of body weight in the very young lamb to about 24 % of body weight in the adult.

5. These results are discussed in relation to differences in carbohydrate metabolism between young lambs and mature sheep.

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The Metabolism of Phenolic Antioxidants

4. THE METABOLITES OF GENTISIC ACID IN THE DOG*

BY B. D. ASTILL, D. W. FASSETT AND R. L. ROUDABUSH
Laboratory of Industrial Medicine, Eastman Kodak Co., Rochester 4, N.Y., U.S.A.

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Gentisic acid and its sodium salt are potentially useful antioxidants in the stabilization of edible fat (Wishnetzky & Stuckey, 1961). Many studies of the fate of gentisic acid after absorption by mice, rats, rabbits and man have been reported (Williams, 1959*a*, p. 369), but its metabolic fate is obscure. As a part of a study to test the suitability for use in foodstuffs of gentisic acid and sodium gentisate, a detailed account of their disposal in a large mammal such as the dog was needed, with particular emphasis being placed on elucidating the nature of the metabolites.

Gentisic acid is known to be rapidly excreted, often largely unchanged, in the urine after oral ingestion, and increases in urinary *O*-sulphate and *O*-glucuronide concentrations may occur. No metabolites were isolated, although chromatographic evidence has been presented for *O*-glucuronide formation with the 5-hydroxyl group (Haberland, Madenwald & Köster, 1957), for dehydroxylation to salicylic acid in fevered humans (Consden & Stanier, 1951) and for methylation of the 5-hydroxyl group in humans (Sakamoto, Inamori & Nasu, 1959). The only previous study in the dog (Likhatscheff, 1895) found an increased urinary *O*-sulphate output to account for 18 % of the dose of gentisic acid.

We now report the isolation and characterization of the products of the metabolism of gentisic acid in the dog, which appear to be formed exclusively by conjugation at the 5-hydroxyl group. Small amounts of the principal metabolites are also excreted as conjugates of gentisic acid after the ingestion of acetylsalicylic acid by the dog.

* Part 3: Astill, Mills, Fassett, Roudabush & Terhaar (1962).

Table 1. *Absorption spectra of gentisic acid and some derivatives*

All spectra were measured in ethanol, except that of potassium 3-carboxy-4-hydroxyphenyl sulphate which was measured in water.

Substance	$\lambda_{max.}$ (mμ)	log ε	$\lambda_{max.}$ (mμ)	log ε
Gentisic acid	236	3·74	335	3·59
2-*O*-Methylgentisic acid	234	3·83	322	3·58
5-*O*-Methylgentisic acid	233	3·74	328	3·59
Potassium 3-carboxy-4-hydroxyphenyl sulphate	224 (inflexion)	3·85	300	3·53
Methyl [*O*-(4-acetoxy-3-methoxycarbonylphenyl)tri-*O*-acetyl-β-D-glucosid]uronate	222 (inflexion)	3·99	289	3·41

METHODS AND MATERIALS

Materials. Melting points are uncorrected.

Gentisic acid, m.p. 209–210°, was kindly supplied by the Tennessee Eastman Co., Kingsport, Tenn., U.S.A. The following were purchased: sodium gentisate (Rhodia Inc., New York, N.Y., U.S.A.), naphtharesorcinol, safranine bluish (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.), and β-glucuronidase (ox-liver; Ketodase; Warner-Chilcott, New York, N.Y., U.S.A.).

5-*O*-Methylgentisic acid, m.p. 147·5°, was obtained by the procedure of Graebe & Martz (1905).

Potassium 3-carboxy-4-hydroxyphenyl sulphate was obtained by the procedure of Baker & Brown (1948), from the oxidation of salicylic acid (14·0 g.) with potassium persulphate (27·0 g.). The ether-extracted aqueous solution (300 ml.) of the *O*-sulphate was evaporated to dryness *in vacuo* at 50° to yield a solid residue. This was washed with ethanol and collected (20·0 g.; 65%). A portion was crystallized repeatedly from aq. 90% (v/v) propan-2-ol to yield lustrous elongated plates, m.p. above 300°, of *potassium 3-carboxy-4-hydroxyphenyl sulphate*, which lost 3·7% on drying at 110° *in vacuo* over P_2O_5 (Found: C, 26·2, 26·4; H, 1·9, 2·1; K, 24·5; S, 9·3. $C_7H_4O_7K_2S,\frac{3}{4}H_2O$ requires C, 26·0; H, 1·7; K, 24·2; S, 9·9; H_2O, 3·7%). The salt was very soluble in water but almost completely insoluble in organic solvents. It gave a stable blue–purple colour with Fe^{3+} ions in aqueous solution. The ascribed structure was confirmed by warming a solution of 1·0 g. in water (2·0 ml.) on the steam bath with conc. HCl (1·0 ml.; 1·5 equiv.). On cooling at 5° for 3 hr. the solution deposited gentisic acid (0·34 g.; 68%), m.p. 208·5° undepressed on admixture with authentic material.

2-*O*-Methylgentisic acid was prepared by the addition of ethereal diazomethane to potassium 3-carboxy-4-hydroxyphenyl sulphate (1·0 g.) dissolved in methanol (150 ml.), followed by a further addition of diazomethane 24 hr. later. An initial blue–purple colour given with $FeCl_3$ solution changed to brown on testing during the methylation. Evaporation of the solution *in vacuo* yielded a yellow oil, which was warmed with 3N-HCl (20 ml.) for 20 min. The solution was cooled, extracted with ether, and the dried ether extract evaporated to a discoloured solid which gave, on crystallization from water, elongated prisms (160 mg.; 30%) of 2-*O*-methylgentisic acid, m.p. 156–157° (Found: C, 56·7; H, 4·7; O·CH_3, 18·0. Calc. for $C_8H_8O_4$: C, 57·1; H, 4·8; O·CH_3, 18·4%). A yellow–brown colour was given in ethanolic solution by $FeCl_3$. These properties agree with those reported by Fischer & Pfeffer (1912).

Methyl [*O*-(4-acetoxy-3-methoxycarbonylphenyl)tri-*O*-acetyl-β-D-glucosid]uronate was obtained as follows. Methyl 2-*O*-acetylgentisate, m.p. 95–100°, was prepared by the procedure of Wagner (1958) who reported m.p. 98–100°. The ester (5·0 g.), mixed with methyl (tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate (6·5 g.), was triturated for 30 min. with silver carbonate (3·0 g.) in quinoline (15·0 ml.) (Bollenback, Long, Benjamin & Lindquist, 1955). The mixture was left for 3 hr. in a desiccator with occasional stirring. Chloroform (150 ml.) was added, the mixture was centrifuged, the sediment resuspended in chloroform, and centrifuged again. The combined supernatants were then washed successively with 3N-HCl (4 × 70 ml.), water (100 ml.), 2N-NaOH (4 × 60 ml.) and water (2 × 150 ml.), and the washings were discarded. The organic phase was dried over anhydrous $MgSO_4$ and evaporated *in vacuo* to a gum. After solution in hot aq. ethanol this yielded on cooling needles (2·1 g.; 16%) of *methyl* [O-(4-*acetoxy*-3-*methoxycarbonylphenyl*) *tri*-O-*acetyl*-β-D-*glucosid*]*uronate*, m.p. 136–137°, $[\alpha]_D^{25}$ −42° (*c* 0·95 in ethanol) (Found: C, 52·8; H, 4·9; O·CH_3, 11·3; O·CO·CH_3, 44·0. $C_{23}H_{26}O_{14}$ requires: C, 52·4; H, 4·9; 2O·CH_3, 11·8; 4O·CO·CH_3, 44·8%).

Absorption spectra are recorded in Table 1 and chromatographic properties in Table 2.

Animals, diets and dosages. Male mongrel dogs of about 14 kg. weight were given Purina Dog Chow and kept singly in metabolism cages. Single doses of gentisic acid and sodium gentisate for the experiments in Table 3 were given orally by ground-meat capsule. Successive daily doses for the isolation and characterisation of metabolites were given by meat capsule at levels of 0·31 g./kg. for gentisic acid and of 0·19 g./kg. for acetylsalicylic acid. Urine was collected from single-dose experiments until metabolites were undetectable, and until dosing ceased in successive dose experiments. Normal daily values were established on urines collected for several days before dosing. Urines were analysed promptly or frozen until required for analysis.

Paper chromatography and electrophoresis. The detection and separation of metabolites in experimental urine and extracts was performed on Whatman no. 1 paper in the descending fashion. Table 1 records R_F values, colour reactions and solvents. Paper electrophoretograms were obtained with Whatman 3MM paper strips in 0·1M-citrate buffer, pH 4·0, with migration times of 4–6 hr. at 0·7–1·0 mA/in. Papers were subsequently dried at 40° and examined in ultraviolet light (Crabtree, Data & Christian, 1958). Mobilities were expressed as $R_{gentisic\ acid}$ values (Table 2).

Determination of gentisic acid in urine. (*a*) From dogs receiving gentisic acid or sodium gentisate. The method of Meade & Smith (1951) for phenolic acids in urine was

modified. Urines were diluted with distilled water. A 1·0 ml. portion containing 0·02 ml. of urine was mixed with 6N-HCl (1·0 ml.) in a 15 ml. stoppered centrifuge tube and shaken with ethyl acetate (8·0 ml.) for 2 min. The mixture was centrifuged for 5 min., 5·0 ml. of the ethyl acetate layer was removed and shaken with 0·5M-$NaHCO_3$ (5·0 ml.) for 2 min. and the mixture was centrifuged for 5 min. A suitable portion of the aqueous layer was removed, mixed with Folin–Ciocalteu solution (1·0 ml.) (Bray & Thorpe, 1954) and 2M-Na_2CO_3 (1·0 ml.), and diluted with water to 10·0 ml. The extinction at 660 mμ was read after 10–20 min. in a Beckmann model DU spectrophotometer. A calibration curve was linear from 5 to 60 μg. of gentisic acid, and recoveries of gentisic acid added to normal urine were 99 ± 5 %. Metabolites of gentisic acid did not interfere in this determination, since chromatography showed them not to be extracted by ethyl acetate from diluted experimental urines.

(*b*) From dogs receiving acetylsalicylic acid. The method of Quilley & Smith (1952) for gentisic acid in the presence of other products of salicylate metabolism was modified. Urine samples (20 μl.) were partitioned on Whatman no. 1 filter-paper strips cut from sheets 18¼ in. × 22½ in. in the machine direction, with solvent II (Table 2) overnight. Gentisic acid spots were located by co-chromatography and in ultraviolet light, cut out and extracted with 0·5M-$NaHCO_3$ (10 ml.). The gentisic acid content of the extracts was measured with the Folin–Ciocalteu reagent as for the 0·5M-$NaHCO_3$ extract in (*a*). A clear separation from other detectable salicylic acid metabolites in experimental urines was obtained. Recoveries of gentisic acid added to urine were 81 ± 2 %.

Estimation of conjugates in experimental urine. Total and inorganic sulphates were determined by Folin's gravimetric procedure (Bray & Thorpe, 1954), and glucuronides by a naphtharesorcinol method (Astill, Fassett, & Roudabush, 1959).

RESULTS

Excretion of metabolites by undosed dogs. Mean daily excretions per dog, from 14 determinations on three undosed dogs, were: free phenolic acids, 0·07 g. (0·02–0·15 g.); glucuronides, 0·47 g. (0·15–0·67 g.); and ethereal sulphates, 0·27 g. (0·17–0·42 g.), all as gentisic acid.

Excretion of gentisic acid and conjugates by the dog. Urines were analysed for free gentisic acid, ethereal sulphate and glucuronide outputs for doses of 0·19 and 0·31 g. of gentisic acid/kg., and 0·19 g. of sodium gentisate/kg., and the results are recorded in Table 3.

Metabolites of gentisic acid in the dog. Chromatograms of urine from experiments with both gentisic acid and sodium gentisate revealed three substances, gentisic acid (R_F 0·85 in solvent I; see Table 2) and compounds L (R_F 0·29 in solvent I) and M (R_F 0·21 in solvent I), which were not present in control urines. The colour reactions of compounds L and M and their fluorescence in ultraviolet light suggested that they were metabolities of gentisic acid. Compound L had an $R_{\text{gentisic acid}}$ that

Table 2. *Paper chromatography and electrophoresis of gentisic acid, its metabolites and some phenolic acids*

Whatman no. 1 paper was used throughout in the descending technique. Solvents were: I, organic phase of freshly mixed butan-1-ol–acetic acid–water (4:1:5, by vol.); II, organic phase of benzene–acetic acid–water (2:2:1, by vol.). Detecting reagents were: *A*, 1 % (w/v) $FeCl_3$ in aq. 50 % (v/v) ethanol; *B*, freshly mixed 1 % (w/v) sulphanilic acid in 3N-HCl–aq. 5 % (w/v) $NaNO_2$ (1:1, v/v), followed by aq. 10 % (w/v) Na_2CO_3. Papers were also sprayed with aq. 1 % (w/v) $AgNO_3$ in aq. 10 % NH_3 soln., with which only gentisic acid reacted, giving an immediate black colour, and with 5 % (w/v) *p*-dimethylaminobenzaldehyde in acetic anhydride (El Masry, Smith & Williams, 1956), which gave no colour reactions with the metabolites. R_F values for compounds M and Q are from aqueous solutions of the appropriate gum at (*a*) pH 4·5 and (*b*) pH 9·5. The mobilities are as $R_{\text{gentisic acid}}$ values on Whatman 3MM paper; spots were located in ultraviolet light.

Substance	R_F		Colour reactions on chromatograms		Fluorescence of spot in ultraviolet light	Mobility in citrate buffer, pH 4·0 ($R_{\text{gentisic acid}}$)
	In solvent I	In solvent II	With reagent *A*	With reagent *B*		
Gentisic acid	0·85	0·10	Blue, rapidly fading to brown	Yellow–orange	Bright green–blue	1·0
Salicylic acid	0·87	0·75	Purple–red	Orange–red	Feeble dark blue	1·35
5-*O*-Methylgentisic acid	0·89	0·75	Blue	Yellow	Blue–green	0·84
2-*O*-Methylgentisic acid	0·87	0·20	Weak brown	Orange–red	Blue	
Metabolites						
Compound K (from ether extract of urine)	0·89	0·75	Blue	Yellow	Blue–green	0·84
Compound L (from pH 9·5 glucuronide gum; 'residual urine')	0·29	0·0	Blue–purple	Orange	Blue	2·1
Compound M (from pH 9·5 glucuronide gum)	0·21 (*b*) 0·58 (*a*)	0·0	Grey–blue	Yellow	Blue–green	0·95
Compound Q (from pH 4·5 glucuronide gum)	0·56 (*a*) 0·21 (*b*)	0·0	Blue–purple	Yellow	Blue	0·87

suggested the presence of an additional strongly ionized moiety such as $-O \cdot SO_2 \cdot O-$. Compound M had properties closely resembling the chromatographic properties described for the metabolite of gentisic acid in the rat believed to be a glucuronide (Haberland *et al.* 1957). No metabolites reduced ammoniacal silver nitrate solution, or gave an Altman reaction for glycine conjugates (El Masry, Smith & Williams, 1956).

Compound L was established as the 5-*O*-sulphate of gentisic acid on isolation from the pH 9·5 glucuronide gum (Kamil, Smith & Williams, 1951) as its safranine bluish salt (Astill, Mills, Fassett, Roudabush & Terhaar, 1962) and subsequent conversion into potassium 3-carboxy-4-hydroxyphenyl sulphate. Further amounts of compound L were isolated from the experimental urine remaining after glucuronide precipitation. Compound M was established as the 5-*O*-glucuronide of gentisic acid when methyl [*O*-(4-acetoxy-3-methoxycarbonylphenyl)tri-*O*-acetyl-β-D-glucosid]uronate was obtained on methylation and acetylation of the pH 9·5 glucuronide gum from which solvent extraction had removed compound L.

Experimental urines contained further metabolites, compounds K and Q. Compound K (R_F 0·89 in solvent I) occurred with gentisic acid in ether extracts of urine from dogs receiving single doses of 0·31 g. of gentisic acid/kg. It was first thought to be salicylic acid (cf. Consden & Stanier, 1951), but on separation by partition chromatography from gentisic acid was isolated and shown to be the 5-*O*-methyl ether of gentisic acid. Neither salicylic acid nor quinol was detected in these extracts.

Compound Q, located on paper chromatography of the experimental pH 4·5 glucuronide gum, had the reactions of a 5-*O*-substituted gentisic acid (Table 2). Its changes in R_F with pH were attributed to a lactone ring in a glucuronide acid moiety (Haberland *et al.* 1957). Methylation and acetylation of crude compound Q yielded the tetra-*O*-acetyl *O*-dimethyl ester of compound M, hence compound Q was formulated as a lactone of the 5-*O*-glucuronide of gentisic acid.

Excretion of metabolites after the ingestion of acetylsalicylic acid. Experimental urines from dogs receiving daily doses of 0·19 g. of acetylsalicylic acid/kg. contained salicylic acid and gentisic acid, and, on working up as for the gentisic acid experiment, traces of compounds L, M and Q. A small quantity of compound L was obtained and characterized as the 5-*O*-sulphate of gentisic acid.

EXPERIMENTAL

Isolation of metabolites

The urine (4·8 l.) from three dogs, each given successive daily doses of 0·31 g. of gentisic acid/kg., was collected for 3 days after the first dose and pooled. A portion (4·2 l.; pH 6·75) was clarified by centrifuging and extracted by slow shaking with ether (1 l. for 20 hr., then 1 l. for 3 hr.), or ethyl acetate (2 × 1 l. for 3 hr.). The pH 4·5 and pH 9·5 glucuronide gums were then obtained from the extracted urine by precipitation with lead acetate (Kamil *et al.* 1951). The 'residual urine' fraction was the concentrated filtrate of the pH 9·5 lead precipitate from which lead had been removed as PbS.

Compound L. This was located in the pH 9·5 glucuronide gum, which was redissolved in water (110 ml.) and washed with ether (3 × 300 ml.) to remove unextracted gentisic acid (1·35 g.). A portion (55 ml.) was neutralized with anhydrous K_2CO_3. The neutralized solution yielded, on

Table 3. *Urinary excretion of gentisic acid and its conjugates by the dog*

A single oral dose was given by meat capsule, and urine was collected daily till metabolites were chromatographically undetectable. Results for the ingestion of gentisic acid are means of four experiments with ranges in parentheses, and those for the ingestion of sodium gentisate are from pooled daily urines from three dosed animals. All results are percentages of the given dose, as gentisic acid.

Dose	Form of gentisic acid excreted	Amount of material excreted (% of given dose): Days after dosing 1	2	3	4	Total recovery
Gentisic acid (0·19 g./kg.)	Free gentisic acid (F)	54 (48–68)	7 (2–12)	2·5 (1–5)	0	63·5
	Ethereal sulphate (E)	24 (17–27)	4 (1–9)	0	0	28
	Glucuronide (G)	6 (3–9)	1 (0–2·3)	0	0	7
	Total (E+G+F)	84	12	2·5	0	98·5
Gentisic acid (0·31 g./kg.)	Free gentisic acid (F)	46 (23–57)	13 (2–28)	2 (1–6)	0	61
	Ethereal sulphate (E)	14 (12–17)	6 (3–12)	0·5 (0–1)	0	20·5
	Glucuronide (G)	11 (7–17)	4 (0–13)	0·5 (0–1)	0	15·5
	Total (E+G+F)	71	23	3·0	0	97
Sodium gentisate (0·19 g./kg.)	Free gentisic acid (F)	45	< 1	4	0	50
	Ethereal sulphate (E)	26	6	0	0	32
	Glucuronide (G)	9	2	4·5	0	15·5
	Total (E+G+F)	80	9	8·5	0	97·5

evaporation at 50° *in vacuo*, 6·35 g. of a gummy residue, which was shaken with aq. 90% (v/v) ethanol (3 × 700 ml.) for 20 min. and finally was refluxed briefly with this solvent (700 ml.). The combined extracts were evaporated *in vacuo* to a yellow gum, from which the safranine bluish salt was prepared as microcrystals (0·66 g.), which by cation exchange on Dowex 50 (K^+ form) yielded a discoloured crystalline residue (0·30 g.). This crystallized from aq. 90% (v/v) propan-2-ol (charcoal) as elongated plates of *potassium 3-carboxy-4-hydroxyphenyl sulphate*, m.p. above 300° (Found: C, 26·0; H, 1·9; K, 24·5%; S, 9·8). The chromatographic properties, $R_{\text{gentisic acid}}$, and ultraviolet-absorption spectrum were identical with those of the synthetic specimen. The infrared-absorption spectrum showed a band at 6·3 μ (C=O stretching vibration of CO_2^-) and at 9·5 μ (S=O stretching vibration). A portion was warmed with warm 3N-HCl for 20 min., when gentisic acid was obtained by ether extraction of the cooled solution, and the aqueous phase gave a SO_4^{2-} ion reaction with aq. 5% (w/v) $BaCl_2$.

Potassium 3-carboxy-4-hydroxyphenyl sulphate (170 mg.) was converted exactly as for the synthetic material into elongated prisms of 2-*O*-methylgentisic acid (28 mg.), m.p. 156–157° not depressed on admixture with an authentic specimen (Found: C, 57·2; H, 4·6%).

The 'residual urine' fraction, which appeared to contain a further quantity of compound L, was shaken with stearic acid-deactivated charcoal (Asatoor & Dalgliesh, 1956) (60 g.) for 1·5 hr. The charcoal was collected and washed to yield filtrate and washings free of compound L, and then was shaken with aq. 7% phenol for 0·5 hr. (2 × 300 ml.). On filtration and evaporation to dryness at 55°/1 mm., the phenol solution yielded an amorphous residue which gave a strong chromatographic reaction for compound L. Partition chromatography on a column of Whatman no. 1 powdered cellulose with solvent I yielded two main fluorescent bands on the column, which were eluted in 5·0 ml. fractions. The first band (fractions 4–18) yielded a small amount of gentisic acid, and the second (fractions 64–84) yielded a semi-solid residue (750 mg.) which gave the chromatographic reactions of compound L and yielded SO_4^{2-} ions on gentle warming with 3N-HCl. The crude product was converted via its safranine bluish salt and cation exchange into *potassium 3-carboxy-4-hydroxyphenyl sulphate*, crystallized as elongated plates (35 mg.) from aq. 90% (v/v) propan-2-ol, m.p. above 300° (Found: C, 26·1; H, 2·0%). The ultraviolet- and infrared-absorption spectra and chromatographic properties of the compound were identical with those of synthetic material.

Compound M. The residue of the pH 9·5 glucuronide gum remaining after ethanol extraction gave a strong chromatographic reaction for compound M and contained only traces of compound L. Incubation of a portion (25 mg.) with Ketodase (4 ml.) in pH 4·2 phosphate buffer (2·0 ml.) at 37·4° for 6 hr., on comparison with a similar solution from which Ketodase was absent, showed that compound M yielded gentisic acid in the presence of β-glucuronidase and was itself no longer detectable, whereas compound L was unaffected. The gum gave a strong positive naphtharesorcinol reaction. It was dissolved in water, the pH adjusted to 9·5 with 6N-ammonia and excess of aq. satd. lead acetate added. The precipitate was collected, washed with water, the lead removed with H_2S and the resulting gum dissolved in aq. 75% (v/v) methanol (250 ml.), to which excess of ethereal diazomethane was then added. The solution gave a strong blue-purple colour with Fe^{3+} ions after 14 hr. (In another experiment several additions of diazomethane failed to affect this colour, but led ultimately to decomposed products.) The solvent was removed *in vacuo* and the residue mixed with acetic anhydride (3·0 ml.) and pyridine (3·0 ml.), yielding, on pouring into water and recrystallizing from aq. ethanol, fine white needles (97 mg.), m.p. 134–135° undepressed on admixture with an authentic specimen, of *methyl [O-4-acetoxy-3-methoxycarbonylphenyl)-tri-O-acetyl-β-D-glucosid]uronate* (Found: C, 52·1; H, 5·1; $O·CH_3$, 12·0%.) The ultraviolet-absorption spectrum was identical with that of the synthetic material, and the infrared-absorption spectrum showed strong absorption at 5·75 μ, and had no OH stretching vibration. The substance gave no colour with Fe^{3+} ions.

Compound Q. The pH 4·5 glucuronide gum was dissolved in water (250 ml.), and on shaking the solution with deactivated charcoal (50 g.) compound Q was completely adsorbed. Elution with aq. 7% (v/v) phenol yielded a brown gum in which compound Q was the only phenolic substance detected chromatographically. On partition on Whatman no. 1 powdered cellulose with solvent I, a wide fluorescent band was eluted (fractions 8–34) to yield an amorphous solid (750 mg.). This gave a deep-blue colour with Fe^{3+} ions and a strong positive reaction with naphtharesorcinol. Incubation of a portion with Ketodase at pH 4·5 liberated gentisic acid. The crude compound Q was dissolved in methanol (100 ml.) and ether (100 ml.) followed by an excess of ethereal diazomethane that had been kept over KOH pellets. After 14 hr. the solvent was removed and the residue acetylated as above, to yield on crystallization from aq. ethanol needles of *methyl [O-(4-acetoxy-3-methoxycarbonylphenyl)tri-O-acetyl-β-D-glucosid]uronate* (280 mg.). The substance had m.p. 135–136° undepressed on admixture with that similarly prepared from compound M and with an authentic specimen (Found: C, 52·4, 52·6; H, 5·0, 5·0; $O·CH_3$, 12·2; $O·CO·CH_3$, 44·6%) $[\alpha]_D^{25}$ −38° (*c* 0·95 in ethanol). The ultraviolet- and infrared-absorption spectra were identical with those of an authentic specimen.

Compound K. This was obtained by a slightly different extraction procedure. Urine (1·5 l.) collected during 24 hr. after dosing from three dogs, each given 0·31 g. of gentisic acid/kg. (total dose, 15 g.), was continuously extracted with ether for 24 hr. The extract contained gentisic acid and compound K (Table 2), and was evaporated to a semicrystalline mass which was extracted with hot ethanol–benzene (1:3, v/v). On cooling, crude gentisic acid (3·5 g.) separated and was collected. A further 1·0 g. of gentisic acid was collected from the filtrate on concentration. Chromatography showed the supernatant liquor to be enriched with compound K. It was extracted with aq. 4% (w/v) $NaHCO_3$, the alkaline solution washed with ether, acidified, and extracted with ether to yield a gum containing gentisic acid and compound K. The gum was dissolved in the organic phase of the mixture, chloroform–methanol–aq. 4% (v/v) formic acid (10:1:1, by vol.) and partitioned in this solvent on a column of Whatman no. 1 powdered cellulose previously equilibrated in the aqueous phase of the mixture. A previous experiment with this system separated gentisic acid and salicylic acid completely. The eluate was automatically collected in 5·0 ml. fractions which were tested with aq. ethanolic $FeCl_3$. The movement of compound K on the column was followed in ultraviolet light. Fractions 43–70 contained gentisic acid.

Fractions 4–14 contained compound K and yielded an intractable gum, which was dissolved in aq. saturated $NaHCO_3$ and re-extracted with ether after acidification. The residue on removing the ether was dissolved in solvent II (10 ml.) and partitioned in solvent II on powdered cellulose. The fluorescent zone attributed to compound K separated cleanly from most impurities, and was eluted to yield a residue (40 mg.) which crystallized after several days *in vacuo*. On twice crystallizing from water, there resulted needles of 5-*O*-methylgentisic acid (13 mg.) m.p. 146–147°, not depressed on admixture with an authentic specimen of m.p. 147·5° (Found: C, 57·5; H, 5·3. Calc. for $C_8H_8O_4$: C, 57·1; H, 4·8%). The natural and synthetic specimens had identical ultraviolet- and infrared-absorption spectra and chromatographic properties (Table 2).

Excretion of compounds L, M, and Q after the ingestion of acetylsalicylic acid by the dog

Urines were analysed for gentisic acid, and metabolites were detected chromatographically. About 5% (2–7%) of the daily dose of 0·19 g. of acetylsalicylic acid/kg. was excreted as gentisic acid, which is in agreement with previous values (Williams, 1959*a*, p. 359). Salicylic acid, gentisic acid, and compounds L and M were detected (Table 2), and salicyluric acid was located by the properties reported by Haberland *et al.* (1957). The pooled experimental urine from a dog receiving three consecutive doses of 3 g. of acetylsalicylic acid/day was shaken with ethyl acetate (2 × 800 ml.) and subsequently worked up, as described for the isolation of the metabolites of gentisic acid. The ethyl acetate extract contained gentisic acid and salicylic acid. The pH 4·5 glucuronide gum contained traces of compound Q, and the pH 9·5 gum contained compounds L and M. Attempts to isolate compound Q as its methylated acetylated derivative yielded intractable products. Compound L was isolated from the pH 9·5 glucuronide gum, which was redissolved in water (30 ml.), an excess of K_2CO_3 was added, the solution was evaporated to dryness *in vacuo* and extracted with aq. 90% (v/v) propan-2-ol (2 × 700 ml.), the extract was evaporated to dryness *in vacuo*, and an impure safranine bluish salt (2·5 g.) was obtained from it and converted into a potassium salt by cation exchange with Dowex 50 (K^+ form) (25 g.) as above, to yield a discoloured crystalline solid. This was extracted with aq. 90% (v/v) propan-2-ol (charcoal); the extract, on concentration to 15 ml. and cooling, yielded elongated needles (63 mg.) of *potassium 3-carboxy-4-hydroxyphenyl sulphate*, having ultraviolet- and infrared-absorption spectra, chromatographic properties and colour with Fe^{3+} ions identical with those of the material obtained similarly from compound L as a metabolite of gentisic acid in dogs (Found: C, 26·3; H, 1·8%). An attempt to isolate the methylated and acetylated derivative of compound M from the residue of the pH 9·5 glucuronide gum was unsuccessful.

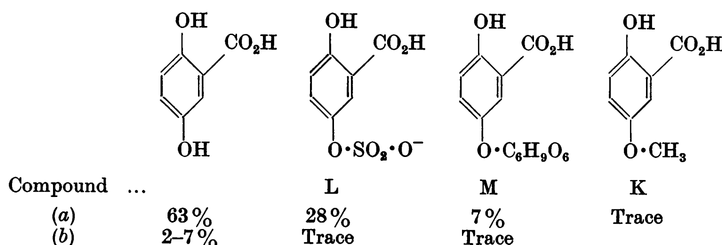
DISCUSSION

Gentisic acid is disposed of by the dog by rapid excretion of more than half of the dose unchanged, and of the remainder as conjugates at the 5-hydroxyl group. Its path of metabolism is usual for phenols and phenolic acids. There is no evidence of conjugation with the 2-hydroxyl or carboxyl groups, and decarboxylation and further hydroxylation are evidently absent. Conjugation of the carboxyl group of salicylic acid with glycine is much depressed in comparison with that of benzoic acid, *m*-hydroxybenzoic acid or *p*-hydroxybenzoic acid, and the absence of glycine conjugation in gentisic acid metabolism can be ascribed to the so-called *ortho* effect of the 2-hydroxyl group, and the added availability of the reactive 5-hydroxyl group for glucuronide and sulphate conjugation.

This study provides confirmation of the various reports of glucuronide formation by gentisic acid, particularly that of Haberland *et al.* (1957) for the rat. We found, in unpublished work, that rats in a prolonged feeding study with gentisic acid excrete substances of chromatographic properties identical with those of the 5-*O*-glucuronide and 5-*O*-sulphate in dogs. Further, the chromatographic properties reported by Haberland *et al.* (1957) closely resemble those of the 5-*O*-glucuronide in the dog. It appears likely that the same glucuronide of gentisic acid is formed by the dog and the rat. As with phenolic acids generally, the extent of glucuronide conjugation of gentisic acid is low. Williams (1959*b*) has pointed out, in studies of several phenolic acids in the rabbit, that with a dose of 0·3 g./kg. this conjugation may be 14–19% for phenolic acids of pK_a 3·9–4·5, whereas salicylic acid, pK_a 2·84, yields only 5% of this dose as a glucuronide. For gentisic acid (pK_a 3·1; Kleckner & Osol, 1952) glucuronide conjugation in the dog lies between these values. Although an appreciable proportion of the 5-*O*-glucuronide was found to exist in the lactone form on processing experimental urines, no metabolite having the reactions of this material was detectable in freshly voided urines, and the lactone is probably an artifact.

The isolation of the 5-*O*-sulphate confirms the various reports that gentisic acid is capable of this conjugation, and the increase of ethereal sulphate output after dosing is of the same order reported by Likhatscheff (1895). The decreased proportion of the etheral sulphate of gentisic acid formed with increased dose and the corresponding increase in glucuronide formation seem to be in agreement with the observed tendency for phenols of glucuronide output to be proportional to the dose, but of ethereal sulphate output to be limited by availability of SO_4^{2-} ion (Bray, Thorpe & White, 1952). The similarity in chromatographic properties between metabolites excreted by the dog and those excreted by the rat, referred to above, suggests that the ethereal sulphate excreted by the rat is also the 5-*O*-sulphate.

The chromatographic properties of 5-*O*-methylgentisic acid and salicylic acid are closely similar. The evidence of Sakamoto *et al.* (1959) in humans



Compound ...		L	M	K
(*a*)	63 %	28 %	7 %	Trace
(*b*)	2–7 %	Trace	Trace	

Scheme 1. Excretions by dogs of gentisic acid and conjugates, after a dose (0·19 g./kg.) of (*a*) gentisic acid or (*b*) acetylsalicylic acid.

and our findings in the dog suggest that the substance detected by Consden & Stanier (1951), and described by them as salicylic acid, was in fact 5-*O*-methylgentisic acid.

The rapid excretion and the almost complete recovery of ingested doses of gentisic acid from the urine appear to eliminate any possibility of a prolonged circulation after dosing, and there appears to be little likelihood of tissue storage. Although experiments with [*carboxy*-^{14}C]gentisic acid in rats show it to be widely disseminated in the organism soon after its ingestion (Crabtree *et al.* 1958), we have found (unpublished work) that prolonged feeding of rats with a diet containing 2 % of gentisic acid produced a negligible storage of gentisic acid in brain, liver, omental and perirenal fat.

Gentisic acid formed during salicylate metabolism is excreted partly unchanged, and partly as conjugates of uncertain structure (Williams, 1959*a*, p. 369). Haberland *et al.* (1957) have presented chromatographic evidence that one of these in the rat is a glucuronide identical with that excreted in much larger amounts after gentisic acid ingestion. The detection in dog urine of traces of the 5-*O*-glucuronide of gentisic acid after the ingestion of acetylsalicylic acid agrees with this evidence. The nature of a further conjugate is established by its isolation as the 5-*O*-sulphate of gentisic acid. Scheme 1 presents the metabolic fate of gentisic acid in the dog, whether administered or formed *in situ*, and it appears likely that the same metabolic pathways are involved.

SUMMARY

1. The fate of gentisic acid in the dog has been studied, and the metabolites were characterized.

2. A single oral dose of 0·19 g. of gentisic acid/kg. is rapidly excreted in the urine within 3 days; most of the excretion occurs on the first day after dosing. Of this dose, 63 % is excreted unchanged; increased *O*-sulphate output accounts for 28 % and *O*-glucuronide output for 7 % of the dose. Corresponding recoveries of a dose of 0·31 g./kg. are 61 % unchanged, 20 % as *O*-sulphate and 15 % of the dose as a glucuronide.

3. A single oral dose of 0·19 g. of sodium gentisate is similarly excreted, with 50 % of the dose excreted unchanged, increased *O*-sulphate output accounting for 30 %, and *O*-glucuronide output for 15 % of the dose.

4. The path of metabolism is apparently exclusively by conjugation at the 5-hydroxyl group.

5. The major metabolites were isolated and shown to be 3-carboxy-4-hydroxyphenyl sulphate and *O*-(3-carboxy-4-hydroxyphenyl)-β-D-glucosiduronic acid. Evidence for the formation of a lactone of the glucuronide, as an artifact, is given. A minor metabolite was shown to be 5-*O*-methylgentisic acid.

6. The conjugates of gentisic acid formed during acetylsalicylic acid metabolism in the dog were the 5-*O*-glucuronide and 5-*O*-sulphate of gentisic acid, and the latter has been isolated.

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A Galactofuranose Disaccharide from the Galactan of *Mycoplasma mycoides*

BY P. PLACKETT AND S. H. BUTTERY
Division of Animal Health, Animal Health Research Laboratory, C.S.I.R.O., Parkville, N. 2, Victoria, Australia

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Mycoplasma mycoides var. *mycoides*, the causal organism of contagious bovine pleuropneumonia, forms an immunologically specific polysaccharide, extractable with warm aqueous phenol (Buttery & Plackett, 1960). Although heterogeneous in immunodiffusion tests and in the ultracentrifuge, the preparations contained 90% by weight of anhydrogalactose in acid-labile combination. Periodate-oxidation data were consistent with a structure composed mainly of (1→4)-linked galactopyranose units, or of (1→5)- or (1→6)-linked galactofuranose units. The optical rotation, $[\alpha]_D$ $-140°$, indicated a high proportion of β-linkages. We have obtained further evidence for (1→6)-galactofuranose linkages by isolating the corresponding disaccharide from a partial acid hydrolysate.

EXPERIMENTAL

Materials

Mycoplasma mycoides galactan was prepared by extraction with warm aq. phenol (phenol–water, 6:1, w/w), and treated with Dowex 1 to remove RNA, as described by Buttery & Plackett (1960). A crude preparation of *Penicillium charlesii* polysaccharides was obtained from the extracellular fluid of cultures grown in Raulin–Thom medium (Haworth, Raistrick & Stacey, 1937). Lactitol and melibi-itol were obtained by reduction of the corresponding disaccharides with $NaBH_4$ in aqueous solution. 5-*O*-D-Galactofuranosylgalactitol was a gift from Dr P. A. J. Gorin.

Methods

Melting points are corrected.

Analytical methods. Total carbohydrate was determined by the anthrone method (Trevelyan & Harrison, 1952), and reducing sugar by the colorimetric procedure of Somogyi (1952), galactose being used as the standard.

Periodate consumption was determined spectrophotometrically at 230 mμ, freshly prepared solutions of $NaIO_4$ and $NaIO_3$ being used as standards.

For the determination of 1,2-glycols, samples (0·05–0·15 μmole in 0·25 ml.) were mixed with sodium metaperiodate (0·15 ml. of 0·1 M solution) and kept at 30° for 7–8 min. Sodium metabisulphite (6%, w/v, in N-H_2SO_4), 0·25 ml., was added to destroy excess of periodate, after which formaldehyde was determined with chromotropic acid reagent according to Hanahan & Olley (1958). Erythritol was used as a standard. Blanks, in which the metabisulphite reagent was added before the periodate, were also run. Many carbohydrate substances gave appreciable colour under these conditions. The blank extinctions were subtracted from the values obtained after periodate oxidation.

Paper chromatography. Whatman no. 1 paper was used with the following solvent systems: (A) butan-1-ol–pyridine–water (6:4:3, by vol.); (B) butan-1-ol–ethanol–water (40:11:19, by vol.); (C) butan-1-ol–acetic acid–water (5:2:3, by vol.); (D) water-saturated phenol (in the presence of aq. NH_3 soln. and KCN).

Reducing sugars and polyols were detected with $AgNO_3$ (Dedonder, 1952), reducing sugars with aniline hydrogen phthalate (Cummins & Harris, 1956) and with aniline–diphenylamine–phosphoric acid (Bailey & Bourne, 1960).