Studies in Detoxication

70. METABOLISM OF HYDRAZIDES AND HYDROXAMIC ACIDS DERIVED FROM SALICYLIC ACID*

By W. M. McISAAC AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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The discovery of the antitubercular action of *isonicotinic acid hydrazide* and of *p*-aminosalicylic acid has aroused interest in the biological properties of the hydrazides and hydroxamic acids derived from salicylic acid, and both salicylohydrazide and salicylohydroxamic acid have been shown to possess antitubercular activity (Ma & Tien, 1953; Urbański, 1950, 1953; Urbański, Hornung, Slopek & Venulet, 1952). However, little is known concerning the metabolic transformations of foreign hydrazides and hydroxamic acids, although the enzymic synthesis of hydroxamic acids has been established (see Waelsch, 1952).

Only one aromatic hydrazide appears to have been extensively investigated, namely, *isonicotinic acid hydrazide*. In man and the rhesus monkey, this compound is mainly acetylated to 1-acetyl-2-isonicotinylhydrazine (Hughes, 1953). In the dog, however, the main metabolite is *isonicotinic acid* and there appears to be no acetylation (Rubin & Burke, 1953). Hydrazones of naturally occurring aldehydes and ketones, such as pyridoxal and pyruvic acid, may also be formed in small amounts (Williams & Abdulian, 1956; Defranceschi & Zamboni, 1954). The simplest aromatic hydrazide, benzohydrazide ($C_6H_5 \cdot CO \cdot NH \cdot NH_2$), is a relatively toxic substance to rabbits (Yard & McKennis, 1955), and other work in this Laboratory (El Masry, Smith & Williams, unpublished results) has shown that nearly 60% of a dose of about 80 mg./kg. is excreted as hippuric acid, only small amounts of 1-acetyl-2-benzoylhydrazine being formed. The likely reactions of aromatic hydrazides *in vivo* are therefore hydrolysis to the corresponding carboxylic acid, acetylation and hydrazone formation. Concerning aromatic hydroxamic acids there is little information. Benzohydroxamic acid ($C_6H_5 \cdot CO \cdot NH \cdot OH$) is rapidly hydrolysed to benzoic acid and hydroxylamine by sheep-liver extracts and yeast autolysates (Virtanen & Berg, 1951), and in rabbits 70% of doses of 90–100 mg./kg. are excreted as hippuric acid (El Masry, Smith & Williams, unpublished results). Lowenthal (1954) has briefly reported that both in the intact rat and in rat-liver extracts salicylohydroxamic acid is

converted into salicylamide. The probable metabolic reactions of aromatic hydroxamic acids are thus hydrolysis to the corresponding carboxylic acids and conversion into the corresponding amides.

MATERIALS AND METHODS

Compounds. Salicylohydrazide, m.p. 145–147°, 1-acetyl-2-salicyloylhydrazine, m.p. 180–182°, salicylohydroxamic acid, m.p. 176–178°, salicyluric acid (*o*-hydroxyhippuric acid), m.p. 166–168°, 5-bromosalicylamide, m.p. 241–243°, and 5-bromosalicylic acid, m.p. 164–165°, were prepared by standard methods. 5-Bromosalicylohydroxamic acid, m.p. 232°, was prepared according to Urbański *et al.* (1952), and salicylamide glucuronide, m.p. 174° (decomp.), according to Bray, Ryman & Thorpe (1948).

Animals. Rabbits, kept on a constant diet, were used for quantitative studies. Experiments in humans, rats and mice were qualitative only. Compounds were administered in water by stomach tube, unless otherwise stated.

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined by the methods of Paul (1951) and Sperber (1948) respectively. 5-Bromosalicylamide was determined fluorimetrically as follows. 5-Bromosalicylamide fluoresces under ultraviolet light in alkaline solutions. Its glucuronide does not fluoresce under these conditions. For the estimation an H 553 Spekker fluorimeter with 20 ml. cuvettes was used, the secondary filters being of Chance O.B. 1 blue glass. The standard curve was prepared with an aqueous solution of 5-bromosalicylamide (0.5 mg./ml.). Known volumes of this solution were diluted to 25 ml. with 0.1 M-glycine buffer, pH 10. The fluorescence of these solutions was measured against a quinine bisulphate standard containing 4 µg./ml. in 0.1 N-H₂SO₄. On plotting the drum readings of the fluorimeter against the concentration of the amide on logarithmic paper, a straight line was obtained for concentrations of 0.5–3.5 mg./25 ml. of the solution. Above this level fluorescence quenching became significant. All solutions subsequently estimated were diluted with buffer so that they contained 20–140 µg. of the amide/ml. Recoveries from aqueous solution were 100–104%, and from urine 95–101% when appropriately diluted.

The determination of the amide in 5-bromosalicylamide glucuronide (see below for its preparation) was carried out after hydrolysis. A solution (5 ml.) of the glucuronide (1 mg./ml.) was heated on the water bath for 3 hr. with 2.5 ml. of 10 N-HCl. The solution was cooled, neutralized with 40% (w/v) NaOH, made up to 50 ml. with glycine buffer, pH 10, and the fluorescence measured. The recovery of the amide was 90–97%.

* Part 69: El Masry, Smith & Williams (1956).

Table 1. R_F values and colour reactions of certain derivatives of salicylic acid

Solvent systems used were: A, propan-1-ol saturated with aq. NH_3 soln. (sp.gr. 0.88), with Whatman no. 1 paper, run for 16 hr.; B, ethyl methyl ketone-2*n*- NH_3 soln. (2:1), with Whatman no. 4 paper, run for 3 hr.; C, benzene-acetic acid-water (1:1:2), with Whatman no. 1 paper, run for 6 hr.; D, benzene-*n*-butanol-aq. NH_3 soln. (sp.gr. 0.88) (2:5:2), with Whatman no. 1 paper, run for 16 hr.; E, *n*-butanol-acetic acid-water (4:1:5), with Whatman no. 4 paper, run for 6 hr. For excitation of fluorescence ultraviolet light from a Hanovia Chromatolite lamp was used; Q means that background fluorescence of the paper was quenched. The sprays used for detecting compounds on paper were: 1% aq. ferric chloride; Gibbs's reagent, consisting of 2% ethanolic soln. of 2,6-dichloroquinonechlorimide, followed by saturated aq. NaHCO_3 ; 0.1% aq. soln. of Bretamine Fast Red B salt, followed by saturated NaHCO_3 ; a mixture of 1% aq. KMnO_4 and 2% aq. NaIO_4 (1:4), which showed up glucuronides as yellow spots on a reddish purple background.

	R_F values in solvent					Colour of spots on paper with				
	A	B	C	D	E	Ferric chloride	Gibbs's reagent	Bretamine Fast Red	$\text{KMnO}_4 + \text{NaIO}_4$	
Salicylic acid	0.85	0.50	0.95	0.41	1.00	Purple	Blue	Pale brown	—	
Salicylicuric acid	0.45	0.08	0.93	0.06	0.95	Purple	Blue	Brown	—	
Gentisic acid	0.66	0.25	0.86	0.14	0.94	Blue	Violet	Pale brown	—	
Salicylohydrazide	0.63	0.30	0.85	0.20	0.90	Purple	Green	Yellow	—	
N^1 -Acetyl- N^2 -salicyloylhydrazine	0.72	0.34	0.96	0.23	0.92	Brown	Blue	Brown	—	
Salicylohydroxamic acid	0.65	0.38	0.17	0.23	0.95	Brown	Green	Brown	—	
Salicylamide	0.75	0.90	0.82	0.61	0.98	Purple	Blue	Red	—	
5-Bromosalicylic acid	0.82	0.70	0.96	0.59	0.95	Purple	Pale green	Pale brown	—	
5-Bromosalicylohydroxamic acid	0.56	0.48	0.25	0.35	0.97	Brown	Pale green	Yellow	—	
5-Bromosalicylamide	0.68	0.83	0.95	0.49	1.0	Purple	Pale blue	Red	—	
Salicylohydrazide glucuronide	0.40	0	—	0	0.55	None	None	None	Yellow	
N^1 -Acetyl- N^2 -salicyloylhydrazine glucuronide	0.42	0	—	0	0.59	None	None	None	Yellow	
Salicylohydroxamic acid glucuronide (toluidine salt)	0.09	0	0	0	0.64	Red	Blue	Brown	Yellow	
Salicylamide glucuronide	0.40	—	—	—	0.59	None	Pale blue	Pale brown	—	
5-Bromosalicylohydroxamic acid glucuronide	0.23	0	0	—	0.57	Pale brown	None	None	Yellow	
5-Bromosalicylamide glucuronide	0.65	0.15	—	—	—	None	None	None	Yellow (delayed)	

The determination of free and conjugated 5-bromosalicylamide in the urine of rabbits receiving 5-bromosalicylohydroxamic acid was carried out as follows. The 24 hr. urine was diluted to 200 ml. with water. A sample was then appropriately diluted with glycine buffer and the free amide determined fluorimetrically. For the determination of total amide the urine was hydrolysed as above with half its volume of 10N-HCl, neutralized with 40% NaOH, appropriately diluted with glycine buffer and the fluorescence measured.

Chromatographic methods. For the detection of metabolites in urine and urine extracts, descending chromatography with Whatman no. 1 or no. 4 paper was used. The solvents, R_f values and colour reactions used are given in Table 1.

RESULTS

Quantitative results are given in Table 2. With benzohydrazide and benzohydroxamic acid the main metabolite is hippuric acid, and the small glucuronic acid conjugation is probably due to benzoylglucuronide. Salicylohydrazide and salicylohydroxamic acid and their derivatives are excreted conjugated mainly with glucuronic acid, and these glucuronides have been isolated (see below). The significant difference between the hydrazides and the hydroxamic acid is in the sulphate conjugation. The hydroxamic acids form appreciable amounts of ethereal sulphates, whereas neither salicylohydrazide nor its acetyl derivative is excreted as sulphate to any significant extent.

Little if any salicylohydroxamic acid is reduced to salicylamide in the rabbit. 5-Bromosalicylohydroxamic acid, however, is mainly converted into 5-bromosalicylamide. In two experiments, in each of which 350 mg. of this acid was fed, 60 and 71% of the dose of hydroxamic acid was excreted as the conjugated amide. A small amount of conjugated 5-bromosalicylohydroxamic acid is also excreted, since the glucuronide of this acid was isolated. The amount of conjugated hydroxamic acid

can be roughly assessed as 10–20% of the dose, from the difference between total conjugation (about 80%; see Table 2) and the conjugated amide excreted.

Isolation and detection of metabolites

From salicylohydrazide. The hydrazide (1 g.) was fed to each of four rabbits. The urine (24 hr. sample) reduced Benedict's reagent (owing to the presence of the hydrazide group), gave an intense naphtharesorcinol reaction and a faint purplish colour with aqueous ferric chloride. Paper chromatography of the urine with propanol-ammonia as solvent (solvent A, Table 1) revealed the presence of appreciable amounts of salicyluric acid (R_f 0.45) and small amounts of salicylic and gentisic acids (R_f 0.86 and 0.66 respectively). The urine was worked up by systematic lead acetate precipitation (cf. Kamil, Smith & Williams, 1951), and the glucuronide was obtained from the basic lead acetate precipitate in the usual manner. The crude glucuronide weighed 1.4 g. and, on repeated recrystallization from ethanol, *o*-aminocarbamoylphenyl β -D-glucosiduronic acid (0.6 g.) was obtained as fine white needles of the monohydrate, m.p. 204–205° and $[\alpha]_D^{20} - 65^\circ$ in water (c, 1) [Found: C, 44.8; H, 5.2; N, 7.9; H₂O, 5.1 (loss at 110°). C₁₃H₁₆O₈N₂·H₂O requires C, 45.1; H, 5.2; N, 8.1; H₂O, 5.2%]. The glucuronide reduced Benedict's reagent by virtue of its hydrazide group, but, unlike salicylohydrazide, was not fluorescent in ultraviolet light and quenched the background fluorescence of filter paper (Whatman). On incubation at 37° in acetate buffer (pH 4.5) with β -glucuronidase (from locust-crop liquor; Robinson, Smith & Williams, 1953) it yielded salicylohydrazide, which was detected chromatographically on paper by its fluorescence in ultraviolet light, its colour with ferric chloride and its R_f value (0.63) with solvent A (Table 1). All chromatographic identifications were checked with a second solvent system (see Table 1).

Table 2. *Quantitative aspects of the conjugation of salicylohydrazide and salicylohydroxamic acid in rabbits*

Results are quoted as the average for three animals, with range in parentheses; the superior figure indicates the number of experiments if other than three. Salicyluric acid was detected by paper chromatography; + indicates its presence and – its absence.

Compound	Dose* (mg./kg.)	Conjugation (% of dose) with			Total amide formation (% of dose)
		Glucuronic acid	Ethereal sulphate	Glycine	
Salicylic acid	138	18 (17–18)	0	+	.
Acetylsalicylic acid	180	11 (6–14)	0	+	.
Benzohydrazide	84	5 (3, 6) ²	0	57 (51–64)†	.
Salicylohydrazide	152	55 (50–60)	1 (0.5–2)	12 (7–17)†	.
N ¹ -Acetyl-N ² -salicyloylhydrazine	97	70 (67–77)	0	–	.
Benzohydroxamic acid	100	11 (7–14)	0	71 (70, 72)†	None
Salicylohydroxamic acid	153	49 (43–54)	19 (14–24)	–	Trace
5-Bromosalicylohydroxamic acid	116	59 (54–63)	20 (19–21)	–	78† (71, 85) ²

* These doses are approximately 0.5 or 1 m-mole/kg. and were given by mouth.

† These figures are for hippuric and salicyluric acids and were determined by A. M. El Masry in another investigation.

² Free amide 12 (11, 14)².

From *N*¹-acetyl-*N*²-salicyloylhydrazine. The acetohydrazide (1 g.) was fed to two rabbits and urine collected for 24 hr. The urine gave a strong naphtharesorcinol reaction, slightly reduced Benedict's reagent but gave no colour with ferric chloride. Paper chromatography of the urine revealed no appreciable amounts of acetylsalicyloylhydrazine, salicylohydrazide or salicylic acid, and the major, if not the only, metabolite appeared to be a glucuronide. The glucuronide (1.4 g.) was isolated as above and was recrystallized from ethanol. The *o*-acetamidocarbamoylphenyl β -D-glucosiduronic acid formed white needles, m.p. 208–210° and $[\alpha]_D^{20} - 63^\circ$ in water (c, 1) (Found: C, 48.3; H, 4.8; N, 7.8. $C_{15}H_{18}O_9N_2$ requires C, 48.65; H, 4.9; N, 7.6%). Hydrolysis with β -glucuronidase, as described above, yielded *N*¹-acetylsalicyloylhydrazine which was detected by colour reactions and its R_F value (0.72) in solvent A (see Table 1). Acid hydrolysis also yielded the acetohydrazide, identified chromatographically.

From *salicylohydroxamic acid*. The hydroxamic acid (1.5 g.) was fed to each of five rabbits and their urine was collected for 24 hr. The urine reduced Benedict's reagent, gave a strong naphtharesorcinol reaction and a reddish colour with ferric chloride. Paper chromatography of the urine revealed two spots, reacting with ferric chloride, of R_F 0.48 and 0.21 in solvent A. An eluate of the spot of R_F 0.48 gave a strong naphtharesorcinol reaction, suggesting that it was a glucuronide containing a free hydroxamic acid residue, since it also gave a colour with ferric chloride. The other spot contained no glucuronic acid and was probably an ethereal sulphate. No spot corresponding to salicylamide, which fluoresces in ultraviolet light, or to salicylamide glucuronide, was found.

The glucuronic acid fraction of the urine was prepared by the lead acetate procedure, and obtained as a gum. This was dissolved in a little water, and acetone (500 ml.) added. A small precipitate of inorganic material was formed and removed. The acetone left a reddish gum (7 g.) on evaporation, which contained both glucuronide and ethereal sulphate. This gum was dissolved in 10 ml. of water and 5 ml. of 2*N*-HCl added, and the solution was heated at 80° for 20 min. to hydrolyse ethereal sulphate which might interfere with the crystallization of the glucuronide. The resulting solution was then evaporated, *in vacuo* at 40°, to a gum, 5 g. of which was dissolved in water (20 ml.) and heated with *p*-toluidine (3 g.) in ethanol (5 ml.). On cooling, a brown crystalline precipitate separated which was repeatedly recrystallized from 90% (v/v) aqueous ethanol. This yielded the *p*-toluidine salt of *o*-hydroxycarbamoylphenyl β -D-glucosiduronic acid (1.7 g.) as colourless plates of the trihydrate, sintering at 100–110° and having $[\alpha]_D^{22} - 56^\circ$ in

water (c, 1) [Found: C, 49.05; H, 6.15; N, 6.0; H_2O , 10.9 (loss at 100° *in vacuo*); *p*-toluidine, 20.5. $C_{15}H_{18}O_9N_2 \cdot C_7H_9N \cdot 3H_2O$ requires C, 49.0; H, 6.2; N, 5.7; H_2O , 11.0; *p*-toluidine, 21.5%]. This salt gave a naphtharesorcinol reaction, a cherry-red colour with ferric chloride, and positive diazo tests for *p*-toluidine, and reduced Benedict's reagent by virtue of its hydroxylamino group. On acid hydrolysis (2*N*-HCl for 5 min.), it yielded a crystalline precipitate of salicylic acid, m.p. and mixed m.p. 155–157° after recrystallization. Paper chromatography of the hydrolysate disclosed the presence of salicylohydroxamic acid (R_F 0.65 in solvent A) and salicylic acid (R_F 0.84). Enzymic hydrolysis with locust-crop liquor glucuronidase gave only salicylohydroxamic acid, detected by paper chromatography in solvent A (R_F 0.65). The salicylohydroxamic acid spot gave a cherry-red colour with ferric chloride and quenched the background fluorescence of the paper under ultraviolet light. The unchanged toluidine salt had R_F 0.09 in solvent A and was also detected in the enzymic hydrolysate by its fluorescence quenching and ferric chloride reaction. This glucuronide is different from *o*-carbamoylphenyl glucosiduronic acid (salicylamide glucuronide), which has R_F 0.40 in the above solvent and does not give a colour with ferric chloride.

From *5-bromosalicylohydroxamic acid*. A total of 7.5 g. of 5-bromosalicylohydroxamic acid was fed to five rabbits and the urine collected for 24 hr. The glucuronide fraction was isolated by the lead acetate procedure. On concentration of the aqueous filtrate obtained after the removal of lead by hydrogen sulphide to about 30–50 ml., and cooling to 0°, glucuronide A (1 g.) separated. Further concentration and cooling yielded a second glucuronide, B, in smaller yields (0.1–0.2 g.).

Glucuronide A was purified by recrystallization from 50% (w/v) aqueous ethanol and formed colourless prisms, m.p. 213–214° (decomp.) and $[\alpha]_D^{20} - 75^\circ$ in 0.1*N*-NaOH (c, 1), which were sparingly soluble in water (Found: C, 39.6; H, 3.7; N, 3.7; Br, 20.2. $C_{13}H_{14}O_8NBr$ requires C, 39.8; H, 3.6; N, 3.6; Br, 20.4%). It was identical with 4-bromo-2-carbamoylphenyl β -D-glucosiduronic acid (mixed m.p. 213–214°) prepared by feeding rabbits with 5-bromosalicylamide (see below). On acid (2*N*-HCl) hydrolysis it yielded 5-bromosalicylamide, m.p. and mixed m.p. 241°. Enzymic hydrolysis (locust-crop liquor β -glucuronidase) also yielded this amide, which was detected chromatographically on paper by its fluorescence in ultraviolet light and R_F values (0.68 in solvent A and 0.83 in solvent B).

The triacetyl derivative of the methyl ester was prepared, and methyl (4-bromo-2-carbamoylphenyl tri-*O*-acetyl- β -D-glucosid)uronate formed fine white needles, m.p. 205–207° with $[\alpha]_D^{20} - 60^\circ$ in $CHCl_3$.

(c, 1) (Found: C, 45.35; H, 4.0; N, 2.8; Br, 14.8. $C_{20}H_{22}O_{11}NBr$ requires C, 45.1; H, 4.2; N, 2.65; Br, 15.0 %).

Glucuronide B formed needles from 50 % aqueous ethanol, with m.p. 191–192° and $[\alpha]_D^{20} - 71^\circ$ in 0.1N-NaOH (c, 1), and was characterized as 4-bromo-2-hydroxycarbamoylphenyl β -D-glucosiduronic acid (Found: C, 36.2; H, 3.6; N, 3.2; H_2O , 4.2. $C_{13}H_{14}O_9NBr \cdot H_2O$ requires C, 36.6; H, 3.8; N, 3.3; H_2O , 4.2 %). On acid (2N-HCl) hydrolysis it yielded 5-bromosalicylohydroxamic acid. With 5N-HCl some 5-bromosalicylic acid was also formed. Both acids were detected chromatographically, 5-bromosalicylohydroxamic acid (R_f 0.56 in solvent A) by its quenching of background fluorescence and its brown colour with ferric chloride and 5-bromosalicylic acid (R_f 0.82) by its dark-blue fluorescence and purple colour with ferric chloride. Enzymic hydrolysis gave only 5-bromosalicylohydroxamic acid, also identified chromatographically.

From 5-bromosalicylamide. The amide (1 g.) was fed to each of four rabbits and urine was collected for 24 hr. Paper chromatography showed the presence of two main metabolites, one a glucuronide (R_f 0.65 in solvent A), which was non-fluorescent, and the other 5-bromosalicylamide (R_f 0.82 in the same solvent), which was fluorescent. The glucuronide (0.5 g.) was isolated in the usual manner and the 4-bromo-2-carbamoylphenyl glucosiduronic acid, m.p. 214–215° (decomp.) and $[\alpha]_D - 74^\circ$ in 0.1N-NaOH (c, 1) was identical with the glucuronide A from 5-bromosalicylohydroxamic acid (Found: C, 39.7; H, 3.1; N, 3.4; Br, 20.3. $C_{13}H_{14}O_8NBr$ requires C, 39.8; H, 3.6; N, 3.6; Br, 20.4 %). Acid and enzymic hydrolysis yielded 5-bromosalicylamide (m.p. and mixed m.p. 241–243° in material from acid hydrolysis), which was also identified chromatographically.

Species differences in the metabolism of salicylohydroxamic acid

Our results indicate that, in the rabbit, there is a significant difference between the metabolism of salicylohydroxamic acid and that of its 5-bromo derivative, since the former formed little or no amide, whereas the latter formed considerable amounts of the amide. Lowenthal (1954), however, found that salicylohydroxamic acid was converted into the amide in rats. We have therefore compared the metabolism of the hydroxamic acid and its bromo derivative in man, rat, mouse and rabbit. Since the fluorescent salicylamide is readily distinguished from the non-fluorescent salicylohydroxamic acid, examination of the urines of the above animals after administration of these compounds could be conveniently carried out by paper chromatography.

In experiments on man, an oral dose of 250 mg. of

salicylohydroxamic acid or of 300 mg. of 5-bromo-salicylohydroxamic acid was taken at night and the morning urine collected for chromatography. During this time these drugs had been largely eliminated. Rats and rabbits were given doses (100 mg./kg.) of both compounds by stomach tube, whereas mice were given doses (100 mg./kg.) of the sodium salts in aqueous solution by intraperitoneal injection. Urines from these animals were collected for 24 hr.

The urines (0.05 ml.) were chromatographed on Whatman no. 1 paper with solvent systems A and B (see Table 1), and examined for the hydroxamic acid, the corresponding amide and carboxylic acid. The urines were also hydrolysed by boiling with 10N-HCl (1 ml. of urine with 0.5 ml. of HCl) for 5 min. and the hydrolysate was similarly chromatographed. This hydrolysis liberated metabolites from their conjugates and partly converted hydroxamic acids into the corresponding carboxylic acids, but did not affect the amides. The authentic compounds in known quantities were run at the same time in the same tank, so that a comparison could be made.

Chromatography of the unhydrolysed urines after administration of salicylohydroxamic acid showed that none of the animals excreted the free hydroxamic acid or salicylic acid. Rat and mouse urine, but not human and rabbit, however, showed traces of free salicylamide. Chromatography of the hydrolysed urines showed small amounts of the hydroxamic acid and large amounts of salicylic acid. In rat, mouse and human urines appreciable amounts of salicylamide were detected, the largest amounts occurring in rat urine. In rabbit urine, only traces of salicylamide were detectable. The rabbit thus differed from the other species.

With 5-bromosalicylohydroxamic acid, none of the hydroxamic acid or 5-bromosalicylic acid could be detected in any of the unhydrolysed urines. Free 5-bromosalicylamide was detected in rat and rabbit urine, traces in mouse urine and none in human urine (in the last-named this might be due to lower dosage). In the hydrolysed urines small amounts of hydroxamic acid and 5-bromosalicylic acid were detected, whereas 5-bromosalicylamide was found in large amounts in all the urines. Thus conjugated 5-bromosalicylamide appeared to be the main metabolite of the hydroxamic acid in all four species.

DISCUSSION

During the course of these experiments we observed that oral doses of 600 mg. of salicylohydrazide/kg. produced convulsions in 50 % of the rabbits (20) used, whereas 800 mg. of N^1 -acetyl- N^2 -salicyloylhydrazine/kg. (the highest dose administered) was well tolerated. Benzohydrazide,

however, was frequently fatal in oral doses of 100 mg./kg. The toxicities of these compounds in rabbits are therefore in the descending order, benzohydrazide, salicylohydrazide, acetylsalicyloylhydrazine. These three compounds also differ in the extent to which they lost their hydrazino groups *in vivo*. More than 60 % of benzohydrazide is converted into benzoic acid and the over-all reaction for its metabolism is probably

With salicylohydrazide there is an alternative metabolic reaction to hydrolysis, namely, glucuronic acid conjugation. This occurs to a large extent, accounting for 50–60 % of the dose. The hydrazino group, however, is lost to some extent since appreciable amounts of salicyluric acid (amounting to 7–17 % as estimated by a variation of the method for hippuric acid by El Masry *et al.* 1956) are excreted. There appears to be only one metabolite of acetylsalicyloylhydrazine, i.e. the glucuronide, and there is no evidence that the hydrazino group is removed. The reactions of the salicyl derivatives are probably those shown in Fig. 1.

These results suggest that the toxicities of these compounds are related to whether or not they release hydrazine *in vivo*. However, if 20 % of a dose of 600 mg. of salicylohydrazide/kg. were hydrolysed *in vivo*, as much hydrazine would be set free as on complete hydrolysis of a dose of 100 mg. of benzohydrazide/kg. If hydrazine is the toxic agent, then, presumably, it is released very much more rapidly from benzohydrazide than from salicylohydrazide. Benzohydrazide, however, appears to possess some toxicity *per se* (Yard & McKennis, 1955). Other work in this Laboratory has shown that *p*-chloro- and *p*-methyl-benzohydrazides are nearly as toxic as benzohydrazide and are completely hydrolysed to the corresponding benzoic acids in the rabbit, whereas *p*-hydroxybenzohydrazide is very much less toxic and is metabolized by direct conjugation of the hydroxyl group.

It is interesting to note (see Table 2) that none of the salicylic acid hydrazides form ethereal sulphates and are thus like salicylic acid itself. This is in contrast with the hydroxamic acids, which form ethereal sulphates and thus behave like salicylamide (Bray *et al.* 1948).

The hydroxamic acids studied in this work appeared to be less toxic than the hydrazides, although hydroxylamine appears to be more toxic than hydrazine (for hydrazine the intravenous LD₅₀ is 34 mg./kg. and the cutaneous LD₅₀ is 91 mg./kg., whereas for hydroxylamine the subcutaneous lethal dose (LD) is 25 mg./kg., for rabbits; see Spector, 1956). Benzohydroxamic acid was tolerated in much higher doses than benzohydrazide despite the fact that it is mainly converted into hippuric acid in

the body and loses its hydroxylamino group. The formation of the corresponding amides from salicylohydroxamic acid and its 5-bromo derivative suggests that benzohydroxamic acid is not hydrolysed to benzoic acid and hydroxylamine but is first reduced to benzamide, which is known to be hydrolysed almost entirely to benzoic acid in the rabbit (Bray, Neale & Thorpe, 1946):

This suggestion could explain the lower toxicity of benzohydroxamic acid compared with benzohydrazide.

Both salicylohydroxamic acid and its 5-bromo derivative are metabolized mainly by direct conjugation with glucuronic and sulphuric acids. In addition to this, they are also reduced to amides in man, the rat and the mouse. In the rabbit, there is the remarkable difference between salicylohydroxamic acid and its 5-bromo derivative, in that the former is not reduced to an amide to any great extent whereas the latter is. This is difficult to explain, but has been checked by repeated experimentation. The fate of these hydroxamic acids in the four species studied can be represented in Fig. 2. (X = H in salicylohydroxamic acid and Br in the 5-bromo derivative; R = C₆H₅O₆ or SO₃H; in the rabbit, when X = H, there is no amide formation.)

Since the above-mentioned salicylohydroxamic acids are partly reduced to amides in man, the question arises whether this reduction is related to their tuberculostatic properties. 5-Bromosalicylamide was tested simultaneously with the corresponding hydroxamic acid against *Mycobacterium tuberculosis* H37Rv in Dubos medium. The amide was found to inhibit growth of the organism in concentrations of 125–250 µg./ml., whereas the hydroxamic acid inhibited at 62.5 µg./ml. (cf. Urbański, 1953). Conversion into the amide thus leads to a reduction, but not an abolition, of activity *in vitro*. In actual practice antitubercular activity must be partly due to the amide.

Fig. 1

Fig. 2

SUMMARY

1. A study has been made of the fate of salicylohydrazide and its *N*-acetyl derivative, and of salicylohydroxamic acid and its 5-bromo derivative, in the rabbit. The last two compounds have also been studied in man, rat and mouse.

2. Salicylohydrazide is metabolized mainly by conjugation with glucuronic acid (55 % of dose), and partly by hydrolysis to salicylic acid, which is excreted largely as salicyluric acid.

3. *N*¹-Acetyl-*N*²-salicyloylhydrazine is not deacetylated and is excreted mainly as its glucuronide (70 % of the dose). These hydrazides do not form ethereal sulphates. Their glucuronides have been isolated.

4. Salicylohydroxamic acid is metabolized by direct conjugation with glucuronic and sulphuric acids in the rabbit, and there is practically no conversion into salicylamide. The glucuronide was isolated. In man, mouse and the rat, it forms considerable amounts of salicylamide, which is excreted conjugated.

5. 5-Bromosalicylohydroxamic acid is excreted by man, mouse, rabbit and rat mainly as conjugates of 5-bromosalicylamide, and to a lesser extent as the glucuronide of 5-bromosalicylohydroxamic acid. The glucuronides of the amide and the hydroxamic acid were isolated from rabbit urine.

6. The *in vitro* tuberculostatic activity of 5-bromosalicylamide is about half that of 5-bromosalicylohydroxamic acid, its precursor *in vivo*.

7. The toxicity of aroyl hydrazides and hydroxamic acids in relation to their metabolism has been discussed.

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Cationic Extracting Agents for Rhodopsin and their Mode of Action

By C. D. B. BRIDGES

*Medical Research Council, Group for Research in the Physiology of Vision,
 Institute of Ophthalmology, Judd Street, London, W.C. 1*

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Many different substances have been found to be capable of extracting rhodopsin from the dark-adapted frog retina. Kühne (1877) used a crude preparation of bile salts, and Tansley (1931) introduced the now widely used extracting agent digitonin, a steroid saponin found in foxglove leaves. Other workers have used sodium cholate (Sugita, 1926), sodium oleate, panaxtoxin and sodium salicylate (Hosoya, 1932).

With few exceptions, all substances capable of extracting rhodopsin dissolve in water yielding foaming solutions with a low air-solution interfacial tension. That is to say, they are surface-active agents.

The extraction of rhodopsin by cationic surface-active agents has not been previously described. Lythgoe (1938), using the cationic hexadecylpyridinium bromide, failed to extract rhodopsin