts of urine. The Pregl (1937) micro-Zeisel method for methoxyl groups was modified so that the flask used for collection of the light petroleum extract could be used as the flask for the reaction with HI in the Pregl apparatus. In control experiments respective recoveries of 93 and 89 ± 3% of *m*- and *p*-nitroanisoles added to urine were obtained. The other anisole derivatives were too volatile in light petroleum vapour to permit removal of the extracting solvent without considerable loss. The application of the method to urine from animals which had received *m*- and *p*-nitroanisole (Table 3) confirmed that only small amounts of methoxy compounds were excreted after administration of these two substituted anisoles. The average amount of methoxy compounds excreted in normal rabbit urine determined by this method was 0·5 mg. CH_3O/day.

General procedure for the examination of urines. The 24 hr. urine, usually from six rabbits each dosed with 0·7 g. of the anisole, was adjusted to pH 6·5 and exhaustively and continuously extracted with ether to give ether extract *A*. The aqueous residue, after addition of an equal volume of 10N-H_2SO_4, was refluxed for 2 hr. A second ether extract, *B*, was obtained by exhaustive extraction of this hydrolysate with ether. The aqueous residue from this extraction was adjusted to pH 7 and again extracted with ether to yield a third ether extract, *C*. The first extraction was omitted for the urines

Table 2. *R_F values for some metabolites of substituted anisoles*

Solvent mixtures (by vol.): *A*, benzene–acetic acid–water (2:2:1); *B*, (3:2:1); *C*, (1:1:2); *D*, chloroform–acetic acid–water (2:2:1); *E*, (3:1:1); *F*, light petroleum (b.p. 90–100°)–*n*-butanol–formic acid (98–100%) (10:1:5); *G*, (10:1:10); *H*, *n*-butanol–ammonia (sp.gr. 0·88) (20:3); *I*, *n*-butanol–sat. aqueous NaCl–pyridine–ammonia (sp.gr. 0·88) (4:5:8:3); *J*, methanol–'amyl alcohol' (A.R., British Drug Houses Ltd.)–benzene–water (2:1:1:1) (Ekman, 1948); *K*, *n*-butanol; *L*, ethanol–water–ammonia (sp.gr. 0·88) (20:4:1).

The ascending method was used with Whatman no. 4 paper at room temp.

Detecting reagents: *a*, 20% (w/v) Na_2CO_3; *b*, ammoniacal $AgNO_3$; *c*, diazotized *p*-nitraniline and 20% Na_2CO_3; *d*, diazotized sulphanilic acid and 20% Na_2CO_3; *e*, 0·2% $FeCl_3$ (*b*, *c*, *d* and *e* as Bray *et al.* 1950*a*); *f*, diazotized with 0·2% $NaNO_2$ + 0·1 N-HCl coupled with 0·1% *N*-(1-naphthyl)ethylenediamine hydrochloride; *g*, 0·04% bromocresol purple.

Compound	Solvent mixture	Time of run (hr.)	R_F	Detecting reagent
m-Aminophenol	*D*	4	0·4	*b*, *c*, *e*, *f*
p-Aminophenol	*B*	1	0·7	*b*, *c*, *e*, *f*
	D	2	0·4	
	F	3	0·1	
m-Anisidine	*D*	2	0·9	*b*, *f*
	H	4	0·9	
p-Anisidine	*D*	2	1·0	*b*, *f*
	H	3	0·9	
	J	4	0·9	
Catechol	*C*	1	0·35	*e*
	D	1	0·8	
p-Chlorophenol*	*K*	2	0·8	None
4-Chlorocatechol	*C*	1	0·45	*e*
	D	1·5	0·7	
p-Cresol*	*K*	3	0·3	None
p-Cyanophenol	*F*	3	0·2	*e*
4-Cyanocatechol	*D*	1	0·75	*e*
p-Methoxyphenol	*G*	4	0·3	*b*
m-Nitrophenol	*C*	1	0·8	*a*
	F	2·5	0·25	
p-Nitrophenol	*B*	1	0·7	*a*
	C	1	0·8	
	F	3	0·1	
3-Nitrocatechol	*C*	1	1·0	*a*, *e*
4-Nitrocatechol	*C*	1	0·15	*a*, *e*
Quinol	*B*	3	0·2	*b*
	E	5	0·25	
Anisic acid	*L*	4	0·77	*g*
Anisuric acid	*L*	4	0·72	*g*
Benzoic acid	*L*	4	0·82	*g*
p-Hydroxybenzoic acid	*A*	3	0·5	*c*, *g*
	I	23	0·5	
	L	4	0·71	
3:4-Dihydroxybenzoic acid	*B*	6	0·15	*e*, *g*
	D	1	0·4	
	L	3	0·3	
Hippuric acid	*L*	4	0·77	*g*
p-Hydroxyhippuric acid	*L*	4	0·67	*g*

* *p*-Chlorophenol was coupled with diazotized *p*-nitroaniline (*c*) and then run on paper which had been soaked in 20% (w/v) Na_2CO_3 and dried (Hossfeld, 1951). *p*-Cresol was coupled with diazotized sulphanilic acid (*d*) but otherwise treated in the same way.

containing only small amounts of free phenol (Table 3). The third extraction was carried out on the nitroanisole urines to collect any aminophenols present. Control experiments had shown that the hydrolysis of the urine with 5N-H_2SO_4 caused less than 0·3% of any of the anisoles to be converted into phenols. About 4% of *p*-cyanoanisole was, however, converted into anisic acid by this treatment. The ether extracts were examined by paper chromatography and the major metabolites isolated by making appropriate derivatives. The estimated excretions recorded are based upon the amounts of recrystallized derivatives obtained and would almost certainly indicate less than the amount actually excreted.

Preparation of tissue slices. The required organs were rapidly removed from freshly killed young buck rabbits and placed in ice-cold Ringer's solution. Tissue slices were cut by the method described by Umbreit, Burris & Stauffer (1949).

Incubation medium. Tissue slices were incubated in Krebs–Ringer bicarbonate solution (Umbreit *et al.* 1949). The substrate was dissolved in the solution of the five salts before the addition of the $NaHCO_3$ solution. The following overall substrate concentrations were used: *p*-methoxyanisole 0·003M; *p*-chloroanisole 0·0018M; *p*-bromoanisole 0·00076M; *p*-methylanisole 0·0068M; *p*-nitroanisole 0·0033M.

Plan of experiments in vitro. The substrate dissolved in the medium (10 ml.) was placed in a 50 ml. flask and the air displaced by a stream of O_2+CO_2 (95:5) passed on to the surface of the liquid. The flask was then closed with a rubber bung and the mixture equilibrated by shaking at 38° for 30 min. The tissue slices were then added and gas was passed again; the flask was closed and shaken in the thermostat for the required time. Appropriate controls were set up for each experiment. The reaction was stopped by the addition of 10% (w/v) trichloroacetic acid (6 ml.) and the tissue slices and precipitated protein removed by filtration and dried to constant weight at 110°. The filtrate was refluxed with 10N-H_2SO_4 (equal vol.) for 1 hr. and the hydrolysate extracted continuously with ether for 6 hr. The extracted material was examined qualitatively or quantitatively for the presence of reaction products.

Examination of hydrolysed filtrates from deproteinized digests. Ether extracts of the hydrolysed filtrates were used for examination for phenols by paper chromatography. An ether extract of the hydrolysate of the filtrate from the medium incubated with tissue slices in the absence of substrate was used as a control. For quantitative analysis, water (10 ml.) was added to the ether extract of the hydrolysed filtrate, ether was removed by gentle evaporation and the residual solution was diluted to 15 ml. with water. Phenols were determined as described for urine.

RESULTS

Quantitative experiments

Normal excretion of metabolites. The ranges and averages of daily outputs of normal metabolites were similar to those recorded in earlier papers (e.g. Bray *et al.* 1952*a*, 1953).

Table 3. *Metabolites excreted in urine after administration of substituted anisoles to rabbits*

Results are expressed as the average percentage of the dose excreted. Ranges are given in parentheses and the superior figures indicate the number of experiments. Dose, 0·7 g./rabbit.

Substituent of anisole	Percentage of dose excreted as							Average percentage of dose accounted for
				Methoxy compounds				
	Ethereal sulphate	Ether glucuronide	Free phenol	Unhydrolysed urine	Hydrolysed urine	Ester glucuronide	Glycine conjugate	
m-NO_2	28 (17–44)[8]	35 (27–43)[2]	9 (4–13)[3]	0·5 (0·5, 0·5)[2]	1·5 (1–2)[2]	—	—	74
p-NO_2	28 (22–36)[10]	35 (29–46)[3]	14 (5–19)[3]	0·5 (0–1)[3]	2 (2, 2)[2]	—	—	79
p-Cl	36 (25–49)[4]	44 (41–46)[2]	4 (0–11)[4]	—	—	—	—	84
p-OMe	27 (16–38)[8]	63 (61–64)[2]	4 (2–5)[2]	—	—	—	—	94
p-CH_3	13 (9–17)[7]	11 (9–14)[3]	3 (0–5)[2]	—	—	20 (9–34)[13]	38 (30–43)[4]	85
p-CN	35 (30–41)[3]	50 (44–65)[4]	0·5 (0–1)[3]	—	—	5 (0–9)[4]	11 (5–16)[4]	101
p-OH	13 (10–15)[2]	69 (65–73)[2]	1 (0–2)[4]	—	—	—	—	82
p-COOH*	—	—	—	—	—	57 (52–59)[3]	38 (33–41)[3]	96

* Results from Bray *et al.* 1954. Percentage excreted as unconjugated acid was 1 (0–3)[3].

Metabolites excreted after administration of substituted anisoles. The quantitative results obtained are summarized in Table 3. The greater part of each of the compounds studied was accounted for. The results, in conjunction with the qualitative findings, suggest that demethylation was the predominant reaction with all the compounds except *p*-methylanisole and anisic acid. Oxidation of the methyl group of *p*-methylanisole occurred and the resulting anisic acid was excreted mainly in conjugated form (p. 230). The higher methoxyl contents of light petroleum extracts of hydrolysed nitroanisole urines compared with those of the unhydrolysed urines suggest that small amounts of methoxy compound were excreted conjugated.

Rate of formation of phenols. The rates of demethylation of the anisole derivatives were determined by measuring the phenols excreted at intervals during experiments lasting 14 hr. (cf. Bray *et al.* 1952*b*). Graphs of the amounts of the dose excreted as ethereal sulphate and ether glucuronide plotted against time were of the form of exponential curves, indicating that the reactions follow first-order reaction kinetics with respect to the anisole derivative. Velocity constants were derived by the usual graphical methods (Bray *et al.* 1951) and are given in Table 4.

Table 4. *Velocity constants for the formation of conjugated phenols from nuclear substituted anisoles in the rabbit*

	Velocity constant ($hr.^{-1}$)	Geometric mean
m-Nitroanisole	0·09, 0·09	0·09
p-Nitroanisole	0·18, 0·10, 0·11	0·13
p-Chloroanisole	0·12, 0·08	0·10
p-Methoxyanisole	0·18, 0·16, 0·22	0·19
p-Methylanisole*	0·10, 0·06, 0·10	0·08
p-Cyanoanisole†	0·26, 0·23, 0·22	0·24

* Mean velocity constant for the oxidation of the methyl group was 0·14 $hr.^{-1}$.

† Velocity constant for the hydrolysis of the cyano group was approximately 0·02 $hr.^{-1}$.

Identification of metabolites

It was important to identify the major phenolic metabolites obtained from the substituted anisoles since these could theoretically be formed either by demethylation of the methoxy group or by the introduction of a hydroxyl group. The former process would yield the phenol corresponding to the anisole administered, whereas hydroxylation would yield a substituted hydroxyanisole. The general procedure for examination of the urines and separation of ether extracts *A*, *B* and *C* has been described in the Methods section.

Nitroanisoles. The quantitative analyses (Table 3) indicated that about 72 and 77 % of doses of *m*- and *p*-nitroanisoles were excreted as phenolic derivatives. Examination by paper chromatography of ether extracts *A* and *B* of the urines showed that these phenols were predominantly the corresponding nitrophenols or their conjugates. 4-Nitrocatechol was detected in *B* extracts of both urines. The nitrophenols were isolated from the *B* extracts in very small yields, but better yields were obtained by conversion into derivatives. *m*-Nitrophenyl toluene-*p*-sulphonate, m.p. 112° unchanged by admixture with an authentic sample (estimated excretion 11 % of dose), and *p*-nitrobenzyl *p*-nitrophenyl ether, m.p. 187° unchanged by admixture with an authentic sample (estimated excretion 14 % of dose), were obtained. Paper chromatography indicated the presence of small amounts of the corresponding aminophenol and anisidine in the ether extracts *C* from both *m*- and *p*-nitrophenol urines. In addition, two unidentified spots, detected with ammoniacal silver nitrate, were observed in chromatograms of the *B* extracts of both *m*- and *p*-nitroanisole urines. The fact that these spots were not revealed by spraying with 20 % Na_2CO_3 suggests that they may have been produced by aminohydroxyanisoles. This would be compatible with the finding of increased amounts of methoxy compounds after hydrolysis of the urines (Table 3). Part of the methoxyl content of hydrolysed *m*-nitroanisole urine is due to *m*-anisidine. The very small methoxyl content of the unhydrolysed urines indicates that very little, if any, unchanged nitroanisole is excreted.

p-*Chloroanisole.* Paper chromatography indicated that *p*-chlorophenol was the main phenolic constituent of the *B* extracts of *p*-chloroanisole urines. There was also a small amount of 4-chlorocatechol. *p*-Chlorophenol was isolated, but a better yield (estimated excretion 50 % of dose) was obtained by isolation as *p*-chlorophenoxyacetic acid, m.p. 156°, unchanged by admixture with an authentic sample.

p-*Cyanoanisole.* There are several theoretically possible metabolites of this compound, since both the cyano and the methoxy groups might be modified. Paper chromatograms from the *B* extracts, however, indicated that *p*-cyanophenol was the chief metabolite and this was isolated (estimated excretion 21 % of the dose) as *p*-cyanophenyl *p*-nitrobenzyl ether, m.p. 164° unchanged by admixture with an authentic sample. An amount of *p*-hydroxybenzoic acid greater than that obtained from normal urine under similar conditions was detected by paper chromatography, but as the preliminary hydrolysis was shown to cause the conversion of about 0·4 % of *p*-cyanoanisole into *p*-hydroxybenzoic acid this acid may have been an artifact rather than a metabolite. It is likely that the glycine conjugate (11 % of dose, Table 3)

consists mainly of anisuric (*p*-methoxyhippuric) or *p*-hydroxyhippuric acid. Paper chromatography revealed a small amount of 4-cyanocatechol and a trace of 3:4-dihydroxybenzoic acid in *B* extracts. A control experiment with 4-cyanocatechol added to normal urine showed that 3:4-dihydroxybenzoic acid was not formed under the conditions used for hydrolysis.

p-*Methoxyanisole.* Paper chromatography revealed the presence of *p*-methoxyphenol and quinol in the *B* extracts of *p*-methoxyanisole urine. *p*-Methoxyphenol was the chief metabolite and was isolated (estimated excretion 34 % of the dose) as *p*-methoxyphenyl *p*-nitrobenzyl ether, m.p. 84°, unchanged by admixture with an authentic sample. From paper chromatograms the amount of quinol seemed to be considerably greater than that present in normal urine. *p*-Methoxyphenol itself gives rise to appreciable amounts of quinol in the rabbit (see below).

p-*Methoxyphenol.* Although this compound is mainly excreted as conjugates of *p*-methoxyphenol an appreciable proportion of a dose is demethylated to quinol. This compound was separated by dissolving in acetone the residue obtained after removal of ether from the *B* extract of the urine from six rabbits and applying this solution to a column (50 cm. long, 3 cm. diameter) packed with hydrocellulose (Imperial Chemical Industries Ltd.). The *p*-methoxyphenol was eluted with 600 ml. of solvent mixture *A* (Table 2) and then the dark band of quinol was eluted with 100 ml. of absolute ethanol. The quinol was separated from the eluate as the ditoluene-*p*-sulphonate, m.p. 157° and mixed m.p. 158° with an authentic sample, m.p. 158° (estimated excretion, 3 % of dose). *p*-Methoxyphenol was isolated from the first eluate as *p*-methoxyphenyl benzoate, m.p. 85° and mixed m.p. 86° with an authentic specimen, m.p. 86° (estimated excretion, 25 % of dose).

p-*Methylanisole.* Paper chromatography of *B* extracts indicated the presence of *p*-cresol and *p*-hydroxybenzoic acid. (Anisic acid could not be identified in this way since the R_F value is the same as that of hippuric acid (Table 2). Similarly, anisuric acid could not be detected with certainty in the presence of *p*-hydroxybenzoic acid.) 3:4-Dihydroxybenzoic acid was not detected. For the isolation of *p*-cresol, the urine was boiled under reflux for 1 hr. with an equal volume of 10N-H_2SO_4. The hydrolysed urine was steam-distilled and the distillate extracted with ether in a continuous extractor. The mobile syrup obtained after removal of ether from the extract smelt strongly of cresol. It was divided into three parts, from which the following derivatives were made: *p*-methylphenoxyacetic acid, m.p. 138°, *p*-cresyl toluene-*p*-sulphonate, m.p. 68°, and *p*-cresyl carbanilate, m.p. 106°. None of these melting points was changed by admixture with an authentic sample. Combined yields were equivalent to an excretion of 3 % of the dose. The amount of *p*-hydroxybenzoic acid was small but undoubtedly greater (judged by the size and intensity of the spots on paper chromatograms) than that found in normal rabbit urine. Anisic acid, m.p. 184°, unchanged by admixture with an authentic sample, was isolated in small amount from the *A* extract and in large amount (corresponding to 24 % of dose) from the *B* extract. Anisuric acid, m.p. 172°, unchanged by admixture with the sample obtained biosynthetically from anisic acid, was isolated from the urine as described below. (Yield 350 mg. from 3·5 g. *p*-methylanisole; 6 % of dose.)

Anisic acid. Urine from three rabbits dosed with anisic acid (1 g. each) was adjusted to pH 1–2 with H_2SO_4 and extracted with ether in a continuous extractor. Ether was removed from the extract and the dry residue was extracted three times with hot toluene (cf. Quick, 1932). The residue was crystallized and recrystallized from aqueous ethanol and anisuric acid, m.p. 172° (Quick gives 170° for this acid isolated from the urine of human subjects dosed with anisic acid), was obtained as colourless platelets (yield 410 mg., 10 % of dose). It depressed the m.p. of both anisic and hippuric acids. (Found: C, 57·7; H, 5·5; N, 6·8. Calc. for $C_{10}H_{11}O_4N$: C, 57·4; H, 5·3; N, 6·7 %.) Anisic acid, m.p. 184° (280 mg., 9 % of dose) was isolated from the toluene extracts. This had probably been formed by decomposition of the ester glucuronide since Bray, Humphris, Thorpe, White & Wood (1954) showed that the excretion of unconjugated acid was negligible (see Table 3). No phenolic metabolites were detected.

Demethylation of substituted anisoles by tissue slices

Identification of products. Rabbit-liver slices were incubated for 2 hr. with *p*-methoxy-, *p*-nitro- and *p*-chloro-anisoles and ether extracts of the hydrolysed incubation mixtures examined for the corresponding phenols by means of paper chromatography. *p*-Methoxyphenol was shown to be formed from *p*-methoxyanisole but no quinol was detected. *p*-Nitrophenol and *p*-chlorophenol were detected in digests with *p*-nitroanisole and *p*-chloroanisole respectively.

Four digests with rabbit-liver slices were made up with the following additions: (1) none; (2) *p*-methylanisole, 0·003M; (3) and (4) formaldehyde, 0·002 %. Digests 1, 2 and 3 were incubated at 37° for 1·5 hr.; digest 4 was worked up immediately. The four digests were deproteinized and portions of the filtrates were examined for formaldehyde by the acetylacetone colour reaction of Nash (1953). Other

portions of the filtrates were treated with dimedone as described by Mackenzie, Johnston & Frisell (1953). Both tests indicated that most formaldehyde was present in digest 2 which gave 5·2 mg. of precipitate compared with 1·1 mg. in digest 1. The dimedone precipitates from digests 3 and 4 (2·3 and 3·6 mg. respectively) suggested that some formaldehyde was lost on incubation for 1·5 hr.

Quantitative experiments. Rabbit liver was more effective than kidney or intestine as a source of the demethylating system. Approximately 10 % (0·36–0·44 mg.) of *p*-methoxyanisole was demethylated by 100 mg. (dry wt.) rabbit-liver slices in 10 ml. of 0·003 M substrate in 2 hr. at 38°. Less than 3 % was demethylated by slices of kidney or intestine. No phenol was formed in control digests and 100 mg. (dry wt.) tissue contained less than 0·01 mg. phenol (calculated as *p*-methoxyphenol). The addition of glycocyamine (0·003 M) or DL-methionine (0·003 M) did not cause a significant increase in the extent of phenol formation. As an indication of the relative initial rates of demethylation, the amount of phenol formed after 20 min. was determined. From eight experiments with *p*-methoxyanisole the average extent of demethylation corresponded to the removal of 17 μg./100 mg. (dry wt.) liver (range 14–21). In similar experiments with other substituted anisoles the average CH_3 removed was 39, 5, 19 and 11 μg. for *p*-bromo-, *p*-chloro-, *p*-methyl- and *p*-nitro-anisole respectively.

Table 5. *Effect of* KCN, *ATP and absence of* O_2 *on the demethylation of* p-*methoxyanisole by rabbit-liver slices*

Slices of rabbit liver were incubated with 0·003 M *p*-methoxyanisole in Krebs–Ringer bicarbonate for 2 hr. at 38°. Additions were made as indicated. Concentrations of KCN and ATP were 0·01 M and 0·001 M respectively. All flasks were gassed with $O_2 + CO_2$ mixture except when N_2 was used. Incubation for 2 hr. at 38°.

Expt.	Addition	*p*-Methoxyanisole demethylated/ 100 mg. (dry wt.) slices (mg.)
1	None	0·41
	N_2	0·00
2	None	0·44
	N_2	0·05
	KCN	0·17
	ATP	0·55
3	None	0·37
	ATP	0·07

It has been shown that in the intact rabbit phenols formed by demethylation of anisoles are excreted largely conjugated with glucuronic and sulphuric acids. Since conjugation of phenols with both these acids has been demonstrated *in vitro* (e.g. Storey, 1950; De Meio & Tkacz, 1952), in some experiments with *p*-methoxyanisole and liver slices the phenols were estimated in the filtrate before as well as after hydrolysis. Although in the earlier stages of incubation a considerable amount of free phenol was present, after incubation for 2 hr. the greater part of the phenol was present in conjugated form and was liberated only after hydrolysis.

Effect of KCN, *adenosine triphosphate, 2:4-dinitrophenol and absence of* O_2. Experiments were performed in which the gas in the flasks was N_2 instead of the $O_2 + CO_2$ mixture. The effect of the addition of 0·01 M-KCN or 0·001 M adenosine triphosphate (ATP) to digests exposed to the $O_2 + CO_2$ mixture was also examined. Some results obtained with *p*-methoxyanisole as substrate are given in Table 5. Both replacement of O_2 by N_2 and the addition of KCN reduced the extent of demethylation, while ATP enhanced it. The formation of *p*-methoxyphenol from *p*-methoxyanisole in digests to which 2:4-dinitrophenol (0·01 M) had been added could not be detected by paper chromatography.

DISCUSSION

This investigation has shown that most of the substituted anisoles examined are excreted by rabbits mainly as phenolic metabolites. *p*-Methylanisole, however, is mainly oxidized to and excreted as conjugates of anisic acid, although about 27 % of the dose is converted into phenols, mainly *p*-cresol. As would be expected, *p*-methoxyphenol is largely excreted as its conjugates with glucuronic and sulphuric acids; a small part of the dose, however, is excreted as quinol. Anisic acid is excreted, as it is by man and the dog (Quick, 1932), conjugated with glucuronic acid and glycine; this was the only compound for which evidence of demethylation was not obtained. This is not surprising, since substituted benzoic acids are usually rapidly excreted, either unchanged or conjugated through the carboxyl group. The identification of the metabolites of the substituted anisoles has made it clear that the predominant phenol-producing reaction is demethylation, although it must not be concluded that hydroxylation of methoxy compounds does not occur. The identification of such metabolites, which could only be present in small amounts, would have required the synthesis of a large number of reference compounds.

Appreciable amounts (about 10 %) of unconjugated *m*- and *p*-nitrophenols were excreted after administration of *m*- and *p*-nitroanisoles. Robinson, Smith & Williams (1951) stated that less than 1 % of doses of *o*-, *m*- and *p*-nitrophenols administered as such was excreted unchanged. It would be expected from kinetic considerations that conjugation processes would be more complete when a

phenol precursor is administered than when the phenol itself is fed. In our experiments with nitroanisoles the extent of sulphate conjugation was greater, although that of glucuronic acid conjugation was less, than that found by Robinson *et al.* (1951) after administration of a comparable dose of the corresponding preformed nitrophenols. We have not, however, examined *m*- and *p*-nitrophenols under the precise conditions which we used for nitroanisoles.

The velocity constants for phenol formation ranged from 0·08 to 0·24 hr.$^{-1}$. There appeared to be no correlation with the electronic nature of the substituents (cf. substituted toluenes, benzoic acids and benzamides, Bray *et al.* 1954). All the substituents studied except the carboxyl group rendered the methoxy group less stable than it is in anisole itself in which fission of the ether linkage was not detected by Bray *et al.* (1953).

The experiments *in vitro* have shown that tissue slices can effect demethylation of the substituted anisoles examined. Liver slices were the most active in this respect. The production of formaldehyde in digests of *p*-methoxyanisole and the absence of demethylation under anaerobic conditions suggests an oxidative mechanism, but there is not at present sufficient experimental evidence to justify any conclusion as to the mechanism of this demethylation. Huggins *et al.* (1948) found that homogenates of rat kidney and liver caused slight cleavage (2–3 % of the ether) of *p*-nitroanisole, *p*-nitrophenetole and *p*-nitrodiphenyl ether.

SUMMARY

1. The fate of some substituted anisoles in the rabbit has been studied and over 74 % of the dose of each compound has been accounted for.

2. The chief metabolites of *m*- and *p*-nitro-, *p*-chloro-, *p*-methoxy- and *p*-cyano-anisoles are the corresponding phenols formed by demethylation. These are excreted mainly as conjugates of glucuronic and sulphuric acids. Dihydric phenols have been detected as metabolites of *m*- and *p*-nitro-, *p*-cyano- and *p*-chloro-anisoles.

3. The greater part of a dose of *p*-methylanisole is oxidized to anisic acid; about 27 % is, however, demethylated and excreted mainly as *p*-cresol.

4. *p*-Methoxyphenol is excreted mainly as its conjugates of glucuronic and sulphuric acids but is partly demethylated to give quinol.

5. Anisic acid is excreted mainly as ester glucuronide and anisuric acid.

6. The velocity constants for the demethylation of substituted anisoles range from 0·08 to 0·24 hr.$^{-1}$.

7. Some substituted anisoles are demethylated by incubation with rabbit-liver slices.

We wish to thank Mr P. B. Wood for assistance with some of the analyses. Elementary microanalyses were done in the Chemistry Department of this University by the kind arrangement of Professor M. Stacey, F.R.S., and Dr R. Belcher.

REFERENCES

Bray, H. G., Clowes, R. C., Thorpe, W. V., White, K. & Wood, P. B. (1952*a*). *Biochem. J.* **50**, 583.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952*b*). *Biochem. J.* **52**, 412.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1954). *Biochem. J.* **59**, 162.
Bray, H. G., James, S. P., Thorpe, W. V. & Wasdell, M. R. (1953). *Biochem. J.* **54**, 547.
Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 134.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
Bray, H. G., Thorpe, W. V. & White, K. (1950*a*). *Biochem. J.* **46**, 271.
Bray, H. G., Thorpe, W. V. & White, K. (1950*b*). *Biochem. J.* **47**, 275.
Bray, H. G., Thorpe, W. V. & White, K. (1951). *Biochem. J.* **48**, 88.
De Meio, R. H. & Tkacz, L. (1952). *J. biol. Chem.* **195**, 175.
Ekman, B. (1948). *Acta. chem. scand.* **2**, 383.
Folin, O. (1905–6). *J. biol. Chem.* **1**, 131.
Frejka, J., Šefránek, B. & Zika, J. (1937). *Coll. Czech. Chem. Comm.* **9**, 238. Cited by *Chem. Abstr.* **41**, 7046.
Henry, L. (1869). *Ber. dtsch. chem. Ges.* **2**, 664.
Hoesch, K. & Zarzecki, T. von (1917). *Ber. dtsch. chem. Ges.* **50**, 462.
Hossfeld, R. L. (1951). *J. Amer. chem. Soc.* **73**, 852.
Huggins, C. H., Jensen, E. V. & Cleveland, A. S. (1948). *Proc. Soc. exp. Biol., N.Y.*, **68**, 477.
Johnson, W. C., Shennan, R. S. & Reed, R. A. (1950). *Organic Reagents for Organic Analysis*, 2nd ed. London: Hopkin and Williams Ltd.
Kossel, A. (1880). *Hoppe-Seyl. Z.* **4**, 296.
Kossel, A. (1883). *Hoppe-Seyl. Z.* **7**, 292.
Lehmann, V. (1889). *Hoppe-Seyl. Z.* **13**, 181.
Mackenzie, C. G., Johnston, J. M. & Frisell, W. R. (1953). *J. biol. Chem.* **203**, 743.
Nash, T. (1953). *Biochem. J.* **55**, 416.
Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 56.
Pregl, F. (1937). *Quantitative Organic Microanalysis*, 3rd ed. p. 171. London: J. and A. Churchill Ltd.
Quick, A. J. (1932). *J. biol. Chem.* **97**, 403.
Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 221.
Smith, J. N. & Williams, R. T. (1949*a*). *Biochem. J.* **44**, 239.
Smith, J. N. & Williams, R. T. (1949*b*). *Biochem. J.* **44**, 250.
Storey, I. D. E. (1950). *Biochem. J.* **47**, 212.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Technique and Tissue Metabolism*, 2nd ed. pp. 110, 119. Minneapolis: Burgess Publishing Co.
Weselsky, P. & Benedikt, R. (1882). *Mh. Chem.* **3**, 386.