vates the enzyme while neither trinitrobenzenesulfonic acid nor 5,5′-dithiobis-(2-nitrobenzoic acid) have any effect points to the likely presence of one or more essential guanidinium groups in or near the catalytic site of L-amino acid oxidase.

D- and L-amino acid oxidase both are known to be capable of utilizing a variety of amino acids as substrates, but their stereospecific requirements appear to be nearly absolute. The only known good substrate that they have in common is L-3,4-dehydroproline[11]. Massey and Curti[12] proposed that the 2 enzymes operate via similar reaction mechanisms, based on kinetic studies. We note that Nishino et al.[13] have recently reported the inactivation of hog kidney D-amino acid oxidase by butanedione. Thus, in spite of stereospecific differences in the substrate specificities of D- and L-amino acid oxidases, it appears likely that they utilize similar reaction mechanisms and may indeed have chemically similar catalytic centers.

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3′-O-Methyl-(+)-catechin glucuronide and 3′-O-methyl-(+)-catechin sulphate: new urinary metabolites of (+)-catechin in the rat and the marmoset

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Summary. The major urinary metabolites of (+)-catechin (cyanidanol-3) in the rat were (+)-catechin glucuronide, 3′-O-methyl-(+)-catechin glucuronide and 3′-O-methyl-(+)-catechin sulphate. The latter conjugate was the major metabolite in marmoset urine.

The naturally occurring flavanol, (+)-catechin, which is known to occur in a number of green plants and plant products of significance in the human diet[3], has recently been reported to possess liver-protective properties[4] and to be beneficial in the therapy of acute viral hepatitis in man[5]. Previous metabolic investigations have shown that oral administration of (+)-catechin (Cyanidanol-3) to rats results in the excretion of 3-hydroxyphenyl-propionic acid, 3-hydroxyhippuric acid[6] and δ-(hydroxyphenyl)-γ-valerolactones[7] in urine. Metabolites extractable from unhydrolyzed urine with diethyl ether (including these compounds) accounted for only ⅙ of the 50–63% of the dose appearing in urine. Our recent finding[8] that 3′-O-methyl-(+)-catechin glucuronide is the major metabolite of (+)-catechin in rat bile led us to examine rat and marmoset urine for the presence of such conjugates.

Materials and methods. [U^{14}C](+)-Catechin (radiochemical purity 95%, sp. act. 698 μCi/mmole) was donated by Zyma S.A., Nyon, Switzerland.

Male albino Wistar rats (S.P.F., 250 g) and male marmosets (300 g) were maintained as previously described[6,9]. [U^{14}C]-(+)-Catechin was administered (40 mg/kg, 2 μCi) to rats by stomach intubation in water and to marmosets orally in Cytacon Syrup (Glaxo Labs., Greenford, Middlesex). Animals were housed in all glass metabolism cages allowing separate collection of urine, faeces (over solid CO_2) and expired CO_2[10].

Measurement of radioactivity, paper chromatography[8,10], enzymic hydrolysis of metabolites[11], HPLC and bile duct cannulation surgery[8] were carried out by techniques previously described. Diazotised 4-nitroaniline[12] was used to locate phenolic compounds on chromatograms. Individual metabolites were quantified following separation on thin layers of cellulose (Type 20, Sigma Chemical Co., Poole, Dorset; butan-2-ol:acetic acid:water 5:1:2) using a Berthold Series 272 radiochromatogram scanner linked to a PET computer. (+)-Catechin and 3′-O-methyl-(+)-catechin were quantified by similar means following separation on silica gel 'G' (300-μm layers) developed in toluene:ethyl acetate:formic acid (5:4:1 v/v/v).

Results and discussion. The urinary excretion of ^{14}C in the rat and marmoset following the oral administration of [^{14}C]-(+)-catechin is shown in table 1. Urinary excretion of ^{14}C (66% of dose) in the rat was close to that observed in earlier studies[7]. In the present study radioscanning of TLC separations of metabolites showed that 3 conjugates accounted for 84% of the ^{14}C of rat urine (table 2). These conjugates were identified by the use of specific enzyme treatments and by co-chromatography of the aglycones liberated (table 2). The evidence obtained indicated that these conjugates were a glucuronide of (+)-catechin and a glucuronide and a sulphate of 3′-O-methyl-(+)-catechin. The latter aglycone was chromatographically identical to the aglycone of the major biliary metabolite of (+)-catechin in the rat which we previously characterized by mass spectrometry[8]. The intact glucuronide conjugate of 3′-O-methyl-(+)-catechin isolated from urine did not however show identical chromatographic properties to that obtained from

bile. Since the polyhydroxylated structure of (+)-catechin gives rise to the possibility of formation of several isomeric glucuronides of 3′-O-methyl-(+)-catechin, this may explain the observed differences between the 2 conjugates.

A further point of interest is that the timing of excretion of urinary metabolites differed. Rat urine collected 0–6 h after administration contained (+)-catechin glucuronide and 3′-O-methyl-catechin glucuronide with traces of the 2 aglycones whereas 6–24-h urine contained predominantly 3′-O-methyl-(+)-catechin sulphate. Small amounts of the previously reported ring fission products[6,7] were detected in urine collected 24–72 h after dosing, and the excretion of $^{14}CO_2$ furnished further evidence of ring fission. Lower levels of ring fission products observed to be excreted in the present study may be attributable to the use of specific pathogen-free (SPF) rats (possessing a restricted microflora) in this investigation and to the smaller dosage level employed facilitating more complete absorption of (+)-catechin from the gastro-intestinal tract and in consequence minimizing microfloral catabolism.

Urinary excretion of ^{14}C in the marmoset was less than $^1/_3$ that in the rat and the levels of conjugates present were also different. In the marmoset 3′-O-methyl-(+)-catechin sulphate accounted for 82% of radioactivity in urine and 3′-O-methyl-(+)-catechin glucuronide for only 7%. (+)-Catechin glucuronide was not detected.

It has previously been demonstrated that ring fission of (+)-catechin is mediated by the intestinal microflora since administration of (+)-catechin to rats pretreated with anti-

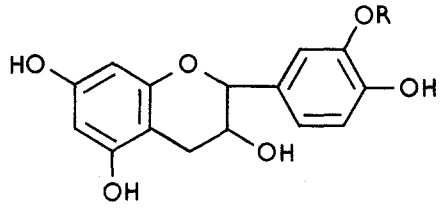

Figure 1. Structures of (+)-catechin (R = H) and 3′-O-methyl-(+)-catechin (R = CH_3).

Table 1. The excretion of ^{14}C in the rat and marmoset following oral administration of [U^{14}C]-(+)-catechin (40 mg/kg orally)

	Rat (% of dose)	Marmoset (% of dose)
Urine		
0–6 h	15.4	5.9
6–24 h	42.7	9.6
24–48 h	6.2	4.1
48–72 h	1.1	2.2
72–96 h	0.04	*
Sub total	65.8 (61.4–68.2)	21.8 (16.9–23.1)
Respired $^{14}CO_2$	7.3 (6.5–8.6)	2.9 (0.7–4.7)
Faeces		
0–24 h	6.3	52.9
24–48 h	12.6	13.7
48–72 h	3.2	3.4
72–96 h	1.6	*
Sub total	23.7 (18.0–28.8)	70.0 (56.2–80.9)
Cage washings	0.7 (0.5–0.9)	1.9 (1.2–3.5)
Total recovery	97.5% (92.7–99.3)	96.6% (89.9–101.4

Values quoted are means of 3 rat experiments and 4 marmoset experiments. Ranges in brackets. *Marmoset experiments continued for 72 h only.

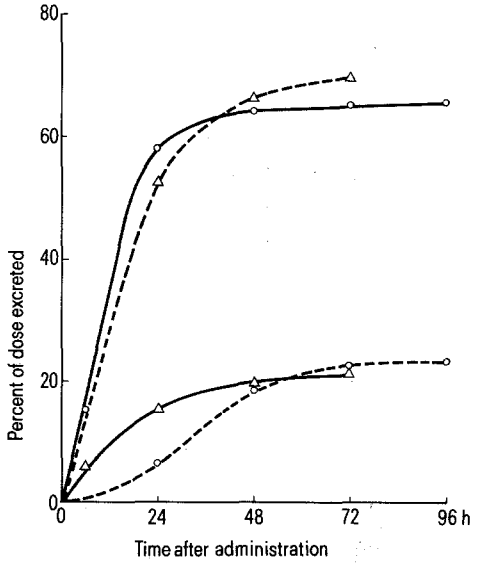

Figure 2. Cumulative urinary and faecal excretion of radioactivity following the oral administration of [^{14}C](+)-catechin to the rat and marmoset. ^{14}C Excretion in marmoset urine: △——△; in rat urine: ○——○; in marmoset faeces: △– – –△; and in rat faeces: ○– – –○.

Table 2. Characteristics of flavanol metabolites detected in rat urine

Metabolite	Retention volume in HPLC system (ml)	R_f* in butan-2-ol: acetic acid: H_2O 5:1:2 (on paper)	R_f* in toluene-ethyl acetate-formic acid 5:4:1 (on silica gel G)	% of urine ^{14}C (rat)	% of urine ^{14}C (marmoset)	Effect of incubation with β-glucuronidase Type H1 (a mixed β-glucuronidase/aryl sulphatase preparation		Identity
						Enzyme only	Enzyme plus D-saccharic acid 1-4 lactone (β-glucuronidase inhibitor)	
1	–	0.09	–	0.8	Not detected	Not incubated	Not incubated	Unidentified
2	–	0.25	–	28.5	Not detected	(+)-Catechin liberated	Not hydrolyzed	(+)-Catechin glucuronide
3	–	0.31	–	25.9	7.2	3′-O-Methyl-(+)-catechin liberated	Not hydrolyzed	3′-O-Methyl-(+)-catechin glucuronide
4	–	0.50	–	29.8	82.0	3′-O-Methyl-(+)-catechin liberated	3′-O-Methyl-(+)-catechin liberated	3′-O-Methyl-(+)-catechin sulphate
5	6.2	0.64	0.47	1.4	Not detected	Not hydrolyzed	Not hydrolyzed	(+)-Catechin
6	7.9	0.75	0.60	3.4	Not detected	Not hydrolyzed	Not hydrolyzed	3′-O-Methyl-(+)-catechin

biotics[6], or to germ free rats[13] reduced or abolished the excretion of phenolic acids and phenylvalerolactones. Recently evidence has been presented[14] that the major biliary metabolite of (+)-catechin, 3′-O-methyl-(+)-catechin glucuronide is formed in liver. The conjugates observed in urine in the present studies may also be formed in liver but the possibility that formation also occurs in other tissues such as the intestine wall should not be excluded.

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Genetic variability of *Apis mellifera ligustica* Spin. in a marginal area of its geographical distribution

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Summary. Marginal populations of *Apis mellifera ligustica* differ from the central populations of this subspecies in allele frequencies at the *Mdh*-1 locus. The difference seems to be due to gene flow from French populations of *A. m. mellifera.*

Apis mellifera L. shows a remarkable variability in several morphological and behavioral properties[1,2] (e.g. hygienic, foraging, stinging behaviour). In turn the level of its biochemical variability has been reported to be very low as compared with other invertebrates[3–5]. Only the malate dehydrogenase (MDH), alcohol dehydrogenase (ADH) and esterase (Est) gene-enzyme systems and the P-3 protein are polymorphic, in some or all of the geographical strains studied[6–9]. The present work analyses the electrophoretic variability of different *A. m. ligustica* Spin. populations from western Piedmont (Italy), which is a marginal area in the geographical distribution of this subspecies. Our investigation was carried out by examining the Est system on polyacrylamide gel (7.5%) and MDH, PGI (phosphoglucose isomerase) PGM (phosphoglucomutase), IDH (isocitrate dehydrogenase), ME (malic enzyme), G6PD (glucose-6-phosphate dehydrogenase), LDH (lactate dehydrogenase) on cellulose acetate strips (Cellogel, Labometrics, Milano). Samples of 58–82 adult workers were randomly collected from 8 spatially separated apiaries, each consisting of 20–50 colonies. The thorax of every bee was homogenized in 0.1 ml of 0.0025 M $MgCl_2$ and centrifuged at 12,000 rpm for 5 min, then the supernatant was used for electrophoresis. The electrode buffers were modified from Shaw and Prasad[10] for MDH, IDH, ME, G6PD, LDH; from Badino[11] for PGI, from Spencer et al.[12] for PGM and from Ayala and Powell[13] for Est. Enzyme activities were demonstrated by classical histological methods[10,14]. With the exception of MDH, none of the examined gene-enzyme systems was polymorphic in the populations studied. Both PGI and ME showed 2 isoenzymatic bands, and PGM, IDH, G6PD, LDH and Est 1 band only.

The MDH electrophoretic pattern (fig. 1) showed 3 enzymatically active regions, one of which migrated to the cathode in our buffer system; only the more anodal region was polymorphic. There was either a 1-banded phenotype or a 3-banded one. The allozymes of this region are produced by 3 codominant alleles at 1 locus (*Mdh*-**1**). These alleles seem to be homologous with fast (F), medium (M), slow (S)[9] and with a, b, c[8,15] of other authors respectively. The allele frequencies we found are shown in the table.

Allele frequencies at the *Mdh*-1 locus in Piedmont populations of *A.m. ligustica.* F, fast; M, medium; S, slow

Apiaries	No. of workers	*Mdh*-1 allele frequencies F	M	S
1 Asti	66	0.265	0.023	0.712
Maira valley				
2 Paschero	58	0.043	0.112	0.845
3 S. Michele	76	0.243	0.020	0.737
4 Acceglio	70	0.301	0.070	0.629
Tanaro valley				
5 Bagnasco	72	0.083	0.174	0.743
6 Ormea	76	0.072	0.572	0.355
7 Upega	78	0.128	0.519	0.353
8 Monesi	82	0.219	0.549	0.232

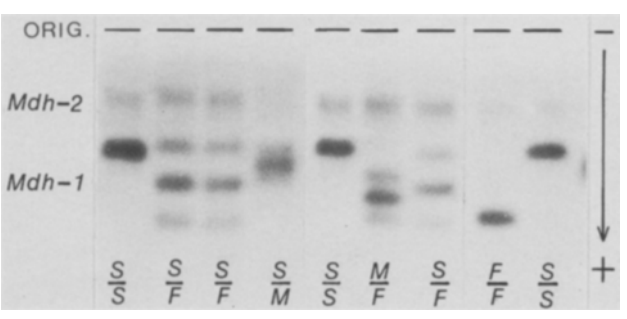

Figure 1. Some MDH-1 phenotypes observed in *Apis mellifera ligustica.* F, fast; M, medium; S, slow.