3. A relation between the stability of the inhibitors to aqueous hydrolysis at physiological pH and temperature and their power as inhibitors of cholinesterase has been found. The more stable the inhibitor, the lower its inhibitory power.

4. The mechanism of inhibition of cholinesterase

by these compounds is discussed in relation to the above facts.

Our thanks are due to Mr B. Topley, Messrs Albright and Wilson Ltd., for providing us with the samples of all the inhibitors used in this work, and to Miss J. I. Wheatley for valuable technical assistance.

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The Metabolism of Aminophenols, *o*-Formamidophenol, Benzoxazole, 2-Methyl- and 2-Phenyl-benzoxazoles and Benzoxazolone in the Rabbit

By H. G. BRAY, R. C. CLOWES AND W. V. THORPE
Department of Physiology, Medical School, University of Birmingham

(Received 28 August 1951)

In this investigation some aspects of the metabolism of *o*-, *m*- and *p*-aminophenols, *o*-formamidophenol (II), benzoxazole (III), 2-methylbenzoxazole (IV), 2-phenylbenzoxazole (V), and benzoxazolone (VI) have been examined. The compounds (II) to (VI)

benzoxazolone, which Gressly & Nencki (1890, 1891) showed to be hydroxylated in the dog.

In this study we have attempted to draw up balance sheets for these compounds and to isolate their principal metabolites. Minor metabolites

(I)

(II)

(III)

(IV)

(V)

(VI)

can be regarded as derivatives of *o*-aminophenol (I), which might be formed from them on hydrolysis. These compounds may, therefore, be considered as having potential centres for conjugation (see Bray, Ryman & Thorpe, 1948; Thorpe, 1950). Williams (1938, 1943) and Smith & Williams (1949) found that the rabbit excretes the aminophenols mainly as *O*-conjugates, i.e. ethereal sulphates and glucuronides. The only other compound of this group which appears to have been investigated previously is

were detected by means of paper chromatography. The results obtained show the influence of substituents upon the stability of the oxazole ring.

EXPERIMENTAL

Materials. The aminophenols were purchased (British Drug Houses Ltd. and Light and Co.). *o*-Formamidophenol was prepared by the action of acetic and formic acids on *o*-aminophenol (Béhal, 1900), benzoxazole and its 2-methyl and 2-phenyl derivatives by dry distillation of *o*-amino-

phenol with formamide, acetamide and benzamide respectively (cf. Bamberger, 1903), benzoxazolone by the method of McDonald & Chechak (1948), *o*-hydroxyphenylurea by the method of Menschutkin (1870) and *o*-aminophenyl sulphate by the method of Burkhardt & Wood (1929). 2:4-Dihydroxybenzamide (m.p. 227–228°) was prepared from 2:4-dihydroxybenzoic acid (British Drug Houses Ltd.) by heating the methyl ester with NH_3 in a sealed tube.

Diet and dosage. The rabbits used (does of 2–3 kg. weight) were maintained throughout the investigation on the diet of rabbit pellets and water customary in this laboratory (Bray, Ryman & Thorpe, 1947). The compounds studied were administered by stomach tube as suspensions or emulsions in water in doses of 1 g. Some rabbits exhibited a mild paralysis of short duration after receiving *o*- and *p*-aminophenol and benzoxazolone. Administration of benzoxazolone was often followed by the excretion of amounts of reducing material far in excess of that which could be formed as metabolites of the compounds given. This was also observed, but less frequently, after giving *o*-aminophenol. Similar excretion of reducing material has been observed with other compounds (see Bray, Hybs & Thorpe, 1951). No toxic symptoms were observed with the other compounds administered.

Methods. None of the compounds studied had any specific property suitable for quantitative estimation of the individual compound. Ethereal sulphate was determined by the method of Folin (1905–6), glucuronic acid by a modification of that of Hanson, Mills & Williams (1944), ether glucuronide by the reducing value method (Bray, Neale & Thorpe, 1946; Bray, Lake, Neale, Thorpe & Wood, 1948), ether-soluble acid as described by Bray, Thorpe & Wood (1951) but using bromothymol blue as indicator, diazotizable compounds by the method of Bratton & Marshall (1939), amino compounds by the Ehrlich reaction (Venkataraman, Venkataraman & Lewis, 1948; Bray, Thorpe & Wood, 1951), phenols by the

Folin & Ciocalteu reaction (Bray, Thorpe & White, 1950*b*), and mercapturic acids by a modification of the method of Stekol (1936). The Hilger Spekker photo-absorptiometer was used for colorimetric estimations.

Urine extracts were examined by paper chromatography as described by Bray, Thorpe & White (1950*a*). The solvent mixtures and the R_F values of the reference compounds used are given in Table 1.

Calculation of results. The chief compounds which might be expected to be present in urine after administration of *o*-, *m*- or *p*-aminophenol are: the unchanged aminophenol, *A*; the acetamidophenol, *B*; the aminophenylsulphate, *C*; the acetamidophenylsulphate, *D*; the aminophenylglucuronide, *E*, and the acetamidophenylglucuronide, *F*. The free aminophenols give no appreciable colour when diazotized and coupled under the Bratton & Marshall (1939) conditions, but they can be estimated by the modified Ehrlich method. *o*-Aminophenylsulphate, while stable in N-HCl in the cold, is decomposed giving sulphate ions on addition of the nitrite for diazotization and gives no colour with the Bratton & Marshall reagent. *p*-Aminophenyl sulphate is more stable and can be diazotized and coupled without losing sulphate (Burkhardt & Wood, 1929). Heating the urine in a boiling-water bath with HCl (0.2 vol. 2*N*) for 30 min. (mild hydrolysis) was found to effect the hydrolysis of the acetamido and sulphate groups, but was not sufficient to liberate glucuronic acid (i.e. these were the conditions which gave maximum diazo values). Hydrolysis of glucuronide was only achieved by heating in a boiling-water bath with HCl (0.33 vol. 10*N*) for 90 min. (complete hydrolysis of conjugates). By carrying out determinations upon the urine as collected, and after mild or complete hydrolysis the following data were obtained from which the amounts of the compounds *A–F* present could be calculated. Diazo determination on urine as collected, *E* or *C + E* (according

Table 1. *Paper chromatography of various phenolic compounds*

(Whatman no. 4 paper. Solvent mixture: *A*, *n*-butanol, 40; pyridine, 20; saturated aqueous NaCl , 25; ammonia, sp.gr. 0.880, 15 (all % v/v); *B*, benzene saturated with formic acid (98–99%); *C*, benzene, 40; glacial acetic acid, 40; water, 20; *D*, chloroform, 40; glacial acetic acid, 40; water, 20. Time 4 hr., except for mixture *B*, 16 hr.)

Compound	R_F value in solvent mixture				Colour given by diazotized <i>p</i> -nitraniline*
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	
<i>o</i> -Aminophenol†	0.8	0	0.05	0.45	Olive green
<i>m</i> -Aminophenol†	0.75	0	0.04	0.40	Red-brown
<i>p</i> -Aminophenol†	0.75	0	0.02	0.40	Dark brown
<i>o</i> -Formamidophenol	0.7	0.5	0.7	1.0	Violet
<i>o</i> -Acetamidophenol	0.75	0.75	0.8	1.0	Violet
<i>o</i> -Benzamidophenol	0.9	0.95	0.9	1.0	Violet
<i>m</i> -Acetamidophenol	0.8	0.1	0.30	0.9	Purple
<i>p</i> -Acetamidophenol	0.9	0.85	0.25	0.9	Blue
<i>m</i> -Hydroxybenzoic acid	0.1	0.2	0.7	0.9	Crimson
<i>p</i> -Hydroxybenzoic acid	0.05	0.2	—	0.9	Crimson
<i>p</i> -Hydroxyphenylacetic acid	0.1	0.15	0.6	0.9	Violet
<i>o</i> -Hydroxyphenylurea	0.65	0.15	0.4	0.95	Mauve
Hydroxybenzoxazolone‡	0.6	0.1	0.2	0.75	Blue-green
2-(3-Hydroxyphenyl)-benzoxazole‡	0.9	0.95	—	—	Purple
Unidentified compound from 2-methylbenzoxazole urine	0.65	0.15	0.3	—	Purple
Chloro-2:4-dihydroxybenzamide	—	—	0.18	—	Purple

* See Bray *et al.* (1950*a*).

† These compounds reduce ammoniacal AgNO_3 .

‡ These compounds were isolated from urine.

to stability of the aminophenylsulphate). Diazo determination on urine after mild hydrolysis, $E + F$. Ehrlich determination on urine as collected, $A + C + E$. Ehrlich determination on urine after complete hydrolysis,

$$A + B + C + D + E + F.$$

Ethereal sulphate determination, $C + D$. Glucuronic acid determination (naphthoresorcinol), $E + F$. It can be seen from Table 2 that glucuronide results are in reasonable agreement with those found by diazo determination on the urine after mild hydrolysis. The relative proportions of E and F were determined by diazo estimations, before and after hydrolysis, on the glucuronide fraction of the pooled urines of ten rabbits obtained by the usual lead procedure (cf. Bray *et al.* 1947). Any significant difference (such differences were only obtained when benzoxazoles were administered) between the $E + F$ value estimated by diazotization and by the naphthoresorcinol method would suggest the existence of a glucuronide in which there is no diazotizable amino group, e.g. that of a hydroxylated benzoxazole. Even if the oxazole ring of such a compound was ruptured during hydrolysis the resulting dihydroxy-aniline would not be expected to diazotize and couple, since the liberated amino group would be in a position *ortho* to hydroxyl.

RESULTS

Excretion of normal metabolites. The average daily excretion of various metabolites by individual rabbits ranged as follows: ethereal sulphate, 26–41 mg. SO_3 ; reducing material, unhydrolysed, 175–210 mg., hydrolysed, 305–480 mg. (calculated as glucuronic acid); ether-soluble acid, 604–900 mg. (calculated as hippuric acid); phenols, free, 25–

60 mg. (using 2-(3-hydroxyphenyl)-benzoxazole as standard) or 17–41 mg. (using hydroxybenzoxazolone as standard), conjugated, 39–97 mg. (calculated as hydroxybenzoxazolone); mercapturic acid, 4–22 mg. (calculated as mercapturic acid of benzoxazolone). The average percentages by which individual daily normal values differed from the weekly average values used for baselines in calculation of final results were ± 14 , ± 10 , ± 10 , ± 11 , ± 29 or 37 , ± 18 and ± 65 respectively.

Metabolism of *o*-, *m*- and *p*-aminophenols

Quantitative. The results of determinations on urines excreted by rabbits which had received *o*-, *m*-, or *p*-aminophenol are summarized in Table 2. From these values the amounts of the different metabolites excreted have been calculated and are given in Table 3. The following illustrates the method of calculation for *o*-aminophenol. From diazo estimation on urine as collected $E = 53\%$. Hence, since $E + F = 65\%$, $F = 12\%$. Calculation from the ratio free : acetylated glucuronide (3.8:1) gives 51 and 14%. Values taken for E and F were, therefore, 52 and 13% respectively. $C + D = 15\%$ and from chromatographic estimation (Table 3) $A = 11\%$. Hence, since

$$A + B + C + D + E + F = 95\%, \quad B = 4\%.$$

Since *o*-aminophenylglucuronide gives only 65% and *o*-aminophenylsulphate only 33% of the colour

Table 2. *Results of quantitative analysis of urines from rabbits dosed with o-, m- and p-aminophenols*

(Results are expressed as average percentage of the dose; ranges in parentheses. Superior figures against ranges indicate the number of experiments. Dose 1 g. throughout.)

Method of estimation	<i>o</i> -Aminophenol	<i>m</i> -Aminophenol	<i>p</i> -Aminophenol
Diazo* on urine as collected (E or $C + E$)	53 (37–60) ¹⁰	59 (54–63) ⁶	53 (45–65) ⁸
Diazo* on urine after mild hydrolysis ($E + F$)	65 (60–68) ⁵	64 (62–67) ⁶	61 (55–65) ⁶
Ehrlich† on urine as collected ($A + C + E$)	50 (41–70) ¹⁰	58 (56–61) ⁶	79 (61–96) ⁸
Ehrlich† on urine after complete hydrolysis ($A + B + C + D + E + F$)	95 (86–108) ¹⁰	98 (85–118) ⁶	104 (85–125) ⁸
Ethereal sulphate ($C + D$)	15 (13–17) ²	15 (14–15) ²	12 (10–13) ²
Glucuronic acid ($E + F$)	70 (62–77) ^{2†}	60 (58–61) ²	62 (60–63) ²
Ratio of free to acetylated glucuronide in glucuronide fraction of urine ($E:F$)	3.8:1	10.8:1	2.8:1

* Corresponding aminophenylglucuronide used as standard.

† The aminophenol used as standard. The *o*-, *m*- and *p*-aminophenylglucuronides gave respectively 65, 105 and 180% of the colour intensity of the corresponding aminophenol.

‡ Pure *o*-aminophenylglucuronide gave only 80% of the theoretical colour value with the naphthoresorcinol reagent. These values have, therefore, been corrected. In these two experiments the dose did not cause the excretion of large amounts of reducing material (see p. 75).

Table 3. *Amounts of metabolites excreted by rabbits after administration of aminophenols*

(Results are expressed as average percentages of the dose and are calculated from data of Table 2 except for values in parentheses which were obtained by paper chromatography. Dose 1 g. throughout.)

Metabolite	<i>o</i> -Aminophenol	<i>m</i> -Aminophenol	<i>p</i> -Aminophenol
Unchanged aminophenol	(11)	0 (0)	(2)
Acetamidophenol	4 (2)	19 (12)	25 (13)
Aminophenylsulphate	15	0	8
Acetamidophenylsulphate	0	15	4
Aminophenylglucuronide	52	59	45
Acetamidophenylglucuronide	13	5	16

intensity of *o*-aminophenol in the Ehrlich method, the determination on urine as collected really gives $A + 0.33C + 0.65E$. Hence

$$11 + 0.33C + 0.65 \times 52 = 50$$

and $C = 15\%$ and $D = 0$. For *p*-aminophenol, C can be obtained from $C + E = 53$, assuming that the intensity of colour given by *p*-aminophenylsulphate in the diazo method is the same as that given by *p*-aminophenylglucuronide. The intensity of colour in the Ehrlich method for *m*-aminophenylglucuronide is the same as that for *m*-aminophenol, hence both A and $C = 0$.

The presence of aminophenol and acetamidophenol in ether extracts of the urines was detected by two-dimensional paper chromatograms using solvent mixture *A* followed by solvent mixture *B* (Table 1). Approximate estimations of the amounts present were made from one-dimensional chromatograms using solvent mixtures *A* (*o*- and *p*-aminophenol), *B* (*m*-acetamidophenol) and *C* (*o*- and *p*-acetamidophenol). The results are given in parentheses in Table 3.

Isolation of metabolites. The urines as collected (pH 7–8) were extracted with ether for 24 hr. in a continuous extractor. The following were isolated and characterized: *o*-aminophenol, m.p. 173° (7.5% of dose), from *o*-aminophenol urine; *m*-acetamidophenol, m.p. 148° (6% of dose), from *m*-aminophenol urine and *p*-acetamidophenol, m.p. 168° (4% of dose), from *p*-aminophenol urine. The amounts isolated provide confirmation of the relative amounts of free and acetylated phenols found by quantitative methods (Table 3).

Crystalline glucuronides were isolated from the urines by means of the usual lead procedure (cf. Bray *et al.* 1947). Their properties, given in Table 4, were essentially in agreement with those recorded by Williams (1943). The *o*-aminophenylglucuronide which we isolated appeared to be anhydrous. (Found: C, 50.1; H, 5.7; N, 5.0, 4.6; equiv. 265. Calc. for $C_{12}H_{15}O_7N$: C, 50.5; H, 5.3; N, 4.9%; equiv. 285.)

Table 4. *Properties of the glucuronides isolated from urine of rabbits after administration of aminophenols*

Crystalline form	<i>o</i> -Aminophenyl- glucuronide. Colourless elongated platelets	<i>m</i> -Aminophenyl- glucuronide. Colourless leaflets	<i>p</i> -Aminophenyl- glucuronide. Colourless needles
Melting point	Blackens from 230°*	Blackens from 220°	218° (decomp.)†
$[\alpha]_D$ in water (<i>c</i> , 0.5)	– 87° at 20°	– 100° at 21.5°	– 93° at 21°
N% (calc. 4.9)	4.8	4.6	5.1
Equiv. by titration (calc. 285)	265	290	293
Yield (% of dose)	24	6	18

* Williams (1943) gives 'blackened without melting on heating to 300°'. Comparison of our glucuronide with a specimen kindly provided by Prof. Williams showed identical behaviour of the two specimens in the melting-point apparatus, namely, no true melting but blackening starting about 230°. Prof. Williams states that by 'blackened without melting on heating to 300°' he meant that the 'limit of heating was 300°', and not that it blackened specifically at 300°.

† Williams (1943) gives 213° (decomp.) for monohydrate. (Calc. for monohydrate: N, 4.6%; equiv. 303.)

Metabolism of o-formamidophenol

Metabolites which might be expected to be excreted after administration of *o*-formamidophenol are *o*-formamidophenylsulphate and *o*-formamidophenylglucuronide, together with the unchanged compound and the metabolites of *o*-aminophenol described in the previous section. The quantitative methods used for aminophenol urines were applied and the results are summarized in Table 5. *o*-Formamidophenol neither diazotizes and couples nor reacts with the Ehrlich reagent, but it forms *o*-aminophenol on mild hydrolysis. The methods do not permit differentiation between the formamido and acetamido compounds. With this limitation the amounts of the various metabolites excreted have been calculated and are given in Table 6.

o-Aminophenol, *o*-formamidophenol and *o*-acetamidophenol were detected in ether extracts of the urine by two-dimensional paper chromatography using solvent mixtures *A* and *B* (Table 1). Approximate estimations indicated an amount of *o*-aminophenol corresponding to 2% of the dose and of *o*-acetamidophenol to 1%. Owing to the difficulty of finding a solvent mixture which separated it clearly from the phenols present in normal urine the amount of *o*-formamidophenol could only be assessed as between 5 and 10% of the dose.

Isolation of metabolites. The urine as collected (pH 7–8) from rabbits which had been given *o*-formamidophenol was continuously extracted with ether. The only metabolite isolated from the extract was *o*-formamidophenol. Yield 2.5% of the dose. *o*-Aminophenylglucuronide was isolated from the urine by the usual lead procedure. Yield 8% of dose.

Metabolism of benzoxazole

If the metabolism of benzoxazole in the rabbit involves rupture of the oxazole ring, *o*-formamidophenol and *o*-aminophenol are likely fission products. The urines from rabbits which had received benzoxazole were, therefore, treated like the

Table 5. *Results of quantitative analysis of urines from rabbits dosed with o-formamidophenol, benzoxazole and 2-methylbenzoxazole*

(Results are expressed as average percentage of dose; ranges given in parentheses. Superior figures indicate number of experiments. Dose 1 g. throughout.)

Method of estimation	<i>o</i> -Formamidophenol	Benzoxazole	2-Methylbenzoxazole
Diazo* on urine as collected	54 (39-62) ⁶	37 (31-47) ¹⁰	7 (5-10) ⁸
Diazo* on urine after mild hydrolysis	74 (68-77) ⁶	50 (42-56) ⁸	35 (26-42) ⁸
Ehrlich† on urine as collected	39 (35-44) ⁶	25 (14-39) ¹⁰	7 (3-8) ⁸
Ehrlich† on urine after complete hydrolysis	107 (90-131) ⁶	98 (87-132) ⁶	99 (83-112) ⁸
Ethereal sulphate	16 ⁸	16 (14-19) ⁴	16 (15-17) ²
Glucuronic acid	60 (57-63) ²	64 (52-83) ⁶	67 (55-76) ⁸
Ratio of free to acetylated glucuronide	2:1	2.3:1	1.4:2.5

* *o*-Aminophenylglucuronide used as standard.

† *o*-Aminophenol used as standard.

Table 6. *Amounts of metabolites excreted by rabbits after administration of o-formamidophenol, benzoxazole and 2-methylbenzoxazole*

(Results are expressed as average percentages of the dose and are calculated from data of Table 5, except for the values in parentheses, obtained by paper chromatography. Dose 1 g. throughout.)

Metabolite	<i>o</i> -Formamidophenol	Benzoxazole	2-Methylbenzoxazole
<i>o</i> -Aminophenol	(2)	(1)	(1)
<i>o</i> -Formamidophenol } <i>o</i> -Acetamidophenol }	8 { (5-10) (1)	17 { (5-10) (2)	15 { (0) (6)
<i>o</i> -Aminophenylsulphate	9	3	3
<i>o</i> -Formamidophenylsulphate and/or <i>o</i> -acetamidophenylsulphate	7	13	13
<i>o</i> -Aminophenylglucuronide	52	36	7
<i>o</i> -Formamidophenylglucuronide and/or <i>o</i> -acetamidophenylglucuronide	22	14	28
Hydroxylated benzoxazole†	—	14	32
Hydroxybenzoxazolone	—	4* (3)	—

* Unconjugated. Found by isolation. This value is additional to the total (98 %) found by the Ehrlich method.

† This value is the difference between that obtained by the naphthoresorcinol and that by the diazo method.

o-formamidophenol urines. The quantitative results are summarized in Table 5. The amounts of the various metabolites calculated from these are given in Table 6. The difference between the glucuronide values determined by the two methods suggests that about 14 % of the dose is excreted as a glucuronide other than that of *o*-aminophenol.

Benzoxazole yields *o*-aminophenol under the conditions for complete hydrolysis of conjugates, so that any unchanged benzoxazole excreted will be included in the estimation of the total amino compounds by the Ehrlich method on completely hydrolysed urines. Since the characteristic smell of benzoxazole was not observed in any of the urines or fractions obtained from them it is probable that the amount of benzoxazole excreted, if any, is very small. There is also the possibility of oxidation of the benzoxazole molecule without rupture of the oxazole ring. This might give benzoxazolone, hydroxybenzoxazole or hydroxybenzoxazolone. Of these only hydroxybenzoxazole would be likely to be estimated along with the total amino compounds (Ehrlich method) and would account for the glucuronide value by the naphthoresorcinol method being larger than the diazo value after mild hydrolysis. Benzoxazolone is not broken down under the

conditions of hydrolysis. The phenolic compounds, if conjugated with sulphuric acid, would be included in the ethereal sulphate excreted.

Two-dimensional paper chromatograms from ether extracts of the urines as collected revealed the presence of *o*-aminophenol, *o*-formamidophenol, *o*-acetamidophenol and a hydroxybenzoxazolone identical with that obtained as a metabolite of benzoxazolone (see below, p. 76). The approximate amounts present were 1, 5-10, 2 and 3 % of the dose respectively.

Isolation of metabolites. The only metabolite isolated from ether extracts of unhydrolysed benzoxazole urines was a hydroxybenzoxazolone which was obtained as a pale-brown powder, yield 4 % of dose, identical with the compound obtained from benzoxazolone urines. (Found: N, 9.1. Calc. for C₇H₅O₃N: N, 9.3 %.) *o*-Aminophenylglucuronide, identical with that obtained from *o*-aminophenol, was isolated from the urine by the usual lead procedure. (Yield 12 % of dose.)

Metabolism of 2-methylbenzoxazole

If the oxazole ring of 2-methylbenzoxazole is broken in the rabbit as is that of benzoxazole, the metabolites expected would be *o*-acetamidophenol

and *o*-aminophenol and their conjugates. The results of the quantitative analysis of the urine from rabbits which had received 2-methylbenzoxazole are summarized in Table 5 and the amounts of the various metabolites calculated from these data are given in Table 6. 2-Methylbenzoxazole is hydrolysed under the conditions for complete hydrolysis of conjugates. The difference between the glucuronide value and that for the diazo after mild hydrolysis suggests that about 32% of the dose is excreted as a hydroxylated methylbenzoxazole. No evidence for the excretion of unchanged 2-methylbenzoxazole, which has a stronger smell than has benzoxazole, was obtained.

Two-dimensional paper chromatograms from ether extracts of the urines showed the presence of *o*-aminophenol and *o*-acetamidophenol in amounts of approximately 1 and 6% of the dose respectively and a small amount of an unidentified metabolite. The unidentified metabolite reacted with Folin & Ciocalteu's reagent and gave a purple spot on the chromatogram when treated with diazotized *p*-nitraniline. *o*-Hydroxyphenylurea, which has the same R_f in solvent mixtures *A* and *B* (Table 1), gives a mauve spot and has a different R_f in solvent mixture *C*. The difficulty of synthesizing hydroxy-methylbenzoxazoles made it impracticable to compare such compounds with the unknown.

Isolation of metabolites. The only metabolite isolated from ether extracts of unhydrolysed 2-methylbenzoxazole urine was *o*-acetamidophenol. Yield 3% of dose. No crystalline glucuronide could be isolated by the usual lead procedure. From hydrolysed urine only *o*-aminophenol was isolated (15% of dose).

Metabolism of 2-phenylbenzoxazole

Preliminary experiments failed to reveal the presence of amino compounds (free or conjugated) in the urine excreted after administration of 2-phenylbenzoxazole to rabbits. This compound is apparently not broken down in the rabbit. The urines were examined quantitatively for ether-soluble acid, phenols, ether-type glucuronide, ethereal sulphate and mercapturic acid. The results obtained are summarized in Table 7. Paper chromatograms from ether extracts of the urines only revealed one phenolic compound in addition to those found in normal rabbit urine. This was the hydroxy-2-phenylbenzoxazole described below. The results suggest that this phenol is excreted free (2% of dose) and conjugated with sulphuric (10%) and glucuronic (48%) acids. The glucuronide is ether-soluble.

Isolation of metabolites. The pooled 24 hr. urines of eight rabbits which had each received 2-phenylbenzoxazole (1 g.) were adjusted to pH 7 and continuously extracted with ether for 48 hr. From the extract 400 mg. of colourless needles, m.p. 215–217°,

were isolated. These gave a positive reaction with Folin & Ciocalteu's reagent but no colour with ferric chloride. A further 120 mg. were obtained by extracting the residual urine with ether at pH 10

Table 7. *Results of quantitative analysis of urines from rabbits dosed with 2-phenylbenzoxazole and benzoxazolone*

(Results are expressed as average percentage of the dose; ranges in parentheses. Superior figures indicate number of experiments. Dose 1 g. throughout.)

Method of estimation	2-Phenylbenzoxazole	Benzoxazolone
Free phenols	2 (0–4) ^{2*}	2 (1–4) ^{5†}
Conjugated phenols	—	75 (68–86) ^{6†}
Ether glucuronide	48 (41–62) ³	28 (11–43) ^{6†}
Ethereal sulphate	10 ²	22 (21–23) ²
Ether-soluble acid	46 (39–55) ⁶	—
Mercapturic acid	2 (0–4) ^{2§}	2 (0–3) ^{2§}

* 2-(3-Hydroxyphenyl)-benzoxazole used as standard.

† Hydroxybenzoxazolone used as standard.

‡ Unreliable since benzoxazolone is a substance which causes excretion of large amounts of reducing material thus giving abnormally high baseline (see Bray, Hybs & Thorpe, 1951).

§ Probably not significant in view of variation in values in normal urine.

(total yield 6% of dose). Some of the compound was boiled under reflux with 10*N*-hydrochloric acid for 2 hr. The reaction mixture was neutralized and continuously extracted with ether. The extract was examined on two-dimensional paper chromatograms using solvent mixture *A* and then *B* (Table 1). Resorcinol, *m*-hydroxybenzoic acid and *o*-aminophenol were identified. This suggests that the compound isolated was 2-(3-hydroxyphenyl)-benzoxazole (VII). (Found: C, 73.5; H, 4.1; N, 6.9. $C_{13}H_9O_2N$ requires C, 73.9; N, 4.3; H, 6.6%.)

(VII)

The residual urine after the separation of the hydroxyphenylbenzoxazole was adjusted to pH 3 and further extracted with ether. The extract was evaporated to dryness and the residue recrystallized from water. Yield 1.3 g., colourless needles, m.p. 202°, which gave a positive naphthoresorcinol reaction. The properties were consistent with those expected of 3-(2-benzoxazolyl)-phenyl glucuronide monohydrate. (Found: C, 55.8; H, 4.6; N, 3.9. $C_{19}H_{17}O_5N \cdot H_2O$ requires C, 56.3; H, 4.7; N, 3.5%. Equiv. 405.) Acid hydrolysis gave resorcinol, *m*-hydroxybenzoic acid and *o*-aminophenol as with the free phenol.

Metabolism of benzoxazolone

This compound is relatively stable to hydrolysis by acids, and the oxazolone ring is not broken under the conditions for complete hydrolysis of conjugates. No evidence of its breakdown *in vivo* was obtained. Urines from rabbits dosed with benzoxazolone were examined quantitatively like those obtained after dosing with 2-phenylbenzoxazole. The results are summarized in Table 7. Only one metabolite of benzoxazolone, a hydroxybenzoxazolone, was detected in ether extracts of the urine by means of paper chromatography. The results suggest that this phenol is excreted free (2% of dose) and conjugated with sulphuric acid (22%) and glucuronic acid (53%). The last figure is derived from the conjugated phenol value since direct determination of glucuronide is unreliable (see footnote to Table 7).

Isolation of metabolites. Continuous extraction with ether of the urine as collected gave a brown powder which separated from the ethereal solution during the extraction. The same substance was obtained by extraction of acidified urine or urine which had been hydrolysed with acid. Yield, 2 g. from 8 g. of benzoxazolone. After crystallization from 50% (v/v) aqueous ethanol it formed very small pale-brown plates, m.p. 297–298° (decomp. with previous blackening). (Gressly & Nencki, 1890, 1891, stated that the substance blackened at 265°.) The substance gave a positive Folin & Ciocalteu reaction, a green colour with ferric chloride changing to brown, and a purple precipitate with Millon's reagent in the cold (unchanged on boiling). These properties are in agreement with those described by Gressly & Nencki (1890, 1891) for their hydroxybenzoxazolone. (Found: C, 55.9; H, 3.5; N, 9.4. Calc. for $C_7H_5O_3N$: C, 55.6; H, 3.3; N, 9.3%.) A *glucuronide* (yield 12% of dose) was isolated from the urine by the usual lead procedure in the form of colourless needles, m.p. 193° (decomp.); $[\alpha]_D^{20} - 79^\circ$ in water (c, 0.5). (Found: C, 43.5; H, 4.5; N, 3.9. Equiv. by reduction, 358; by titration, 336. $C_{13}H_{13}O_9N \cdot 2H_2O$ requires C, 43.0; H, 4.7; N, 3.9%. Equiv. 363.)

Hydrolysis of the hydroxybenzoxazolone by boiling with concentrated sulphuric acid for 3 hr. or heating in a sealed tube with ammonia (sp.gr. 0.880) at 100° for 3 hr. gave a solution which strongly reduced ammoniacal silver nitrate and gave positive Ehrlich and indophenol tests. (For conditions used for the latter see Thorpe, Williams & Shelswell, 1941.) The properties would be consistent with those of a dihydroxyaniline, but neither this compound nor a derivative could be separated for characterization. A positive indophenol reaction is usually indicative of an —OH group in a position *para* to an —NH₂ group. This admittedly slender

evidence suggests that the —OH in hydroxybenzoxazolone is in position 7 (VIII).

An attempt at synthesis of compound VIII from 2:4-dihydroxybenzamide by treatment with sodium hypochlorite (cf. preparation of benzoxazolone from salicylamide, Graebe & Rostovzoff, 1902; Thorpe & Williams, 1941) resulted in the isolation of colourless needles, m.p. 223–226°. (No blue-green spot characteristic of the hydroxybenzoxazole (Table 1) was detected on paper chromatograms.) The compound gave a purple spot on paper chromatograms and a crimson colour with $FeCl_3$ and was identified as a *chloro-2:4-dihydroxybenzamide*. (Found: C, 44.3; H, 3.2; N, 7.5; Cl, 18.9. $C_7H_4O_3NCl$ requires C, 44.8; H, 3.2; N, 7.5; Cl, 18.9%.) This amide was very resistant to hydrolysis by hot acid or alkali (sulphuric acid, 80% (v/v); hydrochloric acid, 10N; phosphoric acid (anhydrous) at 150°; or sodium hydroxide, 40%, w/v). The formation of an acid from the amide was, however, achieved by Bouveault's (1893) method with nitrous acid although this involved nitration. The chloro-2:4-dihydroxynitrobenzoic acid formed pale-yellow needles, m.p. 242° (decomp.), and gave a blood-red colour with ferric chloride. (Found: N, 6.5; Cl, 16.0. $C_7H_4O_5NCl$ requires N, 6.0; Cl, 15.2%.) Attempts to prepare this acid by a similar route starting with 2:4-dihydroxybenzoic acid resulted in decarboxylation by the sodium hypochlorite.

Treatment of the hydroxybenzoxazolone from urine with diazomethane gave a *methoxybenzoxazolone*, colourless plates, m.p. 106–107°. (Found: N, 8.6; OMe, 19.2. $C_8H_7O_3N$ requires N, 8.5; OMe, 18.8%.) No hydroxymethoxyaniline could be isolated after hydrolysis of this compound.

DISCUSSION

The results obtained with the aminophenols are essentially in agreement with those reported by Williams (1938, 1943) and Smith & Williams (1949). In view, however, of some recent observations made in this laboratory upon the conjugation of phenols in the rabbit it is not to be expected that close agreement in quantitative results would be obtained. In that investigation (Bray & White, 1951) it was shown that the percentage of a dose of phenol excreted as ethereal sulphate depends not only upon the available sulphate precursors in the diet and the dose level, but also upon the relation of the time at which the food was consumed to that of dosing. It is,

therefore, unlikely that results obtained in two laboratories will be strictly comparable unless the experimental conditions are very accurately reproduced.

It is clear that in benzoxazole and 2-methylbenzoxazole the oxazole ring may be regarded as a potential centre for conjugation, since the ring is opened and metabolites including *o*-aminophenol are excreted. The results of quantitative analyses suggested the formation also of conjugated hydroxylation products without rupture of the oxazole ring. This is in addition to the small amount of hydroxybenzoxazolone which was isolated from the benzoxazole urine as an unconjugated metabolite and may have been formed by oxidation of a hydroxybenzoxazole.

The formation of *o*-formamidophenol and *o*-aminophenol from benzoxazole suggests that cleavage of the oxazole ring occurs at the C—O linkage. Omitting intermediate stages this could be represented by:

The *o*-acetamidophenol detected was presumably formed by acetylation of the *o*-aminophenol. Attempts to differentiate quantitatively between *o*-formamidophenol and *o*-acetamidophenol in conjugated form by determination of the formic and acetic acids in hydrolysed urines gave unsatisfactory results. It should, perhaps, be mentioned that *o*-formamidophenol was never detected in urines of rabbits which had been given either *o*-aminophenol or *o*-acetamidophenol.

The intermediate expected from 2-methylbenzoxazole would be *o*-acetamidophenol which was actually found in greater amount than from benzoxazole. No *o*-formamidophenol was detected.

In 2-phenylbenzoxazole and benzoxazolone, however, the oxazole ring is stable and no *o*-aminophenol is formed. A new centre for conjugation is formed by hydroxylation. The finding that a considerable percentage of a dose of 2-phenylbenzoxazole or benzoxazolone is hydroxylated is not, therefore, unexpected. The position taken up by the new hydroxyl group introduced is of interest. In 2-phenylbenzoxazole the phenyl group in position 2 is hydroxylated, whereas in benzoxazolone hydroxylation can only occur in the benzene ring fused to the oxazole ring. It would appear that hydroxylation occurs more readily on the ring which is not fused to the oxazole ring, since no evidence of hydroxylation of the benzoxazole moiety of 2-phenylbenzoxazole was obtained.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-aminophenol, *o*-formamidophenol, benzoxazole, 2-methyl- and 2-phenylbenzoxazole and benzoxazolone in the rabbit has been studied.

2. The *o*-, *m*- and *p*-aminophenols were excreted unchanged to the extent of 11, 0 and 2%; they were excreted conjugated with acetic acid 17, 39 and 45%, with sulphuric acid 15, 15 and 12% and with glucuronic acid 65, 64 and 61% of the dose respectively. The properties of the glucuronides isolated were not in complete agreement with those recorded in the literature.

3. *o*-Formamidophenol was excreted as *o*-aminophenol to the extent of 2% of the dose, unchanged 7%, acylated 37%, conjugated with sulphuric acid 16%, and conjugated with glucuronic acid 74%.

4. Benzoxazole was excreted as *o*-aminophenol to the extent of 1% of the dose, as acylated aminophenol 44%, as aminophenol conjugated with sulphuric acid 16%, and as aminophenol conjugated

with glucuronic acid 50%. About 14% of the dose was excreted as undiazotizable glucuronide, i.e. hydroxylated without rupture of the oxazole ring. A hydroxybenzoxazolone (4% of dose) was isolated from urine.

5. 2-Methylbenzoxazole was excreted as *o*-aminophenol to the extent of 1% of the dose and as aminophenol conjugated with acetic acid 56%, with sulphuric acid 16% and with glucuronic acid 35%. About 32% of the dose was excreted as undiazotizable glucuronide, i.e. hydroxylated without rupture of the oxazole ring.

6. 2-Phenylbenzoxazole was excreted as free phenols to the extent of 2%, and conjugated with sulphuric acid 10% and with glucuronic acid 48% of the dose. There was no evidence of fission of the oxazole ring. A metabolite believed to be 2-(3-hydroxyphenyl)-benzoxazole and its glucuronide were isolated.

7. Benzoxazolone was excreted as free phenols to the extent of 2%, and conjugated with sulphuric acid 22% and with glucuronic acid 53% of the dose. There was no evidence of fission of the oxazolone ring. A hydroxybenzoxazolone was isolated from urine and its constitution is discussed.

8. R_f values on paper chromatograms for the phenolic metabolites are recorded.

The naphthoresorcinol glucuronic acid determinations were carried out by Miss B. G. Humphris. The microanalyses were carried out by Weiler and Strauss, Oxford.

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Biochemistry of Organic Phosphorus Insecticides

1. THE MAMMALIAN METABOLISM OF BIS(DIMETHYLAMINO)PHOSPHONOUS ANHYDRIDE (SCHRADAN)

BY J. E. GARDINER AND B. A. KILBY

Department of Biochemistry, University of Leeds

(Received 5 July 1951)

Since the original discovery in 1940 of the high toxicity and powerful anti-cholinesterase activity of dialkyl fluorophosphonates (Adrian, Feldberg & Kilby, 1947), many related organic phosphorus compounds (such as tetraalkyl pyrophosphates, dialkyl *p*-nitrophenyl phosphates) have been shown to have similar properties. Certain of these compounds are now being manufactured for use as insecticides, including bis(dimethylamino)phosphonous anhydride, $(\text{NMe}_2)_2\text{P}=\text{O} \cdot \text{O} \cdot \text{P}(\text{NMe}_2)_2$, which is distributed in this country under the trade name of 'Pestox III', and for which the trivial non-proprietary name of 'Schradan' has been recommended, in recognition of the work of Gerhard Schrader who discovered it (Schrader, 1947, 1951). Schradan has unusual properties as an insecticide, in that it shows only weak contact insecticidal action and no fumigant effect, but is readily absorbed into plants through their roots or leaves when applied as an aqueous solution and is transported around the plant in the sap stream. The whole plant is rendered toxic for several weeks to insects, especially aphides, which may feed upon it (Ripper, Greenslade & Lickerish, 1949).

While the mode of action of organic phosphorus compounds as insecticides is still uncertain, there does appear to be some correlation between high insecticidal action and high potency as inhibitors of insect cholinesterases *in vitro* (Metcalf & March, 1949). Schradan is anomalous in that its inhibitory action on this enzyme is only about a millionth of that of some of the more potent phosphorus insecticides; moreover, its inhibition of mammalian cholinesterases *in vitro* is of a similar low order, but if injected into animals it leads to the development of symptoms similar to those following administration of well known anti-cholinesterase drugs and characteristic of acetylcholine poisoning, although the development of symptoms following Schradan is rather slower and death may be delayed a few hours. There appeared to be the possibility that this compound was being converted in the mammalian body into some much more powerful anti-cholinesterase. A greater knowledge of the mammalian metabolism of Schradan is also desirable in view of the possible health hazards which might arise from the accidental consumption by man or animals of plants which had been treated with the insecticide