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Gut flora and the origin of some urinary aromatic phenolic compounds

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Abstract—The concentration of several phenolic acids and alcohols was measured in urine from germ-free and specific pathogen-free (SPF) rats before and after inoculation with faecal microorganisms, and from conventional rats before and after gut sterilization. The rate of excretion of benzoic acid, phenylacetic acid, and *m*- and *p*-hydroxyphenylpropionic acid in the germ-free animals was markedly increased after inoculation. Some acids showed no increase, including the endogenously generated homovanillic, vanilmandelic and *p*-hydroxyphenyllactic acids. Most others sought showed a small but significant increase. Some of the compounds excreted by the germ-free animals may have been in the food pellets, either as such or as precursors. The pattern was somewhat different in the SPF rats. The excretion of *p*-hydroxyphenylpropionic, *p*-hydroxyphenylacetic and *m*-hydroxyphenylacetic acids was initially much higher than in the germ-free animals and their excretion decreased after inoculation, presumably because of an altered pattern of gut flora. This work quantifies the effect of gut flora in the formation of some of the more important phenolic acids found in rat urine.

Key words: rat; germ-free animals; conventional animals; phenolic acids; metabolism

During a study on the urinary excretion of free and conjugated phenylacetic acid in man [1], it became apparent that the total output was much higher than what might have been expected had the compound arisen from endogenous sources alone. Although the finding pointed to most of the urinary phenylacetic acid being dietary in origin, it also raised the question as to what extent the gut flora are responsible for the production of this and related phenolic compounds from ingested material.

Gut flora interact with a variety of systems in mammalia, both directly and indirectly. Studies with germ-free animals, for instance, have demonstrated altered metabolic patterns of certain xenobiotics [2, 3], and endogenous metabolites [4, 5] and changed activity of the enzyme, monoamine oxidase [6, 7]; whether generation of large amounts of the gut flora derived monoamine oxidase inhibitor, isatin [8] may play any part in the latter effect has still to be evaluated.

Because the situation is complex and the literature unclear, experiments were undertaken in the rat in an attempt to determine the contribution made by intestinal microorganisms to the formation of phenylacetic acid and a number of phenolic metabolites. SPF* rats were used in addition to germ-free rats; their gut flora pattern differs from that in conventional rats in that certain pathogenic flora are not present. These animals might be expected to excrete a different pattern of urinary metabolites from conventional rats as a result of different metabolic pathways in various microorganisms, and hence give a further check on the influence of gut flora.

Materials and Methods

Animals used were conventional female Wistar rats, male Liverpool hooded germ-free and SPF rats of about 200 g weight. The germ-free rats were bred at the MRC Unit at Carshalton, Surrey, U.K. All except the conventional rats were fed Oxoid Breeding Diet, sterilized by γ-irradiation.

Urine samples (24 hr) were collected from animals kept in metabolism cages without food during the collection period, but with water supplied *ad libitum*, into bottles containing 0.5 mL 6 M HCl, and stored at −20° until assay. Conventional rats were then treated with 30 mg of neomycin and 0.5 mg phthalylsulphathiazole per day added to their diet and 24 hr urine samples were again collected on the fourth day. Prior to the second collection, germ-free rats were fed for 2 weeks on diet contaminated with sufficient faeces from SPF rats to ensure rapid colonization of the gut with flora found in SPF rats, and the SPF rats on diet contaminated with faeces from conventional rats.

Urine was assayed for phenylacetic acid, benzoic acid, phenolic acids and phenylglycols by methods previously described [1, 9–12]. Benzoic acid and phenylacetic acid were released from amino acid conjugates by hydrolyzing urine aliquots in 6 M HCl at 100° for 2 hr. Conjugates of phenolic alcohols were hydrolyzed by incubation overnight with a sulphatase-β-glucuronidase ("Glusulase") preparation. Standards were added to separate aliquots which were taken through the extraction and derivatization procedures.

Acids and alcohols were extracted from urine into ethyl acetate and evaporated to dryness. The extracts were evaporated to dryness *in vacuo* and the compounds sought were derivatized to yield volatile derivatives (pentafluorobenzyl esters, ethyl ester-TMS ethers or PFP esters for benzoic and phenylacetic acids, phenolic acids and alcohols respectively). Analysis was carried out by GC using a 100 m × 0.5 mm bore capillary column coated with SE30/OV25 (4 : 1) with flame ionization or electron capture

Table 1. Urinary excretion of phenylacetic acid by conventional rats before and after gut sterilization by oral treatment with 30 mg of neomycin and 0.5 mg phthalylsulphathiazole/day for 4 days

	Before	After
Free acid	0.189 ± 0.077	0.057 ± 0.014
Conjugated acid	31.3 ± 4.9	10.5 ± 3.8

Results (means ± SD; μmol/24 hr) are the means of six animals.

* Abbreviations: GC, gas chromatography; MS, mass spectrometry; PFP, pentafluoropropionyl; SPF, specific pathogen-free; TMS, trimethylsilyl.

Table 2. Urinary excretion of phenolic acids and alcohols by germ-free rats and the effect of gastric inoculation with gut flora (faeces) from SPF rats for 2 weeks

Compound	Before inoculation	After inoculation	P
o-Hydroxyphenylacetic acid	60 ± 22	126 ± 45	<0.001
m-Hydroxyphenylacetic acid	70 ± 16	480 ± 166*	<0.001
p-Hydroxyphenylacetic acid	1380 ± 760	2320 ± 860	<0.01
Homovanillic acid	140 ± 41	139 ± 28	>0.1
p-Hydroxymandelic acid	171 ± 55	220 ± 59	<0.05
m-Hydroxyphenylpropionic acid	25 ± 22	3520 ± 5150*	<0.001
p-Hydroxyphenylpropionic acid	137 ± 15	760 ± 1520	<0.05
p-Hydroxyphenyllactic acid	290 ± 90	320 ± 90	>0.1
m-Hydroxyphenylhydracrylic acid	116 ± 42	133 ± 70†	>0.1
Benzoic acid	2460 ± 3030‡	12,200 ± 7050‡	<0.001
Phenylacetic acid	5070 ± 2060‡	26,300 ± 6300‡	<0.001
p-Hydroxyphenylglycol	10.8 ± 1.8§	26.0 ± 5.1§	<0.005
4-Hydroxy-3-methoxyphenylglycol	121 ± 14§	146 ± 13§	>0.1

Results are given as nmol/24 hr. Each result represents the mean of 12 animals except where indicated: * = 11; and † = 9.

‡ After acid hydrolysis; all other results for acids represent unconjugated compounds. §After enzymatic hydrolysis.

P values were obtained by Student's *t*-test, using a suitable transformation where necessary.

detection, except for benzoic and phenylacetic acids which were measured by GC/MS with selected ion monitoring.

Results and Discussion

The effect of gut sterilization on phenylacetic acid excretion is shown in Table 1. Both free and conjugated acid excretion were decreased by about 70% after treatment, which indicates that the greater proportion was formed by the action of gut flora on dietary components. This finding was confirmed in the germ-free rats, where phenylacetic acid excretion was increased 5-fold after inoculation with faeces (Table 2). A similar pattern was observed with benzoic acid. Statistical analysis was carried out by means of Student's *t*-test. In cases where pairs of results were skewed or showed large differences in standard deviations a suitable (eg. log) transformation was carried out to eliminate as far as possible these effects prior to analysis.

The origin of these two compounds is uncertain. Phenylacetic acid may be generated by gut flora from phenylalanine; the formation of phenylpyruvic acid, phenethylamine and phenylacetamide from phenylalanine by microorganisms has been extensively studied [eg. Refs 13–15]; these are all potential precursors of phenylacetic acid. Benzoic acid may derive from phenylalanine, perhaps via cinnamic acid [16, 17].

In SPF rats, the results for phenylacetic acid output were equivocal (not shown). One batch of six animals exhibited a high excretion of phenylacetic acid, whereas a second failed to do so. The first batch excreted less after ingestion of faeces, presumably because of a change in gut flora.

The relatively large output of benzoic and phenylacetic acids in germ-free animals led us to suspect that these compounds might be present in the diet; and, indeed, 20 μg of benzoic acid and 25 μg of phenylacetic acid were found per g of feed. These values indicate that most of the phenylacetic acid and perhaps all the benzoic acid excreted by this group of rats derived from the diet.

Almost all the *m*-hydroxyphenylpropionic acid output stems from the action of gut flora (Table 2). The normal pathway for its formation involves reduction and *para*-dehydroxylation of caffeic acid, which does not occur in the germ-free rat [18]. Caffeic acid, usually as its conjugate, chlorogenic acid, is found in many plants. The unexpectedly high level of excretion in SPF rats (Table 3) was lowered after inoculation with faeces.

Table 3. Urinary excretion of phenolic acids in SPF rats and the effect of gastric inoculation with gut flora (faeces) from conventional rats for 2 weeks

Compound	Before inoculation	After inoculation	P
o-Hydroxyphenylacetic acid	94 ± 52*	105 ± 44*	>0.1
m-Hydroxyphenylacetic acid	50 ± 23*	600 ± 220	<0.001
p-Hydroxyphenylacetic acid	3550 ± 2170	2170 ± 1850	>0.05
Homovanillic acid	110 ± 22*	164 ± 38*	<0.001
p-Hydroxymandelic acid	182 ± 32*	208 ± 36*	>0.05
m-Hydroxyphenylpropionic acid	4040 ± 2230	2080 ± 1020	<0.005
p-Hydroxyphenylpropionic acid	900 ± 390	220 ± 70	<0.001
p-Hydroxyphenyllactic acid	290 ± 90*	290 ± 60*	>0.1

Results (unconjugated acids) are given in nmol/24 hr; mean ± S.D.

Each result represents the mean of 12 animals except where indicated: * = 11.

P values were obtained by Student's *t*-test, using a suitable transformation where necessary.

p-Hydroxyphenylpropionic acid is excreted to a significant extent only after the establishment of gut flora. Its source is somewhat uncertain, but it may derive, in part, from caffeic acid by *meta*-dehydroxylation [19], or from plant materials structurally or biochemically related to flavonoids.

m-Hydroxyphenylacetic acid excretion increased sharply in both groups of animals following faecal inoculation. It can be formed from 3,4-dihydroxyphenylacetic acid by gut microflora [19], or from quercetin and a range of related flavonoids [20]. In man, substantial amounts may be generated by the action of gut flora on L-dopa, during the treatment of Parkinson's disease [21].

The increase in *p*-hydroxyphenylacetic acid excretion following the establishment of gut flora in germ-free rats probably reflects formation of tyramine from tyrosine by microorganisms [22]. The extent to which tyramine is formed endogenously has been a subject of controversy. Trace amounts are formed, probably, from β-phenylethylamine in brain [23]. However, the possible action of mammalian L-aminoacid decarboxylase on L-tyrosine has proved difficult to establish, because of the extensive decarboxylation of this amino acid by gut flora [22]. Early studies on the mammalian decarboxylase suggested that L-tyrosine was a substrate [24]. Doubts were soon cast on this claim. Both *in vivo* and *in vitro* studies using labelled tyramine failed to demonstrate any decarboxylation [25, 26]. Even now the controversy is not entirely cleared up. For instance, it has recently been claimed [27] that rat kidney decarboxylase will, under extreme conditions, catalyse this reaction, albeit slowly. Most of the excreted *p*-hydroxyphenylacetic acid in germ-free animals probably, in fact, arises by decarboxylation and oxidation of *p*-hydroxyphenylpyruvic acid, the first metabolic product of tyrosine in the pathway that leads to the degradation of this amino acid to CO_2 and water.

The presence of *o*-hydroxyphenylacetic acid in germ-free animals may derive from *o*-tyramine, which is a known component of rat urine [28] and is probably formed in the adrenal gland [29]. Phenylpyruvic acid is another potential source [30]. Coumarin, a possible dietary component, may also be a precursor [31, 32].

p-Hydroxyphenyllactic acid, a reduction product of *p*-hydroxyphenylpyruvic acid, is not formed by gut flora.

Homovanillic acid, which is generated endogenously from dopamine, may be produced by gut flora, but the evidence is equivocal; the small increase in excretion following the inoculation of SPF rats was not observed in germ-free animals.

m-Hydroxyphenylhydracrylic acid is a metabolite of caffeic acid, and gut flora play an important part in its formation. It is therefore surprising that its excretion did not increase after inoculation of germ-free rats. Its presence in germ-free animals may point to its formation from *m*-coumaric acid. This result is unexpected because *m*-hydroxyphenylpropionic acid excretion is greatly enhanced after inoculation, and caffeic acid is the likely common precursor of both compounds. The enzyme for hydration of cinnamic acid double bonds may not, therefore, be very active in the rat.

p-Hydroxymandelic acid excretion is increased slightly after inoculation with faeces, but only at borderline significance. This increase is probably real; *p*-hydroxyphenylglycol is also a metabolite of octopamine and *p*-sympatol, and the excretion of this alcohol is significantly increased, whereas the output of 4-hydroxy-3-methoxyphenylglycol, a major catecholamine metabolite, remains unchanged. Tyramine, generated by gut flora from tyrosine, is the probable precursor; this would then be oxidized to octopamine by dopamine-β-hydroxylase.

These experiments have demonstrated the importance of gut flora in generating urinary aromatic acids and some alcohols in the rat. It is noteworthy that some of the compounds do not have any significant input from gut flora, in particular those that are end-point metabolites of the catecholamine hormones, or, as in the case of *p*-hydroxyphenyllactic acid, they arise from a major endogenous metabolic pathway. Others which are seen traditionally to be metabolites of dietary compounds are, by and large, generated by the action of gut flora, including phenylacetic acid which probably originates from phenylalanine, and others that are known to arise by as yet unstudied enzymes that bring about dehydroxylation of phenolic acids. None of these is absent from urine of germ-free animals; they may be present in food *per se*, or there may be hitherto undescribed pathways by which they may be formed in relatively small amounts, perhaps from dietary materials.

The origin of compounds generated by gut flora is only partially understood. It is possible that some of these compounds may be implicated in the health of the animals.

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