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Decarboxylation and demethylation of some phenolic benzoic acid derivatives by rat caecal contents

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Rat caecal contents decarboxylate phenolic benzoic acid derivatives when a free hydroxyl group is in the *para* position but the presence of substituents adjacent to this group or the carboxyl group reduce or abolish the reaction. Compounds containing a hydroxyl group in the *ortho* or *meta* position but lacking one in the *para* position are not decarboxylated. Some methoxy-derivatives are demethylated. The possible relationship between these findings and urinary phenols is discussed.

URINARY pyrogallol (1,2,3-trihydroxybenzene) is probably derived from gallic acid (3,4,5-trihydroxybenzoic acid) by decarboxylation in the alimentary tract (Tompsett, 1958). Booth, Masri, Robbins, Emerson, Jones & DeEds (1959) reported that rabbits fed a diet containing gallic acid excreted pyrogallol, which was isolated from the acid-hydrolysed urine (see also Watanabe & Oshima, 1965). That the decarboxylation may occur in the alimentary tract is supported by the finding of Booth & Williams (1963) that protocatechuic acid (3,4-dihydroxybenzoic acid) is decarboxylated to catechol (1,2-dihydroxybenzene) by rat faecal and caecal extracts. Gallic acid and protocatechuic acid are partially decarboxylated in the rat when given orally but not when given intraperitoneally (Scheline, 1966). Decarboxylation also occurs when these acids are incubated with extracts of rat intestinal contents or faeces. Since treatment of the rats or the incubation mixtures with oxytetracycline or neomycin greatly reduced or abolished the reaction it was concluded that the decarboxylation of gallic acid and protocatechuic acid was effected by the intestinal microflora.

Other phenolic benzoic acid derivatives might also be decarboxylated. The present report describes a study of the decarboxylation and also the demethylation of some benzoic acid derivatives by the rat caecal microflora.

Experimental

COMPOUNDS

1,3-Dihydroxy-2-methoxybenzene (Schöpf & Winterhalder, 1940), 3-hydroxy-4-methoxybenzoic acid (Perkin & Stoyle, 1923), 3,4-dihydroxy-5-methoxybenzoic acid (Jurd, 1959) and 3,5-dihydroxy-4-methoxybenzoic acid (Geissman & Mojé, 1951) were prepared. Other compounds were obtained commercially. The compounds were checked for purity chromatographically and recrystallised if required.

METHODS

The incubation medium used consisted of 0·5% yeast extract (Difco) and 0·5% peptone (Difco) in 0·1M phosphate buffer (pH 7·4). The

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medium (10 ml) in a 15 × 150 mm test tube was placed in a boiling water-bath for 15 min. The test substances were dissolved in water with the aid of a minimum amount of solid sodium bicarbonate to give a concentration of 10 mg/ml. This solution (1 ml) was added to the cooled medium. Caeca were obtained from adult male albino rats fed a diet obtained from Felleskjöpet, Oslo. The entire caecal contents were well mixed with five volumes of medium and the resulting suspension centrifuged at low speed. The supernatant liquid (1 ml) was added to the sample tube, which was then flushed with nitrogen, stoppered, mixed by inverting a few times and incubated at 37° for 22 hr. The incubation mixture was then acidified with concentrated hydrochloric acid (1 ml) and extracted twice with 25-ml portions of ether. The ether extract was dried over anhydrous sodium sulphate, evaporated to dryness and the residue dissolved in acetone (1 ml). Controls were prepared similarly except that the caecal extract was omitted.

TABLE 1. THIN-LAYER CHROMATOGRAPHY AND COLOUR REACTIONS OF SOME PHENOLIC COMPOUNDS

Compound		Rf		Colour with:	
Chemical name	Trivial name(s)	Solvent 1	Solvent 2	Fast blue B salt	Diazotised sulphanilamide
2-Hydroxybenzoic acid	Salicylic acid	0·94	0·49	Pale yellow-brown	Pale yellow-brown
3-Hydroxybenzoic acid	—	0·35	0·45	Orange	Yellow
4-Hydroxybenzoic acid	—	0·35	0·46	Orange-brown	Yellow-orange
2,3-Dihydroxybenzoic acid	α-Pyrocatechuic acid	0·35	0·42	White-brown	Pink-tan
2,4-Dihydroxybenzoic acid	β-Resorcylic acid	0·24	0·31	Violet	Yellow
2,5-Dihydroxybenzoic acid	Gentisic acid	0·16	0·43	White-grey	Pale-tan
2,6-Dihydroxybenzoic acid	γ-Resorcylic acid	0·18	0·38	Red-brown	Yellow
3,4-Dihydroxybenzoic acid	Protocatechuic acid	0·06	0·34	White-tan	Pink-tan
3,5-Dihydroxybenzoic acid	α-Resorcylic acid	0·02	0·33	Red-purple	Grey-yellow
2,3,4-Trihydroxybenzoic acid	—	0·05	0·28	Pale-brown	Pale-brown
3,4,5-Trihydroxybenzoic acid	Gallic acid	0·00	0·28	Pink-brown	Green-brown
2,4,6-Trihydroxybenzoic acid	—	0·02	0·21	Purple	Yellow-brown
3-Hydroxy-4-methoxybenzoic acid	Isovanillic acid	0·56	0·33	Pink-brown	Orange
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	0·79	0·37	Pink-brown	Orange
3,4-Dihydroxy-5-methoxybenzoic acid	3-*O*-Methylgallic acid	0·10	0·29	White-grey	Pink-grey
3,5-Dihydroxy-4-methoxybenzoic acid	4-*O*-Methylgallic acid	0·07	0·40	Red-purple	Brown-yellow
3,5-Dimethoxy-4-hydroxybenzoic acid	Syringic acid	0·75	0·31	Pink-brown	Red
Phenol	—	0·88	0·69	Red-orange	Yellow-orange
1,2-Dihydroxybenzene	Catechol, pyrocatechol	0·30	0·62	Pink-grey	Pink-grey
1,3-Dihydroxybenzene	Resorcinol	0·11	0·56	Red-purple	Grey-yellow
1,4-Dihydroxybenzene	Hydroquinone, quinol	0·10	0·63	Grey-brown	Brown
1,2,3-Trihydroxybenzene	Pyrogallol	0·04	0·55	Pink-brown	Brown
1,3,5-Trihydroxybenzene	Phloroglucinol	0·00	0·42	Purple	Yellow-brown
2-Methoxyphenol	Guaiacol	0·98	—	Violet	Orange
1,2-Dihydroxy-3-methoxybenzene	1-*O*-Methylpyrogallol	0·54	0·53	White-grey	Pink-grey
1,3-Dihydroxy-2-methoxybenzene	2-*O*-Methylpyrogallol	0·43	0·63	Violet	Grey-yellow
2,6-Dimethoxyphenol	—	0·96	0·54	Purple	Red

Solvent 1: benzene - glacial acetic acid - H_2O (6:7:3, upper layer); Whatman CC.41 cellulose.
Solvent 2: 20% aqueous potassium chloride - glacial acetic acid (100:1); Macherey, Nagel & Co. MN 300 cellulose.

CHROMATOGRAPHY

The above acetone solutions, together with appropriate standards, were examined by thin-layer chromatography on 0·5-mm thick layers of cellulose [CC.41 (Whatman) or MN 300 (Macherey, Nagel and Co.)]. Rf values and colour reactions of the phenolic compounds are shown in Table 1. They were detected by spraying with fast blue B salt and diazotised sulphanilic acid solutions. Whatman CC.41 cellulose was used with solvent 1, as it was found to give sharper separations. Salicylic acid gave a very pale colour with the two spray reagents but was readily detected with ultraviolet light (254 mμ).

Results and discussion

The main findings are summarised in Table 2. Decarboxylation occurred only when a free hydroxyl group was present in the *para* position. This is best shown by 4-hydroxybenzoic acid, which was decarboxylated to phenol under these conditions. Initial experiments using 0·1M

TABLE 2. METABOLISM OF PHENOLIC BENZOIC ACID DERIVATIVES BY RAT CAECAL EXTRACTS

Compound	No. of experiments	Decarb-oxylation	Demethyla-tion	Observations
2-Hydroxybenzoic acid	5*	—**	...	—
3-Hydroxybenzoic acid	4	—	...	—
4-Hydroxybenzoic acid	6	+++	...	Trace of unchanged compound in 2/6, none in 4/6. Large amounts of phenol in 6/6.
2,3-Dihydroxybenzoic acid	4	—	...	—
2,4-Dihydroxybenzoic acid	5	—†	...	—
2,5-Dihydroxybenzoic acid	4	—	...	—
2,6-Dihydroxybenzoic acid	4	—	...	—
3,4-Dihydroxybenzoic acid	5	+++	...	Unchanged compound in 4/5. Large amounts of catechol in 5/5.
3,5-Dihydroxybenzoic acid	4	—	...	—
2,3,4-Trihydroxybenzoic acid	4	—	...	—
3,4,5-Trihydroxybenzoic acid	8	+++	...	Unchanged compound in 4/8. Pyrogallol in 5/8. Resorcinol in 7/8.
2,4,6-Trihydroxybenzoic acid	2	—†	...	—
3-Hydroxy-4-methoxybenzoic acid	6	—†	++	Unchanged compound in 6/6. 3,4-Dihydroxybenzoic acid in 2/6. Catechol in 6/6.
4-Hydroxy-3-methoxybenzoic acid	6	++	++	Unchanged compound in 6/6. Guaiacol in 5/6. 3,4-Dihydroxybenzoic acid in 3/6. Catechol in 6/6.
3,4-Dihydroxy-5-methoxybenzoic acid	5	+	+	Unchanged compound in 5/5. 1-*O*-Methylpyrogallol in 4/5. Resorcinol in 4/5.
3,5-Dihydroxy-4-methoxybenzoic acid	5	—†	+	Unchanged compound in 5/5. Pyrogallol in 1/5. Resorcinol in 4/5.
3,5-Dimethoxy-4-hydroxybenzoic acid	5	—†	+	Unchanged compound in 5/5. 3-*O*-Methylgallic acid in 5/5, Resorcinol in 1/5.

* Equal to number of rats used.
** Symbols: —none, +minor, ++moderate, +++ extensive, ... not applicable.
† See text.

phosphate buffer showed that the reaction was quantitative over the pH range of 6·6–8·0 but fell to 54% at pH 6·2 and 9% at pH 5·8. Other examples of this decarboxylation to the corresponding phenol are seen

with protocatechuic acid, vanillic acid (4-hydroxy-3-methoxybenzoic acid) and 3-*O*-methylgallic acid (3,4-dihydroxy-5-methoxybenzoic acid), which were largely or partly metabolised to catechol, guaiacol and 1-*O*-methylpyrogallol, respectively.

With gallic acid, decarboxylation gave rise to pyrogallol, which was found in most of the samples. However it was sometimes absent and in these instances large amounts of resorcinol were observed on the chromatograms. Dehydroxylation to resorcinol was also seen when pyrogallol itself was incubated with the caecal extract. Pyrogallol was not dehydroxylated to catechol in these experiments. The other trihydric phenols were similarly studied and it was found that hydroxyquinol (1,2,4-trihydroxybenzene) but not phloroglucinol (1,3,5-trihydroxybenzene) was partly dehydroxylated to resorcinol. None of the dihydric phenols (catechol, resorcinol and quinol) were dehydroxylated by the caecal extracts.

The extent of resorcinol formation in the gallic acid experiments was variable and this may reflect differences in the intestinal microflora of the rats used. This variation has also been seen in *in vivo* experiments where a conjugate of resorcinol was found in the urine of some but not all rats given gallic acid orally (Scheline, 1966). A similar finding in man has been reported by Curzon (1957) and Curzon & Pratt (1964) who found that resorcinol sulphate was excreted by some subjects. They suggested that it originated from the action of particular intestinal bacteria on dietary tea polyphenols.

Several of the acids containing a free hydroxyl group in the *para* position underwent little or no decarboxylation and it appears that certain substituents adjacent to either this group or the carboxyl group greatly reduce this reaction. Although decarboxylation was not appreciably affected by adjacent hydroxyl groups as in protocatechuic acid and gallic acid, methoxyl groups adjacent to the hydroxyl group in the *para* position as in vanillic acid, 3-*O*-methylgallic acid and syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), reduced or abolished decarboxylation. Thus moderate amounts of guaiacol were formed from vanillic acid and small amounts of 1-*O*-methylpyrogallol were formed from 3-*O*-methylgallic acid, but decarboxylation did not occur with syringic acid in which two methoxyl groups are adjacent to the hydroxyl group in the *para* position.

The inhibitory effect of substituents adjacent to the carboxyl group was even more pronounced and only the unchanged compound was found in the incubation mixtures containing 2,3,4-trihydroxybenzoic acid. Small amounts of resorcinol were formed from 2,4-dihydroxybenzoic acid but this was not due to the caecal extract, as decarboxylation occurred to the same extent in control samples without caecal extract. This spontaneous decarboxylation was also seen with 2,4,6-trihydroxybenzoic acid, as phloroglucinol was seen in equal amounts in the incubation mixtures with and without caecal extract. Instability of the other compounds was not encountered.

All five of the methylated derivatives in Table 2 underwent demethylation to some extent when incubated with the caecal extracts. Vanillic acid was partly decarboxylated to guaiacol and partly demethylated to

protocatechuic acid, which was then decarboxylated to catechol. Isovanillic acid (3-hydroxy-4-methoxybenzoic acid) was not decarboxylated, as no guaiacol was detected. However, it was partly demethylated but less so than vanillic acid. The presence of a carboxyl group in the molecule was not essential for demethylation as guaiacol was metabolised to catechol to a small extent.

The resorcinol in the incubation mixtures containing 3-*O*-methyl- or 4-*O*-methyl-gallic acid can be accounted for by their demethylation to gallic acid, which would be decarboxylated to pyrogallol and finally dehydroxylated to resorcinol. As demethylation is a minor reaction with these compounds, it is not surprising that the intermediates were usually not detected. Resorcinol could arise similarly from syringic acid, via 3-*O*-methylgallic acid.

The demethylation of several non-phenolic methoxy-acids was examined using *o*-anisic acid (2-methoxybenzoic acid), *m*-anisic acid (3-methoxybenzoic acid), anisic acid (4-methoxybenzoic acid), veratric acid (3,4-dimethoxybenzoic acid) and 3,4,5-trimethoxybenzoic acid. Except when veratric acid was used, no phenolic metabolites were detected when any of these components were incubated with the caecal extracts. Veratric acid was demethylated to a small extent at both the *meta*- and *para*-positions, as traces of vanillic acid were found in three, and traces of isovanillic acid in two of six samples.

The decarboxylation of 4-hydroxybenzoic acid, protocatechuic acid and gallic acid was prevented when oxytetracycline was added to the incubation mixtures at a level of 4 μg/ml.

The present results demonstrate the ability of rat intestinal microflora to decarboxylate and demethylate benzoic acid derivatives. The significance of this effect on the metabolism of such compounds when ingested by man or animals remains to be seen, although two of the compounds, protocatechuic acid and gallic acid, have already been found to be partially decarboxylated when administered orally to rats (Scheline, 1966). The finding by Booth & others (1959) and Watanabe & Oshima (1965) that pyrogallol is excreted in the urine by rabbits given gallic acid orally, indicates that decarboxylation also occurs in this species, possibly by the intestinal flora. Harborne & Simmonds (1964) have stated that many of the simple phenolic acids, including 4-hydroxybenzoic acid, protocatechuic acid and vanillic acid, are widely distributed in plants. These compounds are therefore normal dietary components for man and many animals. From the evidence now available it seems reasonable to assume that decarboxylation and demethylation of phenolic compounds by the intestinal microflora can explain the presence of some of the phenols normally excreted in urine (see Williams, 1959). Scheline (1966) has suggested that decarboxylation of protocatechuic acid contained in plant material is responsible for some of the catechol found in the urine of animals. The present results indicate that vanillic acid may also be a precursor of urinary catechol and suggest that guaiacol may be expected in urine. Urinary phenol is thought to arise from tyrosine in the gastrointestinal tract by the action of bacteria (Bray & Thorpe, 1954; Rogers, Burdick

& Burnett, 1955). The finding that 4-hydroxybenzoic acid is readily decarboxylated to phenol by the caecal microflora indicates that another pathway for phenol formation is available, although the relative importance of these two routes remains to be determined.

Diet can play an important role in influencing the nature of the intestinal flora, for rats fed on different diets showed considerable differences in the types and numbers of micro-organisms found in the alimentary tract (Smith, 1965). Diet could, therefore, influence the pattern of urinary metabolites of those compounds which are susceptible to metabolism by intestinal microflora.

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