Studies in Detoxication

75. FURTHER OBSERVATIONS ON THE METABOLISM OF HYDRAZIDES OF AROMATIC ACIDS*

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In an earlier paper in this series (McIsaac & Williams, 1957) it was shown that the relatively non-toxic salicylohydrazide was metabolized by rabbits mainly by direct conjugation, whereas the relatively toxic benzohydrazide was probably hydrolysed to benzoic acid (excreted as hippuric acid) and hydrazine. It was suggested that the toxicity of benzohydrazide was probably due to the release of hydrazine *in vivo*. Further examples of toxic and non-toxic aroylhydrazines have now been examined in the rabbit, and the results obtained with them seem to support the above suggestion concerning their toxicity. *p*-Chlorobenzohydrazide and *p*-methylbenzohydrazide (*p*-toluoylhydrazine) have been found to be relatively toxic substances whose main metabolites are the glycine conjugates of the corresponding aromatic acids, whereas *m*- and *p*-hydroxybenzohydrazides and their acetyl derivatives are relatively non-toxic and are excreted mainly as *O*-conjugates of the hydrazides.

Under certain conditions the monoacetyl derivatives of the benzohydrazides can be dehydrated and converted into 1-oxa-3:4-diazoles:

$$Ar\cdot CO\cdot NH\cdot NH\cdot CO\cdot CH_3 \rightarrow Ar\cdot C(-O-)N\cdot N:C\cdot CH_3 + H_2O$$

The metabolism of two of these oxadiazoles has been studied, since they could be metabolites of certain benzohydrazides. No evidence, however, was found that benzohydrazides were cyclized *in vivo*.

EXPERIMENTAL

Materials and methods

Reference compounds. Benzohydrazide and its *p*-chloro, *p*-methyl, *m*- and *p*-hydroxy derivatives are known compounds (see *Beilsteins Handbuch*, 4th ed.), and were prepared by the standard procedure of refluxing the corresponding carboxylic acid ethyl esters (1 mole) with hydrazine hydrate (1·5 moles). *p*-Chloro-, *p*-methyl-, *m*- and *p*-hydroxy-hippuric acids were prepared from the corresponding hydrazides according to the method of Fox & Field (1943). 5-Methyl-2-phenyl-1-oxa-3:4-diazole, m.p. 67°, was prepared from 1-acetyl-2-benzoylhydrazine according to Stollé (1912). All these compounds were recrystallized until their melting points agreed with those found in the literature.

Acetylation of p-*hydroxybenzohydrazide.* (*a*) *With acetic anhydride and sulphuric acid.* *p*-Hydroxybenzohydrazide (50 g.) dissolved in acetic anhydride (50 ml.) was treated with concentrated sulphuric acid (2 ml.) and the mixture heated under reflux for 10 min. On cooling and pouring the product into cold water (500 ml.), 2-p-*acetoxyphenyl*-5-*methyl*-1-*oxa*-3:4-*diazole* (66% yield) separated and formed colourless plates from hot water, m.p. 123° (Found: C, 60·1; H, 4·8. $C_{11}H_{10}O_3N_2$ requires C, 60·5; H, 4·6%). The oxadiazole (48 g.) was added to 10% NaOH (w/v) (100 ml.) and the solution boiled for 5 min. On cooling and acidifying the solution, 2-p-*hydroxyphenyl*-5-*methyl*-1-*oxa*-3:4-*diazole* (86% yield) separated and formed white plates from hot water, m.p. 239° (Found: C, 61·7; H, 4·7. $C_9H_8O_2N_2$ requires C, 61·4; H, 4·6%).

(*b*) *With acetic anhydride and sodium carbonate.* *p*-Hydroxybenzohydrazide (50 g.) was suspended in 2N-Na_2CO_3 (100 ml.), and acetic anhydride (25 ml.) was added slowly with stirring and cooling. The mixture was kept overnight, during which time 1-p-*acetoxybenzoyl*-2-*acetylhydrazine* crystallized out. It was recrystallized from hot water and formed white plates of a monohydrate (62% yield), sparingly soluble in water and cold ethanol, m.p. 172° (Found: C, 52·0; H, 5·45; N, 11·6; H_2O, 7·1. $C_{11}H_{12}O_4N_2,H_2O$ requires C, 52·0; H, 5·55; N, 11·0; H_2O, 7·1%). On adding this diacetyl compound (45 g.) to 10% NaOH (w/v) (200 ml.), it dissolved. After the solution had been kept for 0·5 hr. it was acidified with dilute H_2SO_4 and 1-*acetyl*-2-p-*hydroxybenzoylhydrazine* (91% yield) separated, with m.p. 250° after recrystallization from hot water (Found: C, 55·6; H, 5·3; N, 14·1. $C_9H_{10}O_3N_2$ requires C, 55·65; H, 5·2; N, 14·4%).

Acetylation of m-*hydroxybenzohydrazide.* By the procedures outlined above for the *para* isomer, the following compounds were made: 2-m-*acetoxyphenyl*-5-*methyl*-1-*oxa*-3:4-*diazole*, m.p. 116° (Found: C, 60·25; H, 4·7. $C_{11}H_{10}O_3N_2$ requires C, 60·5; H, 4·6%); 2-m-*hydroxyphenyl*-5-*methyl*-1-*oxa*-3:4-*diazole*, m.p. 175° (Found: C, 61·4; H, 4·3. $C_9H_8O_2N_2$ requires C, 61·4; H, 4·6%); 1-m-*acetoxybenzoyl*-2-*acetylhydrazine* monohydrate, m.p. 56° (Found: C, 52·25; H, 5·3; N, 10·8. $C_{11}H_{12}O_4N_2,H_2O$ requires C, 52·0; H, 5·55; N, 11·0%); and 1-*acetyl*-2-m-*hydroxybenzoylhydrazine* monohydrate, m.p. 208° (Found: C, 50·7; H, 5·2; N, 12·7; H_2O, 8·6. $C_9H_{10}O_3N_2,H_2O$ requires C, 50·9; H, 5·7; N, 13·2; H_2O, 8·5%).

* Part 74: Robinson (1958).

p-*Carboxyhippuric acid.* *p*-Ethoxycarbonylbenzoylazide from 2 g. of *p*-ethoxycarbonylbenzohydrazide (Davidis, 1896) was shaken in ether with 2N-Na_2CO_3 (20 ml.) and glycine (2 g.) for 15 min. The ether was evaporated and on acidification the aqueous residue yielded a crystalline precipitate of p-*ethoxycarbonylhippuric acid*, which formed colourless plates from water, m.p. 189° (Found: C, 57·2; H, 5·4; N, 5·9. $C_{12}H_{13}O_5N$ requires C, 57·4; H, 5·2; N, 5·6%).

This ester (0·75 g.) was boiled for 0·5 hr. in 0·5N-NaOH (13 ml.). The solution was cooled and acidified, and the crystalline precipitate which formed was recrystallized from hot water. p-*Carboxyhippuric acid* was obtained as colourless plates, decomposing at 270–280° (Found: C, 54·0; H, 4·2; N, 6·0%; equivalent by titration, 110·9. $C_{10}H_9O_5N$ requires C, 53·8; H, 4·1; N, 6·3%; equiv. 111·5).

The relevant absorption spectra of the compounds used in this work were measured in a Unicam SP. 500 spectrophotometer and are quoted in Table 1.

Paper chromatography. For the detection of metabolites in urine and urine extracts, descending chromatography with Whatman no. 1 paper was used. The three solvent systems used (*A*, *B* and *C*) and R_F values are given in Table 2. The following reagents were used to detect compounds on paper.

Picryl chloride. According to Kul'berg & Cherkesov (1951) hydrazine and picryl chloride react to give a yellow colour which turns deep violet in alkali. The paper was sprayed with a 1% ethanolic solution of picryl chloride, then dried and exposed to fumes of NH_3. Hydrazides showed up as brown spots under this treatment.

Ammoniacal $AgNO_3$. 0·1N-$AgNO_3$ (5 ml.) was treated with one drop of 40% (w/v) NaOH and the precipitate

Table 1. *Ultraviolet absorption spectra of benzohydrazide and its derivatives*

	In ethanol		In 0·1N-HCl		In 0·1N-NaOH	
	$\lambda_{max.}$	$10^{-3}\,\epsilon_{max.}$	$\lambda_{max.}$	$10^{-3}\,\epsilon_{max.}$	$\lambda_{max.}$	$10^{-3}\,\epsilon_{max.}$
Benzohydrazide	—	—	230	10·6	265	5·2
	—	—	270	0·8	—	—
1-Acetyl-2-benzoylhydrazine	—	—	230	10·7	280	11·5
	—	—	268	1·0	—	—
1-Acetyl-2-*p*-hydroxybenzoylhydrazine	254	14·4	256	14·1	255	8·2
	—	—	—	—	297	22·0
1-Acetyl-2-*m*-hydroxybenzoylhydrazine	218	12·2	237	8·0	242	14·2
	228	7·5	—	—	—	—
	295	2·5	290	2·3	280	11·0
5-Methyl-2-phenyl-1-oxa-3:4-diazole	250	18·0	250	27·2	250	22·0
2-*p*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazole	270	22·3	270	18·8	305	46·5
2-*p*-Glucosiduronophenyl-5-methyl-1-oxa-3:4-diazole	265	22·5	262	22·2	262	23·0
2-*m*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazole	254	17·0	252	14·5	238	25·8
	—	—	—	—	265	8·8
	302	4·2	300	2·4	325	3·2
2-*m*-Glucosiduronophenyl-5-methyl-1-oxa-3:4-diazole	252	15·3	252	17·6	252	17·6
	293	2·9	287	3·3	287	3·3
p-Carboxyhippuric acid	240	20	—	—	—	—
	280	~2	—	—	—	—

Table 2. R_F *values of some benzohydrazides and their derivatives*

The solvent systems used were: *A*, propan-2-ol–water (17:3, v/v); *B*, *n*-butanol–acetic acid–water (4:1:5, by vol.); *C*, *n*-propanol–ammonia (sp.gr. 0·88) (7:3, v/v) run on Whatman no. 4 paper, by the descending technique, until the front had moved 10–12 in. from the origin.

Compound	R_F values in solvent		
	A	*B*	*C*
Hippuric acid	0·51	0·85	—
m-Hydroxyhippuric acid	0·40	0·71	—
p-Hydroxyhippuric acid	0·34	0·61	—
p-Methylhippuric acid	—	0·78	0·77
p-Carboxyhippuric acid	—	0·71	0·41
Benzohydrazide	0·59	0·62	—
1-Acetyl-2-benzoylhydrazine	0·63	0·75	—
m-Hydroxybenzohydrazide	0·62	0·86	—
1-Acetyl-2-*m*-hydroxybenzoylhydrazine	0·51	0·59	—
p-Hydroxybenzohydrazide	0·66	0·75	—
1-Acetyl-2-*p*-hydroxybenzoylhydrazine	0·92	0·55	—
2-*m*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazole	0·29	0·48	—
2-*p*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazole	0·80	0·89	—

which formed was just dissolved by adding 2N-NH_3 solution. When sprayed with this solution, free hydrazide spots showed up brown or black.

Veratric aldehyde. A 20% (w/v) solution of veratric aldehyde in ethanolic N-HCl was sprayed on the paper. After drying, the paper was examined under u.v. light (360 mμ) and hydrazides showed up as blue fluorescent spots. If the veratric aldehyde spray is made up in ethanolic 5N-HCl and the paper then heated for 2 min. at 110°, acetylated hydrazides and the oxadiazoles also show up as blue fluorescent spots in u.v. light.

Dimethylaminobenzaldehyde. This reagent was used to detect glycine conjugates according to Gaffney, Schreier, DiFerrante & Altman (1954).

Quantitative methods. Glucuronic acid in urine was determined according to Paul (1951), ethereal sulphates according to Sperber (1948) and hippuric acid according to El Masri, Smith & Williams (1956).

Qualitative experiments

Benzohydrazide. The urine of rabbits which had been dosed orally with 100 mg. of benzohydrazide/kg. was slightly reducing to Benedict's reagent and gave weak positive reactions for glucuronic acid and free hydrazides. Paper chromatography of the urine showed the presence of large amounts of hippuric acid and traces of unchanged benzohydrazide and probably of 1-acetyl-2-benzoylhydrazine.

p-*Chlorobenzohydrazide.* Oral doses slightly greater than 100 mg. of this hydrazide/kg. caused convulsions and death within 2 hr. in rabbits. The urine from animals receiving this compound was non-reducing and gave negative tests for free and acetylated hydrazides. The only metabolite detected chromatographically was *p*-chlorohippuric acid. The 24 hr. urine of ten rabbits which had received collectively 3 g. of *p*-chlorobenzohydrazide was made strongly acid with 10N-HCl and then continuously extracted with ether for 4 days. The residue obtained on evaporation of the ether was recrystallized (charcoal) from hot water and yielded 1·5 g. (corresponding to 40% of the dose of hydrazide) of *p*-chlorohippuric acid, m.p. and mixed m.p. 143°.

p-*Methylbenzohydrazide* (*p*-toluoylhydrazine). This compound was as toxic as *p*-chlorobenzohydrazide. Urine from rabbits receiving oral doses of 0·1 g./kg. was non-reducing; on paper chromatography it was found that it contained two glycine conjugates, one in large amounts and corresponding to *p*-toluric acid (*p*-methylhippuric acid) and the other in smaller amounts identified eventually as *p*-carboxyhippuric acid. No unchanged hydrazide or its acetyl derivative were found. The 24 hr. urine of eight rabbits which had collectively received 2 g. of *p*-methylbenzohydrazide was made strongly acid with 10N-HCl and continuously extracted with ether for 6 days. Removal of the ether from the extract left a residue which on recrystallization from hot water yielded 0·8 g. (31% of the dose) of *p*-toluric acid, m.p. and mixed m.p. 161°. The mother liquor was chromatographed on paper in solvent *C*, and the spot corresponding to *p*-carboxyhippuric acid was eluted with ethanol. The spectrum of the eluate was identical with that of authentic *p*-carboxyhippuric acid and showed a peak at 240 mμ and an inflexion of low extinction at about 280 mμ. A careful search of the urine of a rabbit which had received 1 g. of terephthalic acid failed to show the presence of *p*-carboxyhippuric acid or any other metabolite. It was therefore concluded that the *p*-carboxyhippuric acid was derived by oxidation of *p*-toluric acid.

p-*Hydroxybenzohydrazide.* This hydrazide was relatively non-toxic and doses of 500 mg./kg. could be fed to rabbits without untoward effects. The urine of animals receiving this dose gave an intense naphtharesorcinol reaction, reduced Benedict's reagent and gave an intense reaction with picryl chloride and NH_3. Paper chromatograms showed the presence of some of the unchanged hydrazide, but no acetylated hydrazide or *p*-hydroxyhippuric acid. A spot on the chromatograms reacting with picryl chloride proved also to contain glucuronic acid. This glucuronide was isolated. The aqueous glucuronide fraction (basic lead fraction) of the 24 hr. urine of four rabbits dosed with a total of 6 g. of *p*-hydroxybenzohydrazide was prepared by the lead acetate procedure (Kamil, Smith & Williams, 1951). The fraction was concentrated to 50 ml. and inorganic material removed by precipitation with 250 ml. of ethanol. Addition of a further 100 ml. of ethanol and keeping overnight yielded a crystalline precipitate (0·8 g.) which, on recrystallization from ethanol, yielded p-*aminocarbamoylphenyl* β-D-*glucosiduronic acid* as colourless needles, m.p. 195° and $[\alpha]_D^{21}$ − 89·6° (*c*, 1 in water) (Found: C, 45·0; H, 5·1; N, 8·2; H_2O, 5·0 (loss at 110°). $C_{13}H_{16}O_8N_2,H_2O$ requires C, 45·1; H, 5·2; N, 8·1; H_2O, 5·2%). This glucuronide, besides giving an intense naphtharesorcinol reaction, reduced Benedict's reagent and cold ammoniacal $AgNO_3$ and gave an intense brown colour with picryl chloride and NH_3.

1-Acetyl-2-*p*-hydroxybenzoylhydrazine was also fed (0·5 g./kg.), but although it gave large amounts of a glucuronide the latter could not be isolated.

m-*Hydroxybenzohydrazide.* This compound appeared to be more toxic than its *para* and *ortho* isomers (see McIsaac & Williams, 1957). It was fed at a dose of 250 mg./kg., which appeared to be the maximum dose tolerated without untoward effects. The urine was non-reducing, but gave a strong naphtharesorcinol reaction. Paper chromatography of the urine showed that none of the unchanged hydrazide or its acetyl derivative was present but that *m*-hydroxyhippuric acid was being excreted. Attempts to isolate the glucuronide in a crystalline form failed, but on ether extraction of the aqueous glucuronide fraction (prepared by the lead acetate procedure) *m*-hydroxyhippuric acid was isolated, m.p. and mixed m.p. 191°, after recrystallization from hot water. The yield was 0·3 g. from 12 g. of *m*-hydroxybenzohydrazide, or 2% of the dose.

1-Acetyl-2-*m*-hydroxybenzoylhydrazine was also fed at doses of 300 mg./kg. The urine was non-reducing and contained large amounts of conjugated glucuronic acid. Attempts to isolate the glucuronide failed. Paper chromatography of the urine showed that, besides a glucuronide, small amounts of the unchanged material and *m*-hydroxyhippuric acid were present.

2-m- *and* p-*Hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles.* These two compounds were fed to rabbits in doses of 500 mg./kg. and were found to be relatively non-toxic. They were excreted in a high degree of conjugation with glucuronic acid (see Table 3) and these glucuronides were readily isolated from the urine in the basic lead acetate fraction.

2-p-*Glucosiduronophenyl-5-methyl-1-oxa-3:4-diazole* (3 g. from 9 g. of the original oxadiazole) formed colourless needles from 30% aq. ethanol, m.p. 148° and $[\alpha]_D^{21}$ − 78·2° in water (*c*, 1) (Found: C, 46·7; H, 5·1; N, 7·6. $C_{15}H_{16}O_8N_2,2H_2O$

requires C, 46·4; H, 5·2; N, 7·2%). Water determination was unsatisfactory owing to the hygroscopic nature of the anhydrous glucuronide, and rapid weighing after drying at 110° accounted for 8% of water; calc. H_2O, 9·3%. The *O-triacetyl derivative* was prepared with acetic anhydride and $HClO_4$ and it formed colourless needles from hot water, m.p. 238° and $[\alpha]_D^{23} - 20·1°$ (*c*, 1 in $CHCl_3$) (Found: C, 52·7; H, 4·7; N, 5·8. $C_{21}H_{22}O_{11}N_2$ requires C, 52·7; H, 4·6; N, 5·9%). The corresponding 2-m-*glucosiduronophenyl-5-methyl-1-oxa-3:4-diazole* (5 g. from 9 g. of the oxadiazole fed) formed colourless needles, m.p. 102° and $[\alpha]_D^{21} - 73·8°$ in H_2O (*c*, 1) (Found: C, 45·0; H, 5·4; N, 7·2; H_2O, 10·9. $C_{15}H_{16}O_8N_2$,2·5H_2O requires C, 45·3; H, 5·3; N, 7·1; H_2O, 11·3%). The *O-triacetyl derivative* was also prepared and formed needles from water m.p. 121° and $[\alpha]_D^{19} - 19·1°$ in $CHCl_3$ (*c*, 1) (Found: C, 50·6; H, 4·9; N, 6·0; H_2O, 4·8. $C_{21}H_{22}O_{11}N_2$,H_2O requires C, 50·8; H, 4·9; N, 5·7; H_2O 4·5%).

The absorption spectra of these glucuronides were almost the same in ethanol, 0·1 N-HCl and 0·1 N-NaOH, as would be expected from a perusal of the spectra of 5-methyl-2-phenyl-1-oza-3:4-diazole in the same three solvents (see Table 1). The spectra of 2-*m*- and -*p*-hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles could be expected to be similar in ethanol and HCl, but different in NaOH owing to ionization of the phenolic hydroxyl group; Table 1 shows that this is true.

Both of the above glucuronides were hydrolysed by β-glucuronidase. 2-*p*-Glucosiduronophenyl-5-methyl-1-oxa-3:4-diazole (100 mg.) was dissolved in 3 ml. of water, and the solution titrated to pH 5 with N-NaOH. The solution was then mixed with 1 ml. of Roman-snail gastric juice which contains high amounts of β-glucuronidase (cf. Jarrige & Henry, 1952) and incubated at 37° for 18 hr. By the end of the incubation the solution had crystallized. The crystals (50 mg.) were filtered off and recrystallized from 20% (v/v) aq. ethanol. They were identified as 2-*p*-hydroxyphenyl-5-methyl-1-oxa-3:4-diazole, m.p. and mixed m.p. 239°. The experiment was repeated exactly with 100 mg. of 2-*m*-glucosiduronophenyl-5-methyl-1-oxa-3:4-diazole instead of the *para* isomer. The incubation mixture crystallized as before and 30 mg. of 2-*m*-hydroxyphenyl-5-methyl-1-oxa-3:4-diazole, m.p. and mixed m.p. 175° after recrystallization, were isolated.

Isolation of hydrazine from urine (with D. Robinson). Yard & McKennis (1955) have observed that certain doses of benzohydrazide and of hydrazine cause convulsions and death in rabbits and dogs. High doses of *iso*nicotinic acid hydrazide also cause convulsions. In this work we have observed that oral doses of benzohydrazide, *p*-methyl- and *p*-chloro-benzohydrazide, and picolinic acid hydrazide in the region of 100 mg./kg. invariably cause convulsions and death in rabbits. These are the hydrazides which appear to be hydrolysed *in vivo* to the corresponding aromatic acids and hydrazine. If hydrazine is formed during the metabolism of these compounds, then some hydrazine may be excreted in the urine, since McKennis, Weatherby & Witkin (1955) have shown that when hydrazine (15 mg./kg.) itself is injected in aqueous solution into dogs between 20 and 60% may appear unchanged in the urine. McKennis *et al.* (1955) were able to isolate hydrazine from urine by adding benzaldehyde to form the sparingly soluble derivative, benzalazine (dibenzylidenehydrazine).

Authentic benzalazine, m.p. 93°, was prepared according to Blatt (1943). It showed light-absorption in ethanol with $\lambda_{max.}$ 300–302 mμ, $\epsilon_{max.}$ 36 000. Rabbits were given oral doses of various aroylhydrazines (see Table 3) and the urine was collected for 24 hr. To every 100 ml. of filtered urine, 0·1 g. of benzaldehyde was added with shaking. The aldehyde dissolved and the urine was kept for 6–24 hr. Any crystalline material which had deposited during this time was collected by filtration and recrystallized from aqueous ethanol. In those cases where benzalazine was isolated (see Table 3) it was obtained as yellow crystals, m.p. and mixed m.p. 93°, with an absorption band in ethanol with $\lambda_{max.}$ 300–302 mμ. In the experiments in which benzalazine was not isolated directly, the urine was extracted with ether in which benzalazine is readily soluble. If benzalazine was present, the extract was pale yellow and showed maximum absorption of light at 300–302 mμ and in some cases small amounts of benzalazine were obtained on evaporation of the ether and recrystallization of the residue. Benzalazine was not formed when benzaldehyde was added to solutions of the aroylhydrazines; instead the aroylhydrazones of benzaldehyde were formed and these were readily distinguished from benzalazine.

It is to be noted (see Table 3) that benzalazine was isolated from the urines of rabbits which had received benzohydrazide and the pyridinecarboxylic acid hydr-

Table 3. *Excretion of hydrazine by rabbits receiving certain aroylhydrazines**

Aroylhydrazine administered	Dose (mg./kg.)	Benzalazine isolated from urine (mg.)‡	Remarks
Benzoyl	83	19·5	Equivalent to 5% of dose
p-Toluoyl	83†	0	Benzalazine detected in ether extracts
p-Chlorobenzoyl	83	0	—
m-Hydroxybenzoyl	83	0	—
p-Hydroxybenzoyl	83	0	—
Picolinoyl§	83†	3	Equivalent to 0·8% of dose
Nicotinoyl§	83	0·5	From three animals (0·01% of dose)
*iso*Nicotinoyl§	166	5	Equivalent to 0·6% of dose

* The results are for single animals except in the case of nicotinoylhydrazine where three animals were used.
† In these cases the animals died within 18 hr. of dosing.
‡ These yields are after recrystallization.
§ The urinary metabolites of these three hydrazides in rabbits are the corresponding pyridine carboxylic acids and their glycine conjugates (El Masri, 1956).

azides. It was also detected, after the addition of benzaldehyde, in the urine after dosing with *p*-methylbenzohydrazide, but not after *m*- and *p*-hydroxy- and *p*-chloro-benzohydrazides, although the last compound is relatively lethal. Benzalazine could not be isolated or detected in the urine of four patients receiving 200 and 350 mg. of *iso*nicotinic acid hydrazide.

DISCUSSION

It is clear from Table 4 that benzohydrazide and its *p*-chloro and *p*-methyl derivatives are converted *in vivo* into the corresponding aromatic acids, which are largely excreted as glycine conjugates. None or only traces of the unchanged hydrazides or their acetyl derivatives were excreted. These compounds appear to behave like the corresponding benzamides (Bray, Clowes, Thorpe, White & Wood, 1952; Bray, Thorpe & Wood, 1949). Benzohydrazide is a relatively toxic substance (cf. Yard & McKennis, 1955) and we have observed that *p*-chloro- and *p*-methyl-benzohydrazides are about as toxic, since doses of less than 1 m-mole/kg. caused death with convulsions. It is concluded (cf. McIsaac & Willians, 1957) that this toxicity must be due to release of hydrazine (see Table 3) from these compounds in the body, and that the main route of their metabolism is

$$R\cdot C_6H_4\cdot CO\cdot NH\cdot NH_2 \rightarrow NH_2\cdot NH_2 + R\cdot C_6H_4\cdot CO_2H \rightarrow R\cdot C_6H_4\cdot CO\cdot NH\cdot CH_2\cdot CO_2H$$

(where R = H, CH_3 or Cl)

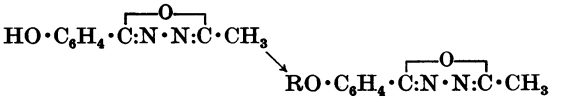
m- and *p*-Hydroxybenzohydrazides and their acetyl derivatives, however, were very much less toxic and, except for *m*-hydroxybenzohydrazide, were well tolerated by rabbits in oral doses of 4 m-moles/kg. (600 mg./kg.). The *meta* derivative, however, was fatal in doses slightly more than 2 m-moles/kg. (300 mg./kg.). These hydroxy compounds were mainly metabolized by direct conjugation with glucuronic and sulphuric acids (see Table 4) and, except for *m*-hydroxybenzohydrazide, they did not give rise to the excretion of the glycine conjugates of the corresponding aromatic acids. Only with *m*-hydroxybenzohydrazide was the corresponding glycine conjugate isolated from the urine. It is concluded that *p*-hydroxybenzohydrazide, its acetyl derivative and N^1-acetyl-N^2-*p*-hydroxybenzoylhydrazine do not release appreciable amounts of hydrazine in the body and are metabolized thus:

$$HO\cdot C_6H_4\cdot CO\cdot NH\cdot NH_2 \rightarrow RO\cdot C_6H_4\cdot CO\cdot NH\cdot NH_2$$

(R = SO_3H or $C_6H_9O_6$)

m-Hydroxybenzohydrazide seems to take an intermediate place between benzohydrazide and *p*-hydroxybenzohydrazide, since it appears to be partly directly conjugated and partly hydrolysed to give hydrazine and the corresponding hippuric acid. It is also of intermediate toxicity.

The cyclic hydrazine derivatives, 2-*m*- and -*p*-hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles possess a stable ring system *in vivo* and are metabolized by direct *O*-conjugation (see Table 4). These cyclic derivatives were not found as metabolites of the benzohydrazides. Their metabolism can be represented as



(R = SO_3H or $C_6H_9O_6$)

Table 4. *Metabolism of benzohydrazide and related compounds*

Results are quoted as the average for three animals, with range in parentheses; superior figures indicate the number of experiments if other than three.

Compound	Dose (mg./kg.)	Conjugations (% of dose) with: Glucuronic acid	Ethereal sulphate	Glycine
Benzohydrazide	80	4 (3, 6)[2]	0	55 (51–57) as hippuric acid
p-Chlorobenzohydrazide	100	0	0	+(40% isolated as *p*-chlorohippuric acid)
p-Methylbenzohydrazide	100	0	0	+(31% isolated as *p*-toluric acid; *p*-carboxyhippuric acid detected)
p-Hydroxybenzohydrazide	300	36 (32–41)	8 (5–10)	None
m-Hydroxybenzohydrazide	300	30 (24–36)	8 (6–11)	+(2% isolated as *m*-hydroxyhippuric acid)
1-Acetyl-2-*p*-hydroxybenzoylhydrazine	390	46 (46–47)	19 (15–23)	None
1-Acetyl-2-*m*-hydroxybenzoylhydrazine	390	57 (55–59)	18 (17–21)	+(*m*-hydroxyhippuric acid detected)
2-*p*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazole	400	79 (70, 88)[2]	5 (4, 7)[2]	None
2-*m*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazole	400	60 (55–67)	7 (6–8)	None

SUMMARY

1. The fate in the rabbit of the following compounds has been investigated: benzohydrazide and its *p*-chloro, *p*-methyl and *m*- and *p*-hydroxy derivatives; 1-acetyl-2-*m*- and -*p*-hydroxybenxoylhydrazines and their anhydrides; 2-*m*- and -*p*-hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles.

2. Benzohydrazide, *p*-chlorobenzohydrazide and *p*-methylbenzohydrazide were lethal to rabbits in oral doses of just over 100 mg./kg. and were metabolized to the corresponding hippuric acids, which were isolated from the urine. Hydroxylation of the benzene ring and acetylation of the hydrazide group were not detected.

3. *p*-Hydroxybenzohydrazide and its acetyl derivative and 1-acetyl-2-*p*-hydroxybenzoylhydrazine were relatively non-toxic and were metabolized by direct conjugation with glucuronic and sulphuric acids.

4. *m*-Hydroxybenzohydrazide was intermediate in toxicity between benzohydrazide and *p*-hydroxybenzohydrazide, and was metabolized mainly by direct conjugation but partly by hydrolysis to *m*-hydroxybenzoic acid.

5. 2-*m*- and -*p*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles were relatively non-toxic and were metabolized by direct conjugation mainly with glucuronic acid.

6. It is suggested that the toxicity of benzohydrazide and its *p*-chloro and *p*-methyl derivatives is due to release of hydrazine *in vivo*.

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The Vitamin B_{12}-binding Systems of Isolated Intestine of the Rat

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It has recently been demonstrated (Schilling & Schloesser, 1957) that pseudovitamin B_{12} does not affect the absorption of vitamin B_{12} by human beings, nor does it compete to any great extent for binding by human gastric juice. They concluded from this that the vitamin B_{12}-binding property of gastric juice was very specific.

It occurred to us that similar observations might be useful in our studies of the uptake of vitamin B_{12} by isolated rat intestine from buffered solutions containing the vitamin. We hoped that it might be possible in this way to obtain information concerning different binding systems. These anticipations have been justified since our observations have led to the demonstration that there are at least two vitamin B_{12}-binding systems in the small intestine of the rat. One of these is susceptible to competition by the analogues we have used, whereas the other seems to be highly specific for cyanocobalamin itself.

MATERIALS AND METHODS

Animals. Black-and-white rats of the Scott–Russ strain were used. The animals were starved overnight before an experiment. They were killed by a blow on the head and the intestine was then removed.

Radioactive vitamin B_{12} and analogues. Radioactive vitamin B_{12}, containing a mixture of ^{58}Co and ^{57}Co was dissolved in Krebs's phosphate buffer at pH 7·4 (Umbreit, Burris & Stauffer, 1949) to give a concentration of 5 μmg./ml. Various analogues of vitamin B_{12} including pseudovitamin B_{12}, a mixture of the ethylamides of vitamin B_{12}