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Studies in Detoxication

77. THE METABOLISM OF PHENYLHYDRAZINE AND SOME PHENYLHYDRAZONES*

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Phenylhydrazine was discovered by Fischer (1875). It is highly toxic and Fischer himself soon became sensitized to it, developing a severe chronic eczema. Although phenylhydrazine is a haemolytic poison, it was used at one time in the treatment of polycythaemia vera. *N*-Acetyl-*N'*-phenylhydrazine (Pyrocin or Hydraceticin), however, was later preferred to the free base in treatment, since it was less toxic and had a greater margin of safety (see Whitby & Britton, 1953).

The metabolism of phenylhydrazine in mammals has not been investigated in detail. Hoppe-Seyler (1885) and Lewin (1901) reported that, in warm-blooded animals, it was rapidly destroyed since its presence in the blood or urine could not be demonstrated after its administration.

In the present work it was possible to demonstrate by the isotope-dilution technique that [^{14}C]-phenylhydrazine was extensively transformed *in vivo*. Its urinary metabolites appeared to be mainly conjugated *p*-hydroxyphenylhydrazine and the phenylhydrazones of the natural keto acids.

Some of the radioactivity of the administered compound, however, remained in the body for longer than 10 days after dosing and this was largely found in the erythrocytes. Observations have also been made on the urinary excretion of metabolites after the administration of acetyl[^{14}C]-phenylhydrazine and of pyruvic acid and α -oxoglutaric acid [^{14}C]phenylhydrazones.

MATERIALS AND METHODS

All melting points are corrected.

Animals. Chinchilla doe rabbits kept on a diet of 50 g. of rat cubes (diet 41: Associated London Flour Millers) per day and water *ad lib.*, were used. The [^{14}C]phenylhydrazine hydrochloride, acetyl[^{14}C]phenylhydrazine, and the sodium salts of pyruvic acid [^{14}C]phenylhydrazone, and α -oxoglutaric acid [^{14}C]phenylhydrazone were administered by stomach tube or by subdermal injection as 10% (w/v) aqueous solutions. Urine was collected daily.

Materials. [^{14}C]Phenylhydrazine hydrochloride, m.p. 242–243° (decomp.), of specific activity 7.65 $\mu\text{C/g.}$, was prepared in 85% yield from randomly labelled [^{14}C]aniline by a standard method of diazotization and reduction (see Gilman & Blatt, 1948). This hydrochloride (1 g.) in water

* Part 76: Jondorf, Parke & Williams (1958).

(10 ml.) was treated with 40% (w/v) NaOH (5 ml.) and the free base extracted with ether. The ether was evaporated and the residue treated with acetic anhydride (1 ml.). The acetyl[¹⁴C]phenylhydrazine, m.p. 128° after recrystallization from hot water, was obtained in 60% yield with a specific activity of 7.25 μ C/g. Pyruvic acid [¹⁴C]phenylhydrazone, m.p. 190°, was obtained in 90% yield by addition of pyruvic acid to a sodium acetate-buffered aqueous solution of phenylhydrazine hydrochloride. It was recrystallized from ethanol and had a specific activity of 6.3 μ C/g. α -Oxoglutaric acid [¹⁴C]phenylhydrazone, m.p. 152° (specific activity, 4.8 μ C/g.), was prepared in a similar manner (Ingold, 1921).

Other non-radioactive reference compounds, prepared by standard methods, were *p*-aminophenyl glucosiduronic acid (m.p. 216°, $[\alpha]_D^{20} - 83^\circ$ in 0.1N-HCl) (Williams, 1943), the phenylhydrazones of acetoacetic acid (decomp. 95–100°) and D-glucose (α -form m.p. 159°) and glucosazone (m.p. 208°). The phenylhydrazones of acetone, oxaloacetic acid, acetoacetic acid and α -oxoglutaric acid were not sufficiently stable for isotopic dilution. The following derivatives were therefore made. Acetone phenylhydrazone (m.p. 38°) was converted into 2-phenyl-1-isopropylidenesemicarbazide (m.p. 138°) with potassium cyanate (Arnold, 1897). Oxaloacetic acid phenylhydrazone (Fenton & Jones, 1900) rapidly decomposes at 95–100° (Fenton, 1901) or, if kept at 30° for several weeks, to pyruvic acid phenylhydrazone. In the presence of acids (pH < 3) it is rapidly converted even at 30° into 1-phenylpyrazol-5-one-3-carboxylic acid (Jones, 1902). The latter, m.p. 255° (decomp.), was prepared by adding aqueous phenylhydrazine hydrochloride to aqueous oxaloacetic acid at 30° and recrystallizing the product from water. 3-Methyl-1-phenylpyrazol-5-one, m.p. 127°, was prepared from ethyl acetoacetate and phenylhydrazine hydrochloride (Michael, 1892) and its 4-nitroso derivative, m.p. 154°, by treating a solution of it in 2N-HCl with an excess of 5% aqueous NaNO₂ and adjusting to pH 7 to precipitate the nitroso derivative (Michaelis, 1905). 1-Phenyl-4,5-dihydropyridazin-6-one-3-carboxylic acid, m.p. 172°, was prepared by refluxing α -oxoglutaric acid phenylhydrazone in 5N-HCl for 1 hr. (Wislicenus & Waldmüller, 1911).

p-Hydroxyphenylhydrazine hydrochloride (Altschul, 1898) was obtained from potassium *p*-hydroxyphenylhydrazine *N*-sulphonate, m.p. 178° (Reisenegger, 1883), as pale-pink crystals which rapidly blackened in air to give a product shown to be largely *p*-aminophenol hydrochloride. *p*-Hydroxyphenylhydrazine oxalate was similarly unstable, and attempts to prepare the acetate and 4-dimethylaminobenzaldehyde 4'-hydroxyphenylhydrazone were unsuccessful. Owing to their instability, *p*-hydroxyphenylhydrazine derivatives were unsatisfactory for isotopic dilution; instead, the facile decomposition of *p*-hydroxyphenylhydrazine into *p*-aminophenol, which proceeds to completion on warming in acid solution, was used in the identification and determination of *p*-hydroxyphenylhydrazine in urine. When potassium *p*-hydroxyphenylhydrazine *N*-sulphonate or freshly prepared *p*-hydroxyphenylhydrazine hydrochloride is warmed to 40° in 5N-HCl in the presence of a little naphtharesorcinol, the solution turns an intense purple colour, which is only slightly extractable by ether (cf. the naphtharesorcinol test for glucuronic acid). On addition of alkali (2N-NaOH) the colour turns to a cherry red. This test appears to be a test for *p*-hydroxyphenyl-

hydrazine and is not given by *p*-aminophenol or phenylhydrazine.

Analytical methods. Glucuronic acid and ethereal sulphates in urine were determined by the methods of Paul (1951; see Mead, Smith & Williams, 1958) and Sperber (1948) respectively.

Measurement of radioactivity. All measurements were carried out on solid or liquid samples of 'infinite thickness' with an end-window counter. The specific activities were determined by comparison with a poly([¹⁴C]methyl methacrylate) reference source (Radiochemical Centre, Amersham), and sufficient counts to give a standard error of $\pm 2\%$ were taken.

Urine and tissues. The total radioactivity in the urine was measured directly, or after evaporation to dryness on planchets under infrared lamps. The radioactivity in the faeces, stomach and intestinal contents and spleen was measured after homogenization of the materials with water. Blood was collected in heparin and the radioactivity was measured directly. The activity of the erythrocytes was determined after haemolysis and drying.

Chromatography

Paper chromatography. The solvent system which most effectively achieved separation of the phenylhydrazones, aminophenols, acetylphenylhydrazine and phenylhydrazine was diisopropyl ether saturated with water. Descending chromatograms were run for 4 hr. on Whatman no. 1 or no. 4 papers. The metabolites were detected as reducing spots after spraying the chromatograms with ammoniacal AgNO₃, as fluorescent spots in u.v. light (254 m μ) after spraying with salicylaldehyde reagent (see Table 1), or by the quenching of the background u.v. fluorescence of the paper. In addition radioautographs were made by placing the chromatograms of radioactive material in contact with X-ray film in light-proof cassettes for 7–14 days.

Zone electrophoresis. The phenylhydrazones of keto acids were also separated by electrophoresis on Whatman no. 1 paper in 0.05N-Na₂CO₃ with a current of about 4 ma and 560 v (see separation of 2,4-dinitrophenylhydrazones of keto acids by Neish, 1953). In 2 hr. 1-phenylpyrazol-5-one-3-carboxylic acid, and the phenylhydrazones of pyruvic acid, α -oxoglutaric acid and oxaloacetic acid, travelled 7.3, 7.6, 13.4 and 13.5 cm. respectively towards the anode.

Isotopic-dilution methods

For the estimations of metabolites by isotopic dilution, urines were collected from each rabbit for 48 or 96 hr. The volumes (5–25 ml.) used depended on the substance estimated.

'Free' phenylhydrazine. Phenylhydrazine hydrochloride (500 mg.) was dissolved in a portion of the urine at 40° and kept for 2 hr. to equilibrate. The solution was then adjusted to pH 10 and the phenylhydrazine extracted with ether. After washing with water the extract was evaporated to dryness and the residue treated with a few drops of 2N-HCl. The phenylhydrazine hydrochloride obtained was recrystallized from water to constant specific activity (m.p. and mixed m.p. 242–243°, decomp.).

'Total' phenylhydrazine. Phenylhydrazine hydrochloride (500 mg.) was added to a portion of the urine, which was then refluxed for 3 hr. with 0.5 vol. of conc. HCl. After

cooling, the procedure adopted was identical with that for 'free' phenylhydrazine.

Acetylphenylhydrazine. Acetylphenylhydrazine (500 mg.) was dissolved in a portion of the urine by slight warming. The solution was kept for 1 hr. and then extracted with chloroform. The extract was washed with water, evaporated to dryness and the residue repeatedly recrystallized from water until its specific activity became insignificant (m.p. and mixed m.p. 128°).

Glucosazone. Glucosazone (250 mg.) was added to a portion of urine, dissolved by warming to 40°, and after keeping for 4 hr. the solution was extracted with ether. The extract was washed with water, evaporated to dryness and the residue recrystallized from 10% (v/v) aqueous ethanol until its specific radioactivity became insignificant (m.p. and mixed m.p. 208°).

Glucose phenylhydrazone. The α -form of D-glucose phenylhydrazone (500 mg.) was dissolved in urine. After 4 hr. it was extracted as in the preceding paragraph. The residue of glucose phenylhydrazone was recrystallized from ethanol until its specific radioactivity became insignificant (m.p. and mixed m.p. 159°).

Acetone phenylhydrazone. Acetone phenylhydrazone (500 mg.) dissolved in 10 ml. of ether was shaken with a portion of the urine for 0.5 hr. to equilibrate. The ethereal layer was separated, and washed with water, 0.2N-HCl, and water again, dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in 0.2 ml. of conc. HCl and 0.2 g. of powdered KOCN was added. The product of 2-phenyl-1-isopropylidene semicarbazide was triturated with water and removed by filtration and recrystallized from ethanol to constant specific activity (m.p. and mixed m.p. 138°).

Phenylhydrazones of pyruvic acid and α -oxoglutaric acid. These phenylhydrazones (300–500 mg.) were added to separate portions of the urine and dissolved by adjusting to pH 8 with 2N- Na_2CO_3 . After 4 hr. the solutions were adjusted to pH 6 with 2N-HCl and continuously extracted with ether for 6 hr. The extracts were washed with water

and the phenylhydrazones transferred to 2N- Na_2CO_3 . The alkaline solutions were then made slightly acid with 2N-HCl and extracted with ether. These extracts were washed with water and evaporated to dryness, and the residual phenylhydrazones recrystallized from aqueous ethanol to constant specific radioactivity (m.p. and mixed m.p. 190° and 152° for pyruvic acid and α -oxoglutaric acid phenylhydrazones respectively). In some cases (see Table 6) the α -oxoglutaric acid phenylhydrazone was refluxed in 5N-HCl to convert it into 1-phenyl-4:5-dihydropyridazin-6-one-3-carboxylic acid, which was recrystallized from ethanol to constant specific radioactivity (m.p. and mixed m.p. 172°).

Confirmation of the identity and isotopic purity of the pyruvic acid and α -oxoglutaric acid [^{14}C]phenylhydrazones were obtained by paper chromatography in diisopropyl ether–water and zone electrophoresis. On paper, the pyruvic acid [^{14}C]phenylhydrazone gave a single radioactive spot (R_F 0.86), and by zone electrophoresis it showed mobility and colour reactions identical with those of authentic pyruvic acid phenylhydrazone. Similarly, the α -oxoglutaric acid [^{14}C]phenylhydrazone gave a single radioactive spot (R_F 0.95) by chromatography and, on zone electrophoresis, it had mobility and colour reactions identical with those of authentic material.

Oxaloacetic acid phenylhydrazone. This phenylhydrazone (250 mg.) dissolved in N- NaHCO_3 was added to a portion of urine, which was then acidified with 2N-HCl and warmed to 60° to convert the phenylhydrazone into 1-phenyl-pyrazol-5-one-3-carboxylic acid. On cooling the solution the pyrazolonecarboxylic acid separated and was filtered off, redissolved in N- NaHCO_3 , treated with charcoal and reprecipitated by acidifying with 2N-HCl. This process was repeated and the pyrazolonecarboxylic acid was finally recrystallized from water and ethanol until the specific radioactivity was constant (m.p. and mixed m.p. 255° decomp.). Isotopic-dilution experiments were also carried out in a similar manner after addition of 1-phenylpyrazol-5-one-3-carboxylic acid dissolved in N- NaHCO_3 to portions of the urine.

Table 1. R_F values and colour reactions on paper of possible metabolites of phenylhydrazine

Descending chromatography on Whatman no. 1 paper. In solvent A (diisopropyl ether saturated with water) values are after 4 hr., and in solvent B (butan-2-one saturated with water) values are after 2 hr. Quenching of the background fluorescence of the paper in u.v. light is denoted by Q.

	R_F values in		Colour reactions		
	A	B	Ammoniacal AgNO_3	Fluorescence	Fluorescence* with salicylaldehyde
Phenylhydrazine	0.05	—	Brown	Q	—
N-Acetylphenylhydrazine	0.23	—	—	Q	—
p-Phenylenediamine	0.18	—	Brown	Q	—
NN'-Diacetyl-p-phenylenediamine	0.01	—	—	Q	—
o-Aminophenol	0.77	—	Brown	—	Yellow
m-Aminophenol	0.60	—	Brown	—	Yellow
p-Aminophenol	0.40	—	Brown	—	Yellow
Glucose phenylhydrazone	0.00	—	Brown	Q	—
Glucosazone	0.00	—	—	Q	—
Pyruvic acid phenylhydrazone	0.86	0.96	Brown	Q	—
α -Oxoglutaric acid phenylhydrazone	0.95	0.93	Brown	Q	—
Oxaloacetic acid phenylhydrazone	0.78	0.90	Brown	Q	—
1-Phenylpyrazol-5-one-3-carboxylic acid	0.38	—	No colour	Blue	—
3-Methyl-1-phenylpyrazol-5-one	0.86	—	Brown	Q	—
Acetone phenylhydrazone	0.96	—	Brown	Q	—

* Fluorescence in u.v. light after treatment with 1% ethanolic salicylaldehyde containing 0.05 vol. of acetic acid.

3-Methyl-1-phenylpyrazol-5-one. The phenylhydrazone of acetoacetic acid is unknown and would probably decompose spontaneously to give acetone phenylhydrazone by loss of CO_2 or 3-methyl-1-phenylpyrazol-5-one by loss of water. 3-Methyl-1-phenylpyrazol-5-one (250 mg.) was added to a portion of the urine. After acidification with 0.1 vol. of 2N-HCl, the urine was cooled to 0° and treated with an excess of a 5% (w/v) aqueous solution of NaNO_2 . The 3-methyl-4-nitroso-1-phenylpyrazolone formed was then extracted with ether. The extract was washed with water and the nitrosophenylpyrazolone was transferred to 2N-NaOH; addition of 2N-HCl precipitated the nitrosophenylpyrazolone, which was filtered off, washed with water and recrystallized from acetic acid or toluene to constant specific activity (m.p. and mixed m.p. 154°).

Aminophenols. The *o*-, *m*- and *p*-aminophenols were determined by the procedure of Parke (1956). The *ON*-dibenzoyl derivatives of *o*-, *m*- and *p*-aminophenols (m.p. and mixed m.p. 183°, 153° and 237° respectively) and the corresponding *o*-, *m*- and *p*-acetamidophenols (m.p. and mixed m.p. 207°, 149° and 169° respectively) were recrystallized from ethanol and aqueous ethanol to constant specific activity or until the radioactivity was insignificant. Under these conditions *p*-hydroxyphenylhydrazine and its conjugates were converted into *p*-aminophenol.

***p*-Phenylenediamine.** A solution of *p*-phenylenediamine (200 mg.) in water (5 ml.) was added to a portion of the urine, which was then refluxed for 3 hr. with 0.5 vol. of conc. HCl. After cooling, the solution was adjusted to pH 12 with 2N-NaOH and extracted into ether. Evaporation of the ether gave a residue of *p*-phenylenediamine, which was dissolved in water (5 ml.), filtered, and acetylated with acetic anhydride (0.2 ml.). The *NN'*-diacetyl-*p*-phenylenediamine was filtered off, washed with water and recrystallized from aqueous ethanol until the specific radioactivity became insignificant (m.p. and mixed m.p. 116°).

***p*-Aminophenylglucuronide.** *p*-Aminophenyl glucosiduronic acid (380 mg.) was dissolved in urine with slight warming and left for 4 hr. The glucuronide was then reisolated from the urine via the basic lead salt (Williams, 1943) and recrystallized from water until the specific radioactivity became insignificant.

Phenols. Phenol and quinol were determined by the method of Parke & Williams (1953).

'Pyridoxal phenylhydrazone'. A sample of this compound was probably obtained by Snell (1944) by the addition of phenylhydrazine to a neutral solution of oxidized pyridoxine, but no melting point or elementary analysis was quoted. This compound was required for isotopic-dilution experiments, but we were unable to obtain a sample which gave a satisfactory analysis, since it appeared to change when attempts were made to purify it. Samples of the compound were prepared as follows: Commercial pyridoxal hydrochloride (0.5 g.; L. Light and Co.) and sodium acetate (0.5 g.) were dissolved in water (20 ml.). This solution was added to a solution of phenylhydrazine hydrochloride (0.35 g.) and sodium acetate (0.5 g.) in water (20 ml.). On mixing the solutions a yellow precipitate formed immediately. This was filtered off and washed with water until free of chloride. It was purified by dissolving it in ethanol and precipitating with water at room temperature. The precipitated material consisted of minute yellow needles, and two of the best samples had decomposition points 216° and 218° (Found: C, 66.3, 64.5; H, 5.7, 5.8; N, 16.2, 16.9.

$\text{C}_{14}\text{H}_{15}\text{O}_4\text{N}_3$ requires C, 65.4; H, 5.9; N, 16.3%). These samples turned pink on keeping in daylight, but became yellow again in the dark. The compound was soluble in acids, alkalis, ethanol and methanol, less soluble in ethyl acetate, acetone and ether and insoluble in water. On recrystallization from hot ethanol, it formed yellow needles (decomp. 212°) with a higher carbon value (Found: C, 67.7; H, 6.3; N, 16.4%).

Proof that our compound contained both pyridoxal and phenylhydrazine was obtained spectroscopically (cf. the study of pyridoxal *N*-dimethylglycylhydrazone by Viscontini, Ebnöther & Karrer, 1951). 'Pyridoxal phenylhydrazone' in 0.1N-HCl showed maximum light-absorption at 251, 338 and 410 $\text{m}\mu$ with ϵ_{max} 16 000, 6000 and 26 000 respectively. After heating under reflux for 2 hr. in 2N-HCl (100 mg. in 20 ml.), these maxima are replaced by two new absorption peaks at 224 and 288 $\text{m}\mu$ (with ϵ_{max} 12 200 and 10 800), which correspond to the maxima of phenylhydrazine hydrochloride and pyridoxal hydrochloride respectively (see Fig. 1). Phenylhydrazine hydrochloride in 0.1N-HCl showed maxima at 224 and 280 $\text{m}\mu$ (ϵ_{max} 8300 and 1000) [Donovick, Rake & Fried (1946) give λ_{max} 230, 275 and 300 $\text{m}\mu$ with ϵ_{max} 5200, 1500 and 500 for phenylhydrazine in very dilute acetic acid] and pyridoxal in 0.1N-HCl had λ_{max} 288 with ϵ_{max} 9000 [Viscontini *et al.* (1951) give λ_{max} 288 $\text{m}\mu$ and ϵ_{max} 6500]. That the extinctions of the peaks of the hydrolysis product are correct for a mixture of phenylhydrazine and pyridoxal in equimolecular proportions is shown in Table 2.

When 'pyridoxal phenylhydrazone' was acetylated it appeared to give a diacetyl derivative. The pyridoxal phenylhydrazone sample decomposing at 218° (0.5 g.) was dissolved in N-NaOH (10 ml.), cooled to 0° and treated with acetic anhydride (1 ml.). The solution became acid almost immediately and was made alkaline with 2N-NaOH and a further 0.5 ml. of acetic anhydride added. After 10 min. the solution was made alkaline with 2N-NaOH and a yellow precipitate separated (0.5 g.). The acetyl derivative was recrystallized from aqueous ethanol and formed fine yellow needles, m.p. 165° (Found: C, 61.6; H, 5.7; N, 12.5. $\text{C}_{18}\text{H}_{19}\text{O}_4\text{N}_3$ requires C, 63.3; H, 5.6; N, 12.3%). A small

Fig. 1. Spectra of pyridoxal phenylhydrazone in 0.1N-HCl (—), and of the acid hydrolysis product of pyridoxal phenylhydrazone in 0.1N-HCl (- - -).

Table 2. *Molecular extinction coefficients of pyridoxal, phenylhydrazine and hydrolysed pyridoxal phenylhydrazone*

Compound in 0.1N-HCl	ϵ_{224}	ϵ_{288}
Pyridoxal hydrochloride alone	4 200	9 000
Phenylhydrazine hydrochloride alone	8 300	500
Pyridoxal and phenylhydrazine hydrochlorides* together in solution	12 800	10 200
Hydrolysis product of pyridoxal phenylhydrazone	12 200	10 800
	Sum 12 500	Sum 9 500

* Equimolecular proportions.

amount of pyridoxal [^{14}C]phenylhydrazine was acetylated in this manner, and from a measurement of the specific activity of the product it was deduced that two acetyl groups had been introduced. Further crystallizations of this compound appear to decompose it, since several yellow products of different melting point were obtained.

It appeared that neither pyridoxal phenylhydrazine nor its acetyl derivative was stable enough for isotope-dilution procedures, and although they were both used the presence of pyridoxal phenylhydrazine in the urine of rabbits dosed with phenylhydrazine could not be proved unequivocally.

Isolation and detection of metabolites.

A. From phenylhydrazine

The urine of rabbits dosed with phenylhydrazine hydrochloride orally (50 mg./kg.) was dark brown in colour, had pH about 9, reduced Benedict's reagent with only slight warming, and gave a strongly positive naphtharesorcinol reaction for glucuronic acid and *p*-hydroxyphenylhydrazine, the latter being detected by the observation that the aqueous layer of the naphtharesorcinol test was a deep-purple colour which turned red with NaOH. The urine also gave a positive benzidine reaction (modified) for blood pigments but contained no intact erythrocytes.

Two-thirds of the 24 hr. urine of two rabbits dosed with a total of 200 mg. of [^{14}C]phenylhydrazine hydrochloride was adjusted to pH 7 and continuously extracted with ether for 6 hr. The extract was evaporated and a sample of the residue chromatographed on paper (Whatman no. 4) with wet diisopropyl ether. Radioautography revealed four radioactive spots (R_F 0.30, 0.38, 0.86 and 0.95). The main spot (R_F 0.30) on elution gave positive naphtharesorcinol tests for glucuronic acid and *p*-hydroxyphenylhydrazine, and was presumably the glucuronide of *p*-hydroxyphenylhydrazine. The minor spots (R_F 0.38, 0.86 and 0.95) were treated with ammoniacal silver nitrate; the first one (a yellow spot slowly turning brown) was not identified and the second and third (black) corresponded with pyruvic acid phenylhydrazine and α -oxoglutaric acid phenylhydrazine respectively.

The other third of the urine was refluxed with 0.5 vol. of 10N-HCl for 3 hr. and then extracted at pH 8 (with NaOH for neutralizing) then at pH 10 with ether (6 hr. continuous extraction). The two extracts were evaporated and chromatographed as above. Both gave the same results. Radioautography revealed two radioactive spots (R_F 0.05 and 0.40) and colour reactions showed the former to correspond with phenylhydrazine, derived by decomposition of phenylhydrazones, and the latter with *p*-aminophenol, derived by decomposition of *p*-hydroxyphenylhydrazine and its glucuronide. Attempts to isolate the glucuronide were unsuccessful.

Isolation of the phenylhydrazones. The 48 hr. urine of 12 rabbits dosed with a total of 1.8 g. of phenylhydrazine hydrochloride was divided. To one half was added pyruvic acid [^{14}C]phenylhydrazine (20 mg.) and, to the other, α -oxoglutaric acid [^{14}C]phenylhydrazine (20 mg.), each dissolved in $\text{n-Na}_2\text{CO}_3$ (5 ml.). The urines, brought to pH 2 with 2N-HCl, were continuously extracted with ether for 20 hr., whereby 85 and 90% of the radioactivities respectively were extracted. Both extracts were treated as follows. The carboxylic acids in the extract were transferred to n-NaHCO_3 (25 ml.), which was then made acid (with 2N-HCl) and re-extracted with ether (3 $\times$ 10 ml.). This second ethereal extract contained about 72% of the original radioactivity in about 3.5 g. of solid material. The whole of this material was chromatographed as a broad band on Whatman no. 1 paper with butan-2-one saturated with water as solvent (see Table 1). The keto acid phenylhydrazones (R_F 0.93–0.96 in this solvent) were allowed to run off the paper and the eluate was evaporated at room temperature. The residue in acetone was again chromatographed in the same way, so that the keto acid phenylhydrazones were separated from 2–3 g. of non-radioactive material. In this way 1.1 g. of material containing 50% of the added radioactivity was obtained. This was dissolved in acetone and chromatographed as a horizontal band on Whatman no. 1 paper with water-saturated diisopropyl ether. The radioactivity was found in a broad yellow band (R_F 0.5–0.95), which was cut out and eluted with acetone. The acetone was evaporated at room temperature, and the residue dissolved in 10 ml. of aq. 2N- NH_3 and filtered off. Aqueous AgNO_3 (10 ml. of 2%, w/v) was added to the filtrate, which was then adjusted to pH 7 by dropwise addition of $\text{n-H}_2\text{SO}_4$. The precipitated silver salts were rapidly centrifuged, washed with water, ethanol and ether and then suspended in water (10 ml.). The suspension was acidified by a few drops of 2N- H_2SO_4 and extracted with ether. The extract (0.25 g. of solid and 21% of the radioactivity) was concentrated and chromatographed on paper with wet diisopropyl ether. On drying, benzoic acid crystallized on the paper and was brushed off. The keto acid phenylhydrazones formed a yellow band (R_F 0.75–0.95). This was cut out and eluted with acetone and the chromatography repeated, to yield 48 mg. of material (12% of the radioactivity), which was submitted to zone electrophoresis. The phenylhydrazones of pyruvic acid and α -oxoglutaric acid just separated, and the pyruvic acid band in one experiment and the α -oxoglutaric acid band in the other were cut out and eluted with 0.05N- Na_2CO_3 . The eluates were acidified with 2N- H_2SO_4 and extracted with ether. The extracts of the two phenylhydrazones were submitted to a second electrophoresis and the elution and extraction repeated. Finally, in the first experiment 9 mg. of crude pyruvic acid phenylhydrazine (7% of added

radioactivity) was obtained, which on repeated recrystallization yielded 1.9 mg. of pure material, m.p. and mixed m.p. 189°. The activity of this suggested that 7.3% of the dose of unlabelled phenylhydrazine was excreted as the pyruvic acid derivative. In another experiment in which no tracer was used, 1.3 mg. of pyruvic acid phenylhydrazine (m.p. and mixed m.p. 189°) was isolated after feeding a total of 2.2 g. of unlabelled phenylhydrazine hydrochloride to 12 rabbits. With α -oxoglutaric acid phenylhydrazine the pure material was not isolated.

p-Aminophenol. If the urine contained *p*-hydroxyphenylhydrazine and its conjugate, acid hydrolysis should yield *p*-aminophenol. The residual urine and residues from the separation of the phenylhydrazones above (equivalent to feeding 4 g. of phenylhydrazine) were refluxed for 3 hr. with an equal volume of conc. HCl. The urine was cooled and adjusted to pH 8.5 by addition of solid Na_2CO_3 and continuously extracted with ether. The ethereal extract (50 ml.) was washed with water (20 ml.) and extracted with 2×20 ml. of 2*N*-HCl. The acid extracts were adjusted to pH 12 by addition of 2*N*-NaOH and extracted with 2×20 ml. of ether. Evaporation of the ethereal extracts and benzoylation of the residue yielded no crystalline material. The alkaline aqueous layer was then quickly adjusted to pH 8.5 and extracted with 2×20 ml. of ether. The ethereal extract was shaken with 10 ml. of 2*N*-HCl and the acid layer benzoylated by the addition of 0.5 ml. of benzoyl chloride in the presence of an excess of 2*N*-NaOH. The benzoylated mixture was poured into water and the small amount of solid material which separated was removed by filtration. Recrystallization from aqueous ethanol eventually gave *p*-aminophenol *ON*-dibenzoate, m.p. and mixed m.p. 235°.

B. From pyruvic acid and α -oxoglutaric acid [^{14}C]phenylhydrazones

After oral administration. The urine from rabbits receiving pyruvic acid [^{14}C]phenylhydrazine orally at a dose level of 60 mg./kg. was normal in colour, had pH 8–9, reduced Benedict's reagent on warming and gave a strongly positive naphtharesorcinol reaction for glucuronic acid and for *p*-hydroxyphenylhydrazine.

Two rabbits were each given 0.5 g. of pyruvic acid phenylhydrazine orally and the urines were collected for 2 days and added to that obtained from the rabbit which had received 0.15 g. of pyruvic acid [^{14}C]phenylhydrazine. The bulked urines were adjusted to pH 6 with acetic acid and continuously extracted with ether for 16 hr. Evaporation of the ethereal extract gave a radioactive gum which was dissolved in 5 ml. of 50% (v/v) aqueous ethanol. The ethanolic solution was extracted with ether, and the ethereal extract was washed with water and the ether removed by evaporation. The ethanolic residue after treatment with charcoal gave a crystalline product (19 mg.), which after recrystallization from aqueous ethanol had m.p. and mixed m.p. 188–190° with authentic pyruvic acid phenylhydrazine.

Paper chromatography in diisopropyl ether–water of an ethereal extract of the pyruvic acid phenylhydrazine urine (at pH 3) revealed 4 radioactive spots with R_F values 0.28 (*p*-hydroxyphenylhydrazine glucuronide), 0.34 (unidentified), 0.83 (pyruvic acid phenylhydrazine) and 0.93 (α -

oxoglutaric acid phenylhydrazine). Similarly, α -oxoglutaric acid phenylhydrazine urine gave 5 radioactive spots with R_F values 0.28, 0.35, 0.54 (unidentified), 0.85 and 0.95.

After injection. The urines from rabbits receiving pyruvic acid [^{14}C]phenylhydrazine (30 mg./kg.) and α -oxoglutaric acid [^{14}C]phenylhydrazine (50 mg./kg.) by subdermal injection were normal in colour, had pH 7.5, reduced Benedict's reagent slightly on prolonged boiling, and gave a weakly positive naphtharesorcinol reaction for glucuronic acid and a negative reaction for *p*-hydroxyphenylhydrazine. Paper chromatography in diisopropyl ether–water of an ethereal extract of pyruvic acid phenylhydrazine urine (pH 3) gave 2 radioactive spots with R_F values 0.84 (pyruvic acid phenylhydrazine) and 0.95 (α -oxoglutaric acid phenylhydrazine) and present in amounts of about 10:1 respectively. Similarly, the urine from rabbits receiving α -oxoglutaric acid phenylhydrazine gave 4 radioactive spots with R_F values 0.34, 0.54 (unidentified), 0.85 (pyruvic acid phenylhydrazine) and 0.95 (α -oxoglutaric acid phenylhydrazine) in the ratio of about 1:1.5:25 respectively. Paper electrophoresis revealed only 2 bands in each case, these moving at the same rates as pyruvic acid and α -oxoglutaric acid phenylhydrazones. It is significant that the spot due to *p*-hydroxyphenylhydrazine glucuronide (R_F 0.28) was absent in both cases.

RESULTS

The distribution of radioactivity in the tissues and excreta of two rabbits 4 days after a single oral dose of 50 mg. of [^{14}C]phenylhydrazine hydrochloride/kg. is shown in Table 3. Although not all the tissues were examined because of the low specific activity of the compound administered, we were able to account for 75% of the dose. About 60% of the dose was eliminated from the body, mainly in the urine. The rest was probably still in the body, some in the alimentary tract, but about 10% was found in the erythrocytes. The large amount of radioactivity associated with the erythrocytes (possibly present as a phenylhydrazine) is of considerable interest in view of the known action of phenylhydrazine on the red cell and the production of the light-refracting granules called Heinz bodies.

Table 4 gives the results of experiments in which the excretion of radioactivity has been followed for four days, and in one case for 10 days. About one-third of the radioactivity is excreted in the urine in the first 24 hr.; thereafter, radioactivity is slowly eliminated and continues for at least 10 days. The results for the animal which was kept for 10 days are shown in Fig. 2 and in this particular animal about 63% of the dose has appeared in the urine in that time.

The quantitative aspects of the excretion of metabolites were examined during 2 days in four animals and the results are shown in Table 5. As far as we could ascertain with the radioactivity available, there were three main metabolites of

Fig. 2. Excretion of radioactivity in the urine of a rabbit which received an oral dose (50 mg./kg.) of [^{14}C]phenylhydrazine hydrochloride. x, Daily excretion; ●, total excretion.

Table 3. Distribution of radioactivity in rabbits receiving [^{14}C]phenylhydrazine hydrochloride orally

Dose of phenylhydrazine hydrochloride, 50 mg./kg.

Material analysed (Dose of radioactivity)	Radioactivity found in 4 days after dosing (% of dose)	
	Expt. 1 (0.9 μC)	Expt. 2 (1.1 μC)
Erythrocytes	10	10
Plasma	<1	<1
Spleen	0.8	1.0
Stomach contents	3.6	3.2
Intestinal contents	<1	<1
Caecum contents	<1	<1
Faeces	0.8*	1.7*
Urine	58.8	59.1
Total	77	78

* The small activity of the faeces is probably due to contamination with urine.

Table 4. Urinary excretion of radioactivity by rabbits dosed with [^{14}C]phenylhydrazine hydrochloride

Dose of phenylhydrazine hydrochloride; 50 mg./kg. Average figures; the ranges are given in parentheses and the superior figures indicate the number of animals.

Day after dosing	Radioactivity found in urine (% of dose)
1	34 (13-43) ⁷
2	8.5 (5.8-16.5) ⁷
3	4.5 (2.4-9.6) ⁴
4	3.5 (2.2-7.0) ⁷
5	1.5 (0.3-3.4) ³
6-10	5.8 ¹
Total	57.8
Total for 4 days	50 (40-58) ⁷

Table 5. Urinary metabolites of [^{14}C]phenylhydrazine hydrochloride

Dose of phenylhydrazine hydrochloride, 50 mg./kg. orally; dose of radioactivity 0.8-1.2 μC per rabbit. Urine was collected for 2 days after dosing and the metabolites were determined by isotope dilution. The figures quoted are the mean values for 4 experiments for the main metabolites and 2 experiments for the others. The figures in parentheses give the ranges of values and the superior figures the number of experiments. Zero values mean less than 0.05 % of the dose, except for oxaloacetic acid phenylhydrazone, phenol and quinol, where they mean less than 0.1 % of the dose.

Metabolites examined	Found (% of dose)
<i>p</i> -Hydroxyphenylhydrazine*	17.2 (10.4-21.2) ⁴
Pyruvic acid phenylhydrazone	8.5 (5.9-12.8) ⁴
α -Oxoglutaric acid phenylhydrazone	5.2 (4.0, 6.5) ²
['Free' phenylhydrazine]†	11.8 (11.3, 12.3) ²
['Total' phenylhydrazine]†	12.3 (9.3-14.6) ⁴
Acetoacetic acid phenylhydrazone‡	> 0.2
Acetone phenylhydrazone	> 0.2
Pyridoxal phenylhydrazone§	0
Oxaloacetic acid phenylhydrazone	0
Glucose phenylhydrazone	0
Glucosazone	0
Acetylphenylhydrazine	0
Phenol	0
Quinol	0
<i>o</i> -Aminophenol	0
<i>m</i> -Aminophenol	0
<i>p</i> -Aminophenylglucuronide	0
<i>p</i> -Phenylenediamine	0
Sum of metabolites	30.7
Total radioactivity of urine	38.8 (29-45) ⁴

* Determined as *p*-aminophenol (see text).

† Artifacts arising from the hydrolysis of phenylhydrazones.

‡ Determined as 3-methyl-1-phenylpyrazol-5-one.

§ See text.

Table 6. Urinary excretion of the metabolites of acetyl[^{14}C]phenylhydrazine

Dose of acetylphenylhydrazine, 50 mg./kg. Metabolites excreted in 2 days after dosing were measured by isotope dilution.

Metabolite excreted	Amount (% of dose)
<i>p</i> -Hydroxyphenylhydrazine	13.8
Acetylphenylhydrazine	4.7
Pyruvic acid phenylhydrazone	1.4
Sum of above metabolites	20
Total radioactivity	29

phenylhydrazine. The major one was conjugated *p*-hydroxyphenylhydrazine, which, by isotope dilution, accounted for about 17 % of the dose. The conjugate is probably a glucuronide, as shown by paper chromatography. Quantitative determinations of glucuronide and ethereal sulphate excretion showed that, after a 50 mg./kg. oral dose of phenylhydrazine hydrochloride, there was an increase of glucuronic acid excretion corresponding to 33 % of the dose (range in three experiments, 22-42 %) and

a doubtful increase in ethereal sulphates of $>2\%$ of the dose (range 1.3–2.1%). The other urinary metabolites were the phenylhydrazones of the keto acids, pyruvic acid and α -oxoglutaric acid. Together these accounted for about 13.7% of the dose, there being somewhat more of the pyruvic acid derivative than the other. A determination of the 'total' phenylhydrazine of the urine amounted to about 12.3%, and this should roughly equal the amount of keto acid phenylhydrazones (13.7%). It appeared probable that traces of other phenylhydrazones occurred in the urine, e.g. acetoacetic acid, acetone and pyridoxal, but we were unable to prove this unequivocally. The sum (about 31%) of *p*-hydroxyphenylhydrazine and pyruvic acid and α -oxoglutaric acid phenylhydrazones is not far short of the total radioactivity of the urine (about 39%) excreted in two days.

Phenylhydrazine could decompose to aniline and to benzene, but it is probable that this does not occur *in vivo*, since none of the metabolites (e.g. phenol and aminophenols) of these decomposition products were found in the urine (see Table 5).

Table 6 shows the result of one experiment with acetylphenylhydrazine. It appears probable that it is converted into the same metabolites as phenylhydrazine. Table 5 shows that acetylphenylhydrazine is probably not a metabolite of phenylhydrazine.

Table 7 shows the results of experiments in which the two phenylhydrazone metabolites of phenylhydrazine were administered. When given orally, pyruvic acid phenylhydrazone is partly decomposed, since about one-third of it is excreted as *p*-hydroxyphenylhydrazine, the main metabolite of phenylhydrazine itself. About one-third of the hydrazone is apparently excreted as such, but it is partly converted into α -oxoglutaric acid phenylhydrazone. When injected intradermally, however, it is largely excreted unchanged. These results suggest that pyruvic acid phenylhydrazone is partly converted in the gut into free phenylhydrazine, which is then mainly hydroxylated and

partly combined with α -oxoglutaric acid and pyruvic acid. The two experiments with α -oxoglutaric acid phenylhydrazone (Table 7) suggest that its fate is similar to that of the pyruvic acid derivative. During these experiments we observed that pyruvic acid phenylhydrazone was less toxic than phenylhydrazine, for oral doses of 180 mg./kg. of this phenylhydrazone were well tolerated by five rabbits, whereas ten rabbits which had received 75 mg. of phenylhydrazine hydrochloride/kg. orally died within 48 hr. of dosing.

Phenylhydrazine and erythrocytes

The erythrocytes from 100 ml. of human blood (citrated; group B, Rh negative), suspended in 99 ml. of physiological saline, were incubated at

Fig. 3. Uptake of $[^{14}\text{C}]$ phenylhydrazine by erythrocytes. ●, Specific activity of the erythrocytes; x, specific activity of the supernatant solution.

Table 7. *Excretion of the metabolites of $[^{14}\text{C}]$ phenylhydrazones*

Metabolites were measured by isotope dilution. o, oral administration; i, administered by subdermal injection; n.m., not measured.

Compound administered ...	Pyruvic acid phenylhydrazone			α -Oxoglutaric acid phenylhydrazone	
	1	2	3	4	5
Expt. no. ...	...	...	...	...	...
Dose of hydrazone (mg./kg.)	60 (o)	6 (o)	30 (i)	6 (o)	50 (i)
Dose of radioactivity (μC)	1.1	0.1	0.6	0.1	0.7
Duration of experiment (days)	2	2	1	2	1
Percentage of dose excreted in urine					
Metabolites					
<i>p</i> -Hydroxyphenylhydrazine	33	n.m.	0.2	n.m.	<0.2
Pyruvic acid phenylhydrazone	31	31	93	12	12
α -Oxoglutaric acid phenylhydrazone	5	6	7	12	65
Sum of above metabolites	69	(37)	100	(24)	77
Total radioactivity of urine	92	99	93	105	89

37° with 1 ml. of a 5% (w/v) solution of [^{14}C]-phenylhydrazine in saline. The suspension was continuously shaken, and samples (1 ml.) were removed at intervals of 5 min. The erythrocytes were deposited from the samples by centrifuging and the specific activities of these unwashed cells and of the supernatant were determined. From Fig. 3 it can be seen that the phenylhydrazine is taken up rapidly by the erythrocytes and that equilibrium is reached in 10 min. After this time the specific activity of the erythrocytes becomes greater than that of the supernatant and the haemolysis increases. After 1 hr., 50% of the erythrocytes have been haemolysed and the specific activities of the cells and supernatant are again in equilibrium.

DISCUSSION

The results of the present experiments show that the main metabolic reactions of phenylhydrazine in the rabbit consist of (1) hydroxylation of the aromatic ring, probably followed by conjugation as with aniline (Smith & Williams, 1949), and (2) phenylhydrazone formation with the natural keto acids. The excretory products found were conjugated *p*-hydrazinophenol, most probably the glucuronide, pyruvic acid phenylhydrazone and α -oxoglutaric acid phenylhydrazone. Together these metabolites account for about 90% of the material excreted during two days after dosing, which amounts to one-third of the dose.

Acetylation of phenylhydrazine did not occur, although it has been claimed by Scevola (1953) that phenylhydrazine is acetylated by yeast. Acetylphenylhydrazine itself when fed to rabbits appears to give rise to the same metabolites as phenylhydrazine and is presumably first deacetylated.

Beaven & White (1954) have reported that, in the presence of oxygen and oxyhaemoglobin, both phenylhydrazine and its acetyl derivative give rise to benzene. If this occurred *in vivo*, then the main metabolites of benzene, i.e. phenol and quinol, could be expected in the urine (Parke & Williams, 1953). However, neither of these compounds was labelled with ^{14}C and it can be concluded that, if phenylhydrazine is oxidized to benzene *in vivo*, it occurs only to a very small extent.

Aniline is a decomposition product of phenylhydrazine, but there was no evidence of such a decomposition *in vivo*, since *o*- and *p*-aminophenols, the metabolites of aniline in the rabbit (Smith & Williams, 1949; Parke & Williams, 1956), were not found. Phenylhydrazine could isomerize to *p*-phenylenediamine, but we found no evidence for this reaction *in vivo*.

The formation of the phenylhydrazones of the keto acids is of considerable interest since pyruvic

acid phenylhydrazone was subsequently found to be much less toxic than phenylhydrazine. Their formation is presumably non-enzymic since it occurs in pure solution at room temperature on mixing the ingredients. If phenylhydrazone formation also involved pyridoxal, it could explain some of the toxic effects of phenylhydrazine as being due to removal of pyridoxal from the body and producing a deficiency. In fact, Williams & Abdulian (1956) have shown that, in man and animals, certain hydrazides (e.g. semicarbazide, isonicotinic acid hydrazide) produce convulsions which can be prevented by pyridoxine. Pyridoxine has been used to prevent neuritis in patients receiving high doses of isonicotinic acid hydrazide (Biehl & Vilter, 1954).

The phenylhydrazones of pyruvic acid and α -oxoglutaric acid are metabolically inert when injected and are rapidly excreted as such. When given by mouth, however, they give rise to *p*-hydroxyphenylhydrazine and presumably they are partly hydrolysed to free phenylhydrazine in the gut (see Table 7).

Phenylhydrazine is not readily removed from the body and only about 60% of a 50 mg./kg. dose is eliminated in 10 days. Much of the material remaining in the body is associated with the red cell. Since phenylhydrazine is a highly reactive compound, it probably attaches itself firmly to structures containing suitable groupings such as aldehyde and keto groups. This suggests that the red cells are rich in such structures and that, once the phenylhydrazine combines with the cell, it remains there until the cell is destroyed. Since the ^{14}C -labelled phenylhydrazine was rapidly incorporated, it is not unreasonable to assume that it is present in the red cell as a kind of phenylhydrazone.

SUMMARY

1. [^{14}C]Phenylhydrazine hydrochloride administered orally to rabbits in doses of 50 mg./kg. is slowly metabolized. In 4 days 50% of the dose is excreted in the urine, the rest being found in the body, particularly the erythrocytes (10%). There is a slow excretion of radioactivity for at least 10 days after dosing, and in this time the excreted radioactivity accounts for about 60% of the dose.

2. The principal urinary metabolites have been identified as *p*-hydrazinophenol (17%), probably present as the glucuronide, pyruvic acid phenylhydrazone (8.5%) and α -oxoglutaric acid phenylhydrazone (5.2%). Traces of the phenylhydrazones of acetone, pyridoxal and acetoacetic acid may also be present in the urine.

3. The phenylhydrazones of pyruvic acid and α -oxoglutaric acid are less toxic than phenylhydrazine and when administered are quickly excreted.

When pyruvic acid [^{14}C]phenylhydrazone is injected, it is excreted in the urine largely as such (93 %) but partly as α -oxoglutaric acid phenylhydrazone (7 %). When administered orally it is hydrolysed in the gut and excreted in the urine as conjugated *p*-hydroxyphenylhydrazone (33 %) together with the phenylhydrazones of pyruvic acid (31 %) and α -oxoglutaric acid (5 %). Similar results were obtained with α -oxoglutaric acid [^{14}C]phenylhydrazone.

4. Acetyl[^{14}C]phenylhydrazone, when administered orally to rabbits, is slowly excreted as metabolites and in 2 days about 29 % of the dose is found in the urine as conjugated *p*-hydroxyphenylhydrazone (14 %), unchanged acetylphenylhydrazone (4.7 %) and pyruvic acid phenylhydrazone (1.4 %).

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The Incorporation of Phosphorus into Fractions of *Escherichia coli* made by Centrifuging and by Chemical Means

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Experiments described earlier (Wade & Morgan, 1957) suggested that the increase in ribonucleic acid concentration which occurs in resting cells of *Escherichia coli*, when they are inoculated into fresh medium and start growing and dividing rapidly, does not represent a general increase in all ribonucleic acid-containing constituents but an increase in a fraction which sediments at 25 000–100 000 g. It was postulated that the 'additional ribonucleic acid' which develops has some role in cell division or deoxyribonucleic acid synthesis and the term 'fluctuating ribonucleic acid' was applied to it to distinguish it from other ribonucleic acid constituents ('constitutional ribonucleic acid'), the concentrations of which remain constant.

Sedimentation fractionation cannot provide unequivocal proof for the existence of biologically distinct ribonucleic acid constituents since changes in their states of aggregation may occur during the transition from the resting to the dividing state. The marked influence of magnesium upon the aggregation of ribonucleic acid (Mallette & Lamanna, 1954) may be of importance in this connexion for equivalent sedimentation fractions in resting cells and dividing cells have different ratios of magnesium concentration to ribonucleic acid concentration (Wade & Morgan, 1957). Other evidence is required to confirm the heterogeneity of the ribonucleic acid in this bacterium and the individuality of the 'fluctuating ribonucleic acid' in its dividing cells.