Studies on Flavonoid Metabolism

METABOLISM OF (+)-CATECHIN IN THE GUINEA PIG

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1. Administration of (+)-catechin to the guinea pig gives rise to a number of phenolic acids and lactones, which have been identified by chromatographic and spectrophotometric methods. The major phenolic acid metabolite is *m*-hydroxybenzoic acid and the major lactone metabolite is δ -(3-hydroxyphenyl)- γ -valerolactone. 2. The phenolic acid and lactone metabolites are excreted in both free and conjugated forms, including their glucuronides and to a lesser degree their ethereal sulphates. 3. Administration of certain of the metabolites isolated has permitted certain sequential relationships of these intermediates to be established. 4. Degradation of (+)-catechin in the guinea pig is effected at least in part by the gut microflora and is suppressed by aureomycin plus phthaloyl-sulphathiazole.

The main phenolic acid metabolites of (+)-catechin in the rat are *m*-hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid (Griffiths, 1964). In the rabbit, however, the metabolites are vanillic acid, *m*-hydroxybenzoic acid and protocatechuic acid together with certain phenyl- γ -valerolactones (Oshima, Watanabe & Isakari, 1958; Oshima & Watanabe, 1958; Watanabe, 1959*a,b*). In view of these species differences it was of interest to examine the pattern of (+)-catechin metabolism in the guinea pig. Gero (1946) reported that 65% of a dose of epicatechin injected into guinea pigs intraperitoneally was excreted unchanged, but the urine was not examined for breakdown products.

EXPERIMENTAL

Animals. Male guinea pigs (approx. 300g.) were used. The animals were housed in separate metabolism cages, designed to permit the collection of urine separately from faeces.

Diet. Animals were given the diet described by Das & Griffiths (1966) at 24 hr. intervals during the period of urine collection, but unrestricted water intake was allowed throughout the experimental period.

(+)-Catechin. This was obtained from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Since the m.p. of (+)-catechin is influenced by the conditions of crystallization and drying (Hergert & Kurth, 1953), the (+)-catechin was carefully recrystallized under standard conditions (Das & Griffiths, 1967). The crystals obtained possessed m.p. 176–177°, in agreement with that reported by Perkin

& Yoshitake (1902). The compound was shown to be chromatographically pure and the infrared spectrum was identical with that of an authentic specimen of (+)-catechin.

Collection and extraction of urine. These procedures were as described by Griffiths (1964).

Colorimetric determination of phenolic acids. This was carried out by the method of Griffiths (1964), but extinctions were measured at 660 m μ in a Hilger spectrophotometer.

Spectra. Ultraviolet spectra were determined in ethanol in a Bausch and Lomb Spectronic 505 recording spectrophotometer. Infrared spectra were determined either in KBr disks or in Nujol mull, in a Unicam SP.200 spectrophotometer.

Determination of glucuronides. The method of Hanson, Mills & Williams (1944) as modified by Paul (1951) was used. A standard curve with a range of 0–80 μ g. of glucuronide/2 ml. was prepared on each occasion. Measurements of the extinction at 565 m μ were made in a Hilger spectrophotometer.

Determination of ethereal sulphate. The turbidimetric method of Sperber (1948), with a standard curve of 0.05–0.8 mg. of SO₃/10 ml., was used. Turbidity was measured on a Hilger spectrophotometer at 605 m μ .

Paper chromatography. Whatman no. 1 paper was employed for normal qualitative chromatography, but, for the separation of larger quantities of metabolites by band chromatography, either Whatman 3MM or Whatman 17MM paper was used. The paper was pre-washed by the procedure of Griffiths (1964) where indicated. The solvent mixtures were: *A*, propan-2-ol–aq. NH₃ (sp.gr. 0.88)–water (8:1:1, by vol.); *B*, benzene–acetic acid–water (6:7:3, by vol.); *C*, chloroform–acetic acid–water (2:1:1, by vol.); *D*, aq. 20% (w/v) KCl; *E*, aq. 2% (v/v) acetic acid; *F*, water-saturated

benzene-acetic acid (9:1, v/v); *G*, butan-1-ol-acetic acid-water (4:1:5, by vol.); *H*, phenol-water (73:27, w/v). The spray reagents were described by Griffiths (1964).

Conditions for enzymic hydrolysis. (a) Glucuronidase. To 30 ml. of ether-extracted urine, sufficient NaH_2PO_4 was added to give a final concentration of 0.2M, and the pH was adjusted to 4.6. To one-half of the urine was added 3000 Fishman units of β -glucuronidase (EC 3.2.1.31; molluscan; Koch-Light Laboratories Ltd., Colnbrook, Bucks.). The other half of the urine served as control. The urine samples were incubated for 24 hr. at 37° (Elliott, Parke & Williams, 1959).

(b) Arylsulphatase. Each urine sample (30 ml.) was freed from inhibitory ions by the method of Dodgson & Spencer (1953). Saccharic acid (0.1M, final concentration) was added to each treated urine sample and the pH was adjusted to 5. To one half of the urine 12.6 units of arylsulphatase (EC 3.1.6.1; type II from limpets; Sigma Chemical Co., St Louis, Mo., U.S.A.) was added. The other half served as control. The urine was carefully adjusted to pH 2.25 with conc. HCl, kept for 5 min. and then readjusted to pH 5.0 with 2N-NaOH and incubated for 24 hr. at 37°.

Acid hydrolysis of conjugates in urine. Conc. HCl (5 ml.) was added to 10 ml. of urine and the mixture was heated under reflux in a boiling-water bath for 3 hr.

Isolation of valerolactone derivatives. Six guinea pigs maintained on the standard diet were each given 100 mg. of (+)-catechin daily for 6 days. The urine samples collected on 7 consecutive days were hydrolysed with HCl and then extracted continuously with ether for 24 hr. The pooled concentrated extracts were applied as bands to eight sheets of Whatman 3MM paper and developed in solvent *C* overnight. The appropriate band was eluted with methanol, and the eluate was concentrated and applied to a second series of six sheets of pre-washed Whatman 3MM paper, which were then developed with solvent *B*. The appropriate bands were eluted with methanol and concentrated to 5 ml. Water (15 ml.) was added, whereupon the lactones separated as crystalline precipitates. These were recrystallized twice from ethanol-benzene (1:9, v/v) and dried for 6 hr. under reduced pressure at 60° over P_2O_5 . The crystalline compounds were characterized by comparison of their infrared- and ultraviolet-absorption spectra, melting points and chromatographic properties with those of the authentic compounds as indicated in the text.

RESULTS

Phenolic constituents of urine. Use of the standard diet resulted in a marked decrease in the excretion of phenolic compounds. These include traces of *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyl-lactic acid and *m*-hydroxyhippuric acid (Das & Griffiths, 1966).

Metabolites of (+)-catechin. Six guinea pigs that had been maintained on the standard diet for 2 weeks were each given a single dose of 50 mg. of (+)-catechin orally; six guinea pigs maintained in the same way but without the dose of catechin served as controls. The 24 hr. urine samples collected over 4 days were each extracted with ether. Examination of the extracts by two-

dimensional paper chromatography with solvent *A* followed by solvent *B* revealed several metabolites of catechin, which were absent from, or present in much smaller amounts on, the control chromatograms.

Compound A. This was shown by co-chromatography to be indistinguishable from *m*-hydroxybenzoic acid (Table 1). Compound A was isolated by band chromatography in solvent *A* followed by elution and chromatography in solvent *B*. Pre-washed Whatman 3MM paper was used. Compound A and *m*-hydroxybenzoic acid had identical ultraviolet-absorption maxima (Table 2). The bathochromic displacement observed on the addition of aluminium chloride to the methanolic solution of the compound was stated by Nakagawa, Shetlar & Wender (1964) to be a characteristic feature of hydroxybenzoic acids, which distinguishes them from hydroxyphenylacetic acids and hydroxyphenylpropionic acids.

Compound B. Co-chromatography (Table 1) showed the chromatographic behaviour of compound B to be identical with that of *m*-hydroxyhippuric acid. The colour reaction obtained with the dimethylaminobenzaldehyde reagent is specific for aroylglycines (Smith, 1960). Hydrolysis of compound B with concentrated hydrochloric acid and chromatographic analysis of the hydrolysate revealed the presence of *m*-hydroxybenzoic acid, which had the chromatographic and ultraviolet-absorption characteristics shown in Table 1. Glycine in the hydrolysate was identified by two-dimensional co-chromatography with solvent *G* followed by solvent *H*. Glycine was detected by ninhydrin and by the *o*-phthalaldehyde reagent, which gives a specific yellow-green colour with this amino acid (Patton & Foreman, 1949). Compound B, isolated by band chromatography in solvents *A* and *B*, had ultraviolet-absorption maxima identical with those of *m*-hydroxyhippuric acid (Table 2).

Compound G. Co-chromatography showed that the chromatographic behaviour of compound G, which was present in trace amounts, was identical with that of *m*-hydroxyphenylpropionic acid (Table 1). Isolation of the compound by band chromatography in solvents *A* and *B* followed by determination of the ultraviolet-absorption spectra showed that these were identical with those of *m*-hydroxyphenylpropionic acid (Table 2).

Compound D. This gave positive reactions with the phenolic reagents (Table 1). That compound D was a neutral substance was indicated both by the absence of reaction with the bromocresol green spray reagent and by its failure to be extracted into sodium hydrogen carbonate solution from diethyl ether. Crystalline compound D (m.p. 105–107°) isolated from guinea-pig urine had infrared- and

Table 1. R_F values and colour reactions of (+)-catechin metabolites

The solvents are described in the text. Chromatograms were run until the solvent front was 30 cm. from the origin. All compounds gave a blue colour with $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$. *m*-Hydroxybenzoic acid gave a violet and *m*-hydroxyhippuric acid a blue fluorescence in ultraviolet light; compound F absorbed.

Solvent ...	R_F						Colour	
	A	B	C	D	E	F	With diazotized <i>p</i> -nitroaniline	With diazotized sulphanilic acid
<i>m</i> -Hydroxybenzoic acid	0.46	0.44	0.61	0.66	0.73	0.38	Red	Orange-yellow
<i>m</i> -Hydroxyhippuric acid	0.36	0.11	0.13	0.78	0.88	0.12	Red	Yellow
<i>m</i> -Hydroxyphenylpropionic acid	0.60	0.66	0.69	0.79	0.88	0.43	Purple	Orange-red
δ -(3-Hydroxyphenyl)- γ -valerolactone	0.98	0.60	0.84	0.67	0.85	0.75	Red	Orange-brown
δ -(3,4-Dihydroxyphenyl)- γ -valerolactone	Streaks	0.18	0.70	0.90	0.76	0.16	Red-brown	Orange-brown
δ -(4-Hydroxy-3-methoxyphenyl)- γ -valerolactone	0.98	0.77	0.92	0.85	0.82	0.90	Red	Orange-brown
Compound F	0.96	0.03	—	—	—	—	Red-purple	Orange-brown

Table 2. Ultraviolet-absorption maxima of metabolites of (+)-catechin

Solvent ...	Absorption maxima (m μ)			
	0.1 N-HCl	0.1 N-NaOH	Methanol	Methanol-AlCl ₃
<i>m</i> -Hydroxybenzoic acid	296	312	233, 294	243, 304
<i>m</i> -Hydroxyhippuric acid	290	315	293	295
<i>m</i> -Hydroxyphenylpropionic acid	272	290	274	274
δ -(3-Hydroxyphenyl)- γ -valerolactone	271	289	276	—
δ -(3,4-Dihydroxyphenyl)- γ -valerolactone	274	—	283	—
δ -(4-hydroxy-3-methoxyphenyl)- γ -valerolactone	274	—	283	—

ultraviolet-absorption spectra (Table 2) and R_F values (Table 1) identical with those of an authentic specimen of δ -(3-hydroxyphenyl)- γ -valerolactone supplied by Professor Y. Oshima. Compound D was also shown to be identical with 'compound 4' detected but not identified in the urine of rats fed with (+)-catechin (Griffiths, 1964).

Compound E. This gave positive reactions with the phenolic reagents and an immediate orange-brown colour with saturated sodium molybdate solution. The latter reaction was stated by Pridham (1959) to be characteristic of *o*-dihydroxyphenols. Failure of the compound to react with bromocresol green or to be extracted into ether from sodium hydrogen carbonate solution indicated that the compound was a neutral substance. The compound had chromatographic properties (Table 1) identical with those of δ -(3,4-dihydroxyphenyl)- γ -valerolactone, isolated by Watanabe (1959b). Compound E was isolated as described in the Experimental section as white needles, m.p. 145–147°; its ultraviolet-absorption spectrum (Table 2) and infrared spectrum (over the range 2.5–16 μ) were identical with those of a specimen of δ -(3,4-dihydroxyphenyl)- γ -valerolactone.

Compound C. This was a neutral phenolic compound chromatographically identical with δ -(4-hydroxy-3-methoxyphenyl)- γ -valerolactone (Watanabe, 1959a) (Table 1). Since compound C was present in trace amounts only, it was not possible to characterize it further.

Compound F. This was an artifact formed from δ -(4-hydroxyphenyl)- γ -valerolactone on exposure to the ammoniacal solvent A. It is thought that compound F is probably an acid amide. Compound F had chromatographic properties similar to those of 'compound 5' detected in the urine of rats fed with (+)-catechin (Griffiths, 1964).

Examination of urine for conjugated metabolites of (+)-catechin. After the administration of 50 mg. of (+)-catechin to six guinea pigs (another group of six animals being used as controls), the urine excreted in each 24 hr. period was collected for 4 days. To remove all free phenolic metabolites, the acidified urine was submitted to exhaustive ether extraction for 24 hr. The extracted urine samples were then hydrolysed with acid under the standard conditions (see the Experimental section). Extracts from the acid-hydrolysed urine were then examined by two-dimensional chromatography in solvents A

and *B*. The chromatograms of the 0–24 hr. and 24–48 hr. extracts showed increased amounts of *m*-hydroxyphenylpropionic acid, δ -(3-hydroxyphenyl)- γ -valerolactone, δ -(3,4-dihydroxyphenyl)- γ -valerolactone and (+)-catechin, indicating their presence in conjugated form. From the intensity and area of the coloured spots formed on spraying the chromatograms with the diazotized *p*-nitroaniline reagent it was evident that δ -(3,4-dihydroxyphenyl)- γ -valerolactone and (+)-catechin were excreted mainly in the 0–24 hr. urine samples, whereas *m*-hydroxyphenylpropionic acid and δ -(3-hydroxyphenyl)- γ -valerolactone were maximal in the 24–48 hr. urine samples.

Evidence for the presence of these compounds in urine as glucuronides and ethereal sulphates was obtained by the use of enzymes specific for β -glucuronide or aryl sulphate linkages. Urine obtained from control animals and animals fed with catechin (as described above) was acidified and exhaustively extracted with ether for 24 hr. to remove all free phenolic compounds. The urine samples were then incubated with β -glucuronidase (EC 3.2.1.31). The presence of sodium dihydrogen phosphate (0.2M) suppressed any arylsulphatase activity present (Dodgson & Spencer, 1953). Ethereal extracts of the centrifuged urine were then examined by two-dimensional chromatography with solvents *A* and *B*. The presence, in the enzyme-treated urine of animals fed with catechin, of δ -(3,4-dihydroxyphenyl)- γ -valerolactone, δ -(3-hydroxyphenyl)- γ -valerolactone, δ -(4-hydroxy-3-methoxyphenyl)- γ -valerolactone, *m*-hydroxyphenylpropionic acid and (+)-catechin showed that these compounds were all in part present as glucuronides. These compounds were absent from the untreated urine samples and those of the control animals. Traces of *p*-hydroxybenzoic acid and *p*-hydroxyphenylacetic acid were present in the enzyme-treated urine samples of both animals fed with catechin and control animals.

Ether-extracted urine from both animals fed with catechin and control animals was hydrolysed with limpet arylsulphatase (EC 3.1.6.1). The incubations were carried out in the presence of saccharic acid (0.1mM) and with temporary adjustment of the hydrogen ion concentration to pH 2.2. Under these conditions any glucuronidase activity present would be arrested (Dodgson & Spencer, 1953). Ethereal extracts of the centrifuged urine were examined as before by two-dimensional chromatography. The presence of δ -(3-hydroxyphenyl)- γ -valerolactone, (+)-catechin and trace amounts of *m*-hydroxyphenylpropionic acid in the arylsulphatase-treated urine samples of guinea pigs fed with catechin provided evidence for their presence in part as ethereal sulphates. Traces of *p*-hydroxybenzoic acid and *p*-hydroxyphenylacetic

acid were present in the enzyme-treated urine of both animals fed with catechin and control animals.

Although not extracted from urine by diethyl ether, the glucuronide conjugates were extractable by butan-1-ol. The urine samples of six animals that had each received 100mg. of catechin, and those of six untreated animals, were separately subjected to exhaustive ether extraction. The ether-extracted urine was then submitted to butan-1-ol extraction and portions were taken for two-dimensional chromatographic analysis in solvents *A* and *B*. The developed chromatograms were sprayed with the naphtharesorcinol reagent but only one large area (subsequently shown to contain several substances) gave a positive reaction with the spray reagent. The R_F values of this area were: solvent *A*, 0.61; solvent *B*, 0.01; solvent *C*, 0.09; solvent *D*, 0.97. After band chromatography in solvent *C*, the naphtharesorcinol-positive area was eluted and hydrolysed under the standard conditions with concentrated hydrochloric acid. Two-dimensional chromatography in solvents *A* and *B* gave, with the diazotized *p*-nitroaniline reagent, four spots corresponding to δ -(3-hydroxyphenyl)- γ -valerolactone, δ -(3,4-dihydroxyphenyl)- γ -valerolactone, *m*-hydroxyphenylpropionic acid (trace) and (+)-catechin. Attempts to separate the glucuronide conjugates by chromatography with a range of solvent systems and by the method of Kamil, Smith & Williams (1951) were unsuccessful.

Determination of total glucuronide and ethereal sulphates. After three groups of three guinea pigs had been kept for 2 weeks on the standard diet, the urine was examined on 2 consecutive days for total glucuronide and total ethereal sulphate excretion. The animals then received (+)-catechin at three dosages, and the total glucuronide and total ethereal sulphate were determined on each of the 4 succeeding days (Table 3).

Determination of free phenolic acid metabolites. The results (Table 4) of this determination show that *m*-hydroxybenzoic acid is the major free phenolic acid metabolite of (+)-catechin in the guinea pig.

Participation of the intestinal microflora in the catabolism of catechin. A group of six guinea pigs maintained for the preceding 14 days on the standard diet were each fed with 25mg. of (+)-catechin on 3 consecutive days. This dosage was maintained for a further 4 days, when in addition to the (+)-catechin the animals received daily phthaloylsulphathiazole (70mg.) and aureomycin (50mg.). All the compounds were administered orally. A control group received only (+)-catechin over this period. Examination of the urine samples by two-dimensional chromatography showed that in the treated group metabolite formation from (+)-catechin was completely suppressed by the

Table 3. *Excretion of glucuronide and ethereal sulphate before and after administration of (+)-catechin to guinea pigs*

The results are expressed as mg. of glucuronide or SO₃/24 hr., and are the means of three experiments with the ranges in parentheses. At the end of the first 2 days of the experiment each animal received a single dose of (+)-catechin as follows: group 1, 25 mg.; group 2, 50 mg.; group 3, 200 mg.

Day	Group 1		Group 2		Group 3	
	Glucuronide	Ethereal sulphate	Glucuronide	Ethereal sulphate	Glucuronide	Ethereal sulphate
1	14.3 (13.9-14.6)	0.40 (0.30-0.50)	13.4 (12.8-14.4)	2.34 (1.50-3.40)	11.1 (10.5-12.1)	1.09 (0.96-1.30)
2	13.7 (12.7-14.4)	0.63 (0.51-0.73)	11.6 (10.8-12.6)	2.45 (0.64-4.21)	12.5 (11.0-14.3)	1.17 (0.97-1.53)
3	15.4 (12.8-19.1)	1.04 (0.84-1.24)	16.9 (16.4-17.8)	3.80 (3.22-3.90)	26.3 (22.8-29.1)	3.13 (2.12-4.20)
4	17.3 (15.9-19.5)	2.76 (2.66-2.83)	15.4 (14.9-16.4)	4.33 (3.60-5.43)	18.4 (17.0-19.2)	4.44 (3.53-5.01)
5	13.9 (11.5-16.5)	1.61 (1.40-1.80)	12.4 (10.6-14.8)	2.32 (1.95-2.76)	13.9 (12.1-15.5)	0.93 (0.62-1.18)
6	13.2 (11.2-14.4)	0.80 (0.60-1.00)	11.5 (10.6-12.8)	1.92 (0.92-2.88)	12.2 (11.9-12.6)	1.48 (0.74-2.30)

Table 4. *Excretion of free phenolic acid metabolites of (+)-catechin in the guinea pig*

The results are expressed as μ g. of the acid determined, and are the means of six experiments with the ranges in parentheses. Each animal received a single dose of 25 mg. of (+)-catechin at the end of the first day.

Day	<i>m</i> -Hydroxyphenylpropionic acid (μ g.)	<i>m</i> -Hydroxybenzoic acid (μ g.)	<i>m</i> -Hydroxyhippuric acid (μ g.)
1	24.8 (24.3-35.0)	41.6 (30.0-50.0)	36.6 (30.0-45.0)
2	52.2 (50.0-55.0)	497 (470-520)	235 (215-265)
3	35.9 (30.0-42.9)	88.5 (75.0-112)	95.2 (60.0-140)
4	35.6 (20.0-52.5)	66.6 (55.0-72.5)	117 (60.0-140)
5	34.2 (20.2-52.5)	40.0 (35.0-45.0)	55.6 (50.0-62.5)

Table 5. *Effect of antibacterial compounds on the excretion of phenolic metabolites of (+)-catechin*

Each animal of groups I and II was, after the first day, given (+)-catechin (25 mg.) daily. After the first 4 days each animal of group I was also given phthaloylsulphathiazole (70 mg.) and aureomycin (50 mg.) daily. Relative amounts of metabolites excreted are indicated by the number of + signs.

Days	Group I			Group II		
	<i>m</i> -Hydroxybenzoic acid	<i>m</i> -Hydroxyhippuric acid	Valerolactones	<i>m</i> -Hydroxybenzoic acid	<i>m</i> -Hydroxyhippuric acid	Valerolactones
1	0	+	0	0	+	0
2	++	++	+++	++	++	+++
3	+++	+++	+++	+++	+++	++++
4	++++	++++	++++	++++	++++	++++
5	++	++	+	++++	++++	++++
6	+	0	0	++++	++++	++++
7	0	0	0	++++	++++	++++
8	0	0	0	++++	++++	++++
9	0	0	0	++++	++++	++++

seventh day, whereas in the control group high metabolite excretion was observed over the whole of the experimental period (Table 5).

Inter-relationships of (+)-catechin metabolites. To establish the sequential relationships of the metabolites of catechin in urine, certain of the major metabolites were fed separately to guinea pigs, and the products were identified.

(a) Administration of *m*-hydroxyphenylpropionic acid. A group of six guinea pigs maintained on the standard diet received *m*-hydroxyphenylpropionic acid (25mg.) by oral administration. Examination of the urine revealed the presence of unchanged *m*-hydroxyphenylpropionic acid, *m*-hydroxybenzoic acid and *m*-hydroxyhippuric acid. Identifications were made by co-chromatography and determination of ultraviolet-absorption spectra by the standard method. The amounts are given in Table 6.

(b) Administration of δ -(3-hydroxyphenyl)- γ -valerolactone. Pure δ -(3-hydroxyphenyl)- γ -valerolactone (8mg.) isolated from urine was administered under conditions similar to those of the experiment with *m*-hydroxyphenylpropionic acid, to each of a group of six guinea pigs. The urine samples were examined for free phenolic compounds by direct ether extraction and for conjugated phenolic substances by hydrolysis of exhaustively ether-extracted urine. Examination of the chromatograms of urine collected during the first 24 hr. period after administration of the lactone revealed the presence of unchanged δ -(3-hydroxyphenyl)- γ -valerolactone in both the free and the conjugated form, and also free and conjugated *m*-hydroxyphenylpropionic acid. In the 24–48 hr. urine increased amounts of *m*-hydroxybenzoic acid and of *m*-hydroxyhippuric acid were noted. A control group of guinea pigs fed with the standard diet only showed no increases over this period.

(c) Administration of δ -(3,4-dihydroxyphenyl)- γ -valerolactone. Pure δ -(3,4-dihydroxyphenyl)- γ -valerolactone (7mg.), isolated from urine, was

administered to each of a group of six guinea pigs. Chromatographic evidence was obtained of the presence in 0–24 hr. urine of unchanged δ -(3,4-dihydroxyphenyl)- γ -valerolactone, mainly in the conjugated form. Traces of δ -(3-hydroxyphenyl)- γ -valerolactone and δ -(4-hydroxy-3-methoxyphenyl)- γ -valerolactone were present in the conjugated form. In the 24–48 hr. urine no lactones were detected, but an increase was noted in the *m*-hydroxybenzoic acid present. No increases were noted in a control group fed with the standard diet only.

DISCUSSION

In the guinea pig the major phenolic acid metabolite of (+)-catechin has been shown to be *m*-hydroxybenzoic acid. *m*-Hydroxyphenylpropionic acid, the major (+)-catechin metabolite in the rat (Griffiths, 1964), was present only in trace amounts. Vanillic acid and protocatechuic acid, detected in the urine of rabbits fed with catechin (Oshima *et al.* 1958), were not detected in guinea-pig urine. Several phenyl- γ -valerolactones detected by Oshima & Watanabe (1958) in rabbit urine were also formed from (+)-catechin in the guinea pig. The major lactone was δ -(3-hydroxyphenyl)- γ -valerolactone, which suggests a possible origin for the *m*-hydroxybenzoic acid detected. The lactone, δ -(4-hydroxy-3-methoxyphenyl)- γ -valerolactone, corresponding to vanillic acid, which was not detected in the urine, was present only in trace amounts.

Administration of certain of the metabolites of (+)-catechin provided evidence of their sequential relationships, which supports the pathway of (+)-catechin metabolism in the guinea pig shown in Scheme 1.

It is noteworthy that the conversion of δ -(3-hydroxyphenyl)- γ -valerolactone into *m*-hydroxyphenylpropionic acid occurs slowly, as the former substance when orally administered is itself excreted

Table 6. *Excretion of metabolites of m-hydroxyphenylpropionic acid in the guinea pig*

The results are expressed as μ g. of the acid determined and are the means of six experiments with the ranges in parentheses. Each animal received a single dose of *m*-hydroxyphenylpropionic acid (25mg.) at the end of the first day.

Day	<i>m</i> -Hydroxyphenylpropionic acid (μ g.)	<i>m</i> -Hydroxybenzoic acid (μ g.)	<i>m</i> -Hydroxyhippuric acid (μ g.)
1	26.7 (20.1–32.3)	39.8 (36.1–42.2)	43.3 (41.1–48.2)
2	409 (396–415)	416 (396–420)	398 (366–409)
3	119 (96.9–136)	201 (191–214)	121 (112–129)
4	36.9 (29.0–37.8)	48.9 (40.9–58.0)	60.3 (59.8–68.1)

Scheme 1. Pathway of the metabolism of (+)-catechin in the guinea pig.

in larger amounts than its phenolic acid metabolites. The presence of δ -(3-hydroxyphenyl)- γ -valerolactone and the absence of 3,4-dihydroxyphenylpropionic acid indicates that *p*-dehydroxylation occurs at the phenyl- γ -valerolactone stage rather than at the phenylacetyl stage, although orally administered 3,4-dihydroxyphenylpropionic acid is known to undergo *p*-dehydroxylation in the rat (Booth, Emerson, Jones & DeEds, 1957).

On the basis of his experiments on catechin metabolism in the rabbit, Watanabe (1959b) also suggested that the phenolic acids detected were derived from the corresponding phenyl- γ -valerolactones. His suggestions were, however, wholly based on their structural relationships, as the intermediate metabolites were not administered to the animals (Watanabe, 1959b).

Since the formation of phenylvalerolactones as well as *m*-hydroxyphenyl acids was arrested when antibacterial compounds were administered with (+)-catechin, it is evident that the intestinal bacteria are involved at an early point in the degradative pathway. Whether these organisms

are also active in the opening of the lactone ring was not, however, investigated. In addition to involvement in catechin metabolism in the rat (Griffiths, 1964) and guinea pig, the intestinal microflora have been shown to be involved in the metabolism of the related flavonol, quercetin (Nakagawa, Shetler & Wender, 1965). The pathway of metabolism may, however, be dissimilar as Nakagawa, Shetler & Wender (1965) did not detect phenylvalerolactones.

Although several phenolic acids, including *m*-coumaric acid (Booth *et al.* 1957), are known to give rise to glucuronide conjugates the formation of glucuronides of hydroxyphenyl- γ -valerolactones and of (+)-catechin has not previously been reported. The observation that conjugates of (+)-catechin and δ -(3,4-dihydroxyphenyl)- γ -valerolactone were maximal in the 0-24 hr. urine sample, whereas conjugates of 3-(hydroxyphenyl)- γ -valerolactone and *m*-hydroxyphenylpropionic acid were maximal in the 24-48 hr. sample provides additional support for the metabolic sequence proposed (Scheme 1).

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