XENOBIOTICA, 2001, VOL. 31, NO. 12, 879–890

Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-*O*-methyltransferase

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Received 28 February 2001

1. The metabolic *O*-methylation of several catechol-containing tea polyphenols by rat liver cytosolic catechol-*O*-methyltransferase (COMT) has been studied.

2. When (−)-epicatechin was used as substrate, its *O*-methylation showed dependence on incubation time, cytosolic protein concentration, incubation pH and concentration of *S*-adenosyl-L-methionine. The *O*-methylation of increasing concentrations of (−)-epicatechin followed typical Michaelis–Menten kinetics, and the apparent K_m and V_{max} were 51 μM and 2882 pmol mg protein^{-1} min^{-1}, respectively, at pH 7.4, and were 17 μM and 2093 pmol mg protein^{-1} min^{-1}, respectively, at pH 10.0.

3. Under optimized conditions for *in vitro* *O*-methylation, (−)-epicatechin, (+)-epicatechin and (−)-epigallocatechin were rapidly *O*-methylated by rat liver cytosol. In comparison, (−)-epicatechin gallate and (−)-epigallocatechin gallate were *O*-methylated at significantly lower rates under the same reaction conditions.

4. COMT-catalysed *O*-methylation of (−)-epicatechin and (−)-epigallocatechin was inhibited in a concentration-dependent manner by *S*-adenosyl-L-homocysteine, a demethylated product of *S*-adenosyl-L-methionine. The IC_{50} was ~10 μM.

5. In summary, the results showed that several catechol-containing tea polyphenols were rapidly *O*-methylated by rat liver cytosolic COMT. These observations raise the possibility that some of the biological effects of tea polyphenols may be exerted by their *O*-methylated products or may result from their potential inhibition of the COMT-catalysed *O*-methylation of endogenous catecholamines and catechol oestrogens.

Introduction

Catechol-*O*-methyltransferase (COMT), an enzyme ubiquitously present in high activity in rodents and humans, catalyses the metabolic *O*-methylation of endogenous catecholamines and catechol oestrogens (Axelrod and Tomchick 1958, Axelrod 1966, Guldberg and Marsden 1975, Thakker and Creveling 1990, Zhu and Conney 1998). In addition to the endogenous catechol substrates, COMT also catalyses metabolic *O*-methylation of many catechol-containing xenobiotics (Axelrod and Tomchick 1958, Axelrod 1966, Guldberg and Marsden 1975, Thakker and Creveling 1990, Zhu *et al*. 1994). Zhu *et al*. (1994) reported that quercetin and fisetin, two catechol-containing dietary flavonoids, are rapidly *O*-methylated by cytosolic COMT, with rates two to three orders of magnitude higher than for endogenous catecholamines.

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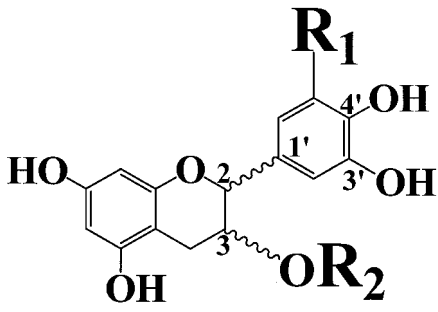
Xenobiotica ISSN 0049–8254 print/ISSN 1366–5928 online © 2001 Taylor &
http://www.tandf.co.uk/journals
DOI: 10.1080/00498250110079798



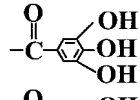
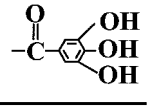
Tea polyphenols	Stereochemistry	R_1	R_2
(−)-Epicatechin	2*R*, 3*R*	—H	—H
(+)-Epicatechin	2*S*, 3*S*	—H	—H
(−)-Epigallocatechin	2*R*, 3*R*	—OH	—H
(−)-Epicatechin gallate	2*R*, 3*R*	—H	
(−)-Epigallocatechin gallate	2*R*, 3*R*	—OH	

Figure 1. Chemical structures of several catechol-containing dietary tea polyphenols used.

Like dietary quercetin and fisetin, several of the common tea polyphenols, such as (−)-epicatechin, (+)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate (figure 1), also contain a catechol structure and share certain degrees of structural similarity to them. These catechol-containing tea components constitute a major fraction of the water-extractable polyphenols contained in the tea leaves (Balentine 1992). Recent studies showed that several of these tea polyphenols (e.g. (−)-epigallocatechin and epicatechins) can be readily absorbed in human subjects after drinking tea, and some of them can reach submicromolar peak concentrations (ranging from 0.1 to 0.4 μM) in the plasma (Lee *et al*. 1995, Unno *et al*. 1996, Yang *et al*. 1998, van het Hof *et al*. 1998, Li *et al*. 2000). Another earlier study using ^{3}H-labelled (−)-epigallocatechin gallate showed that this tea catechin is widely distributed in mouse tissues (Suganuma *et al*. 1998).

We recently reported that the catechol-containing tea polyphenols are rapidly metabolized to monomethyl ethers by the cytosolic COMT present in human term placenta (Zhu *et al*. 2000). We describe here our additional results on the rapid *O*-methylation of tea catechins by rat liver cytosol. These observations raise the possibility that some of the biological effects of tea polyphenols may be caused by their methylated products or may result from their potential inhibition of COMT-catalysed *O*-methylation of endogenous catecholamines and catechol oestrogens.

Materials and methods

Chemicals

(−)-Epicatechin, (+)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, *S*-adenosyl-L-methionine, dithiothreitol and Tris-HCl were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The purities for the tea polyphenols used were 95–98% as determined by

HPLC analysis. [Methyl-^{3}H]*S*-adenosyl-L-methionine (specific activity 11.2–13.5 Ci mmol^{-1}) was obtained from Perkin Elmer Life Science Products (Boston, MA, USA). All organic solvents used in this study were of HPLC grade and obtained from the Fisher Scientific Co. (Springfield, NJ, USA).

Preparation of rat liver cytosol

Six-week-old female Sprague–Dawley rats (body weight 125–150 g) were purchased from Harlan Sprague–Dawley Laboratory (Houston, TX, USA). After arrival, they were allowed to acclimatize for 1 week before use. The animals were euthanized under CO_2 inhalation, and the livers from five female rats were collected and pooled. Rat livers were immediately minced in 3 vols ice-cold 0.1 M Tris-HCl/KCl solution (pH 7.4) and homogenized with a Polytron homogenizer for 2–3 min. Tissue homogenates were centrifuged at 9000*g* for 15 min, and the supernatants were pooled and filtered through two layers of cheesecloth to remove lipid clots. The filtrates were recentrifuged at 105 000*g* (4°C) for 90 min. The supernatants (cytosolic preparations) were stored in 1-ml aliquots at −80°C. The protein concentration was determined by using the BioRad protein assay kit with bovine serum albumin (BSA) as a standard.

Rat liver COMT-catalysed O-methylation of catechol-containing tea polyphenols

The reaction mixture was prepared at ~4 °C and consisted of the desired concentration of a tea polyphenol substrate, 0.5 mg rat liver cytosolic protein ml^{-1}, 50 μM *S*-adenosyl-L-methionine (containing 0.25 μCi [methyl-^{3}H]*S*-adenosyl-L-methionine), 1.2 mM magnesium chloride, and 1.0 mM dithiothreitol in a final volume of 1 ml Tris-HCl buffer (10 mM, pH 7.4). The reaction was initiated by addition of rat liver cytosolic protein and was carried out at 37 °C for 20 min under constant mild shaking. The reaction was arrested by placing the tubes on ice followed immediately by addition of 0.5 ml ice-cold water and extraction of the ^{3}H-containing methylated catechin metabolites with 5 ml ethyl acetate. After centrifugation for 20 min at ~3000*g*, portions of the upper ethyl acetate phase (2 ml) were removed for measurement of radioactivity in a scintillation counter (Beckman Instruments, model LS 5000TD). Blank values obtained from incubations without rat liver cytosolic protein were determined in each individual assay and subtracted. The blank radioactivity counts were usually <20% of the values obtained from incubations in the presence of rat liver cytosolic protein. By using the ^{3}H-labelled monomethylated products of (−)-epicatechin and (−)-epigallocatechin (isolated by HPLC), the extraction efficiency for the *O*-methylated (−)-epicatechin and (−)-epigallocatechin was >94%. The rate (velocity) for the *O*-methylation of various tea polyphenol substrates by rat liver cytosolic COMT was expressed as pmol methylated tea polyphenol formed per mg protein per^{-1} min^{-1}.

Liquid chromatography-mass spectrometry analysis of methylated tea polyphenols

To determine the structures of the methylated tea polyphenols, 50 μM (−)-epicatechin or (−)-epigallocatechin was first incubated *in vitro* for 30 min with 1.0 mg rat liver cytosolic protein ml^{-1}, 100 μM *S*-adenosyl-L-methionine, 1.2 mM magnesium chloride, and 1.0 mM dithiothreitol in a final volume of 1 ml Tris-HCl buffer (10 mM, pH 7.4). The reaction mixture was extracted twice with 10 ml ethyl acetate. The organic solvent extracts were combined and dried under a stream of nitrogen, and the residues were redissolved in 100 μl 20% acetonitrile in water by vortex mixing. The mixture was then centrifuged at ~15 000*g* for 10 min, and a 50-μl aliquot of the supernatant was injected into the HPLC for analysis of methylated tea polyphenol metabolites according to the method for tea catechins described by Lee *et al.* (1995). The incubations described above were first done with tea catechins and [methyl-^{3}H]-*S*-adenosyl-L-methionine to determine the retention times of the radioactive metabolites. Parallel incubations of the catechins with only non-radioactive *S*-adenosyl-L-methionine were performed for collection of metabolites for mass spectrometric analysis.

The peaks corresponding to methylated metabolites of (−)-epicatechin or (−)-epigallocatechin were collected from the HPLC column and were dried under a stream of nitrogen. They were then analysed by liquid chromatography-mass spectrometry (Finnegan LCQ; Thermoquest Corp., San Jose, CA, USA) using electrospray-mass spectrometry with an ion trap operated in the negative-ion mode.

Results

Confirmation by mass spectrometry of metabolic formation of monomethylated tea catechins

Incubation of (−)-epigallocatechin with rat liver cytosol in the presence of [methyl-^{3}H]*S*-adenosyl-L-methionine (0.25μ Ci) resulted in formation of a less polar radioactive metabolite peak with a retention time of 22.0 min (figure 2A, B). Similarly, incubation of (−)-epicatechin with rat liver cytosol in the presence of

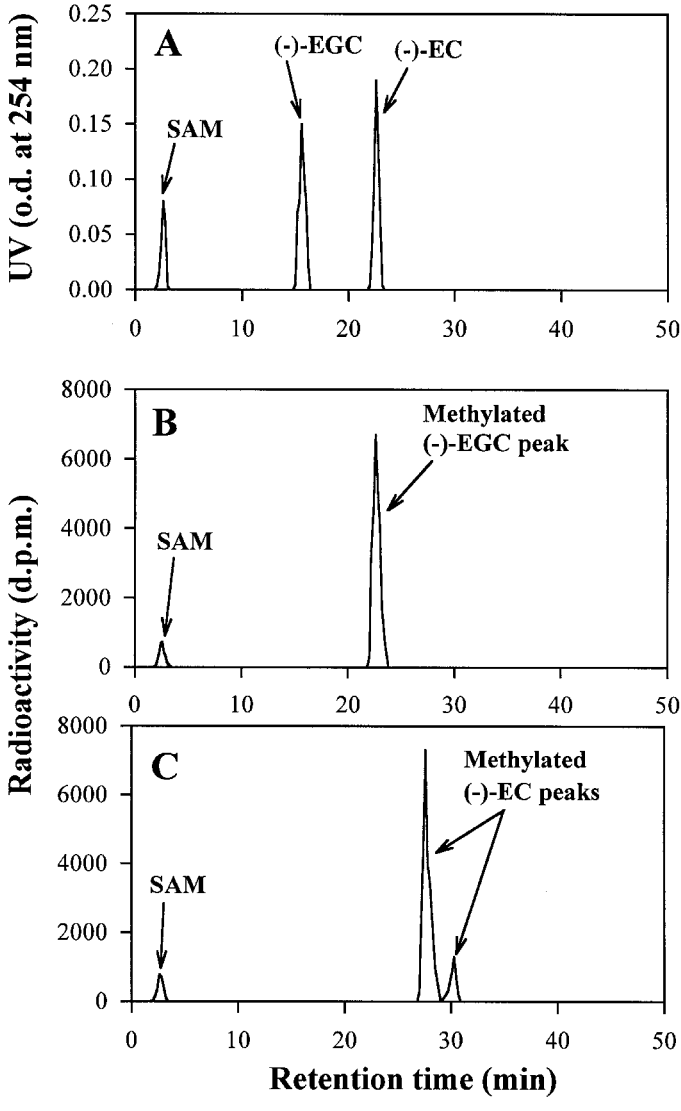


Figure 2. HPLC separation of the methylated metabolites of (−)-epicatechin and (−)-epigallocatechin formed by rat liver cytosol. (A) Representative UV trace for authentic *S*-adenosyl-L-methionine (SAM), (−)-epigallocatechin (EGC) and (−)-epicatechin (EC). (B, C) Representative radioactive traces for the methylated metabolites of (−)-epigallocatechin and (−)-epicatechin, respectively. The enzymatic formation and analysis of methylated tea catechins were as described in the Materials and methods.

[methyl-^{3}H]*S*-adenosyl-L-methionine (0.25 μCi) resulted in formation of two less polar radioactive metabolite peaks, with retention times of 27.2 and 30.0 min, respectively (figure 2A, C). The ratio of the two radioactive peaks was ~5:1.

For mass spectrometric analysis of the methylated metabolites of tea catechins, we first used the HPLC to isolate sufficient amount of the 22.0-min metabolite peak formed from (−)-epigallocatechin and the 27.2-min metabolite peak formed from (−)-epicatechin. Analysis by liquid chromatography-mass spectrometry (LC/MS) of the metabolite derived from (−)-epigallocatechin (MW = 306) revealed a

base peak (deprotonated ion) with a mass of 319, consistent with the identity of this metabolite as a monomethylated metabolite of (−)-epigallocatechin. Similarly, analysis of the major metabolite derived from (−)-epicatechin (MW = 290) revealed a base peak (deprotonated ion) with a mass of 303, consistent with the identity of this major metabolite as a monomethylated metabolite of (−)-epicatechin.

Kinetic studies on the O-methylation of catechol-containing tea polyphenols by rat liver cytosol

When (−)-epicatechin (30 μM) was used as substrate for metabolic *O*-methylation by rat liver cytosol, formation of methylated products was dependent on incubation time (figure 3, upper) and cytosolic protein concentration (figure 3, lower). The dependence of *O*-methylation of a tea catechin on the concentrations

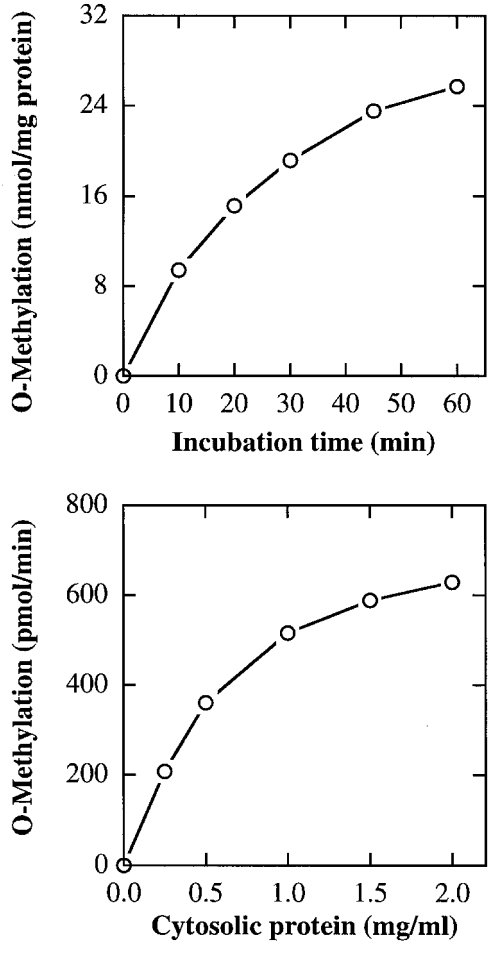


Figure 3. Dependence of hepatic *O*-methylation of (−)-epicatechin on incubation time (upper) and cytosolic protein concentration (lower). The incubation conditions and HPLC analyses were as described in the Materials and methods. Data are the mean of triplicate determinations.

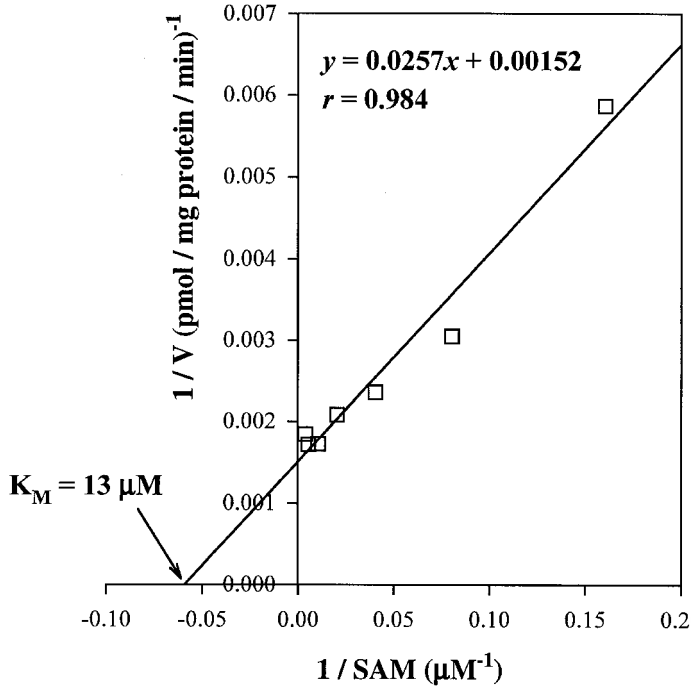


Figure 4. Double-reciprocal plot of increasing concentrations of *S*-adenosyl-L-methionine (SAM) versus the rate of *O*-methylation of (−)-epicatechin. The incubation conditions and HPLC analyses were as described in the Materials and methods. Each point is the mean of triplicate determinations.

of *S*-adenosyl-L-methionine follows typical Michaelis–Menten kinetics, with a $K_m = \sim 13\ \mu M$ as calculated from a double-reciprocal plot (figure 4).

We also determined the pH dependence (from 4.5 to 13) for metabolic *O*-methylation of (−)-epicatechin by rat liver cytosol (figure 5). Rates of (−)-epicatechin *O*-methylation by rat liver cytosol were highest at pH > 8.0. This pH dependence pattern is very different from that when human placental cytosol was used as the enzyme source, which showed a characteristic narrow pH optimum at ~7.4 (data not shown).

Under optimized conditions for *in vitro O*-methylation, (−)-epicatechin, (+)-epicatechin and (−)-epigallocatechin at a 10 or 100 μM concentration were all rapidly *O*-methylated by rat liver cytosol (table 1). Note that similar rates observed for the enzymatic *O*-methylation of (−)-epicatechin and (+)-epicatechin suggest that different stereochemistry (2*R*,3*R* versus 2*S*,3*S*; figure 1) between the two isomers does not significantly affect the rate of their enzymatic *O*-methylation *in vitro*. In comparison, (−)-epicatechin gallate and (−)-epigallocatechin gallate were *O*-methylated at markedly lower rates under the same reaction conditions (table 1).

We also determined the kinetic parameters (apparent K_m and V_{max}) for the *O*-methylation of (−)-epicatechin by rat liver cytosolic COMT. The rate of metabolic *O*-methylation of increasing concentrations of (−)-epicatechin at pH 7.4 or 10.0 showed typical Michaelis–Menten curve patterns (figure 6). Double-reciprocal plotting of this data showed the apparent K_m and V_{max} were 51 μM

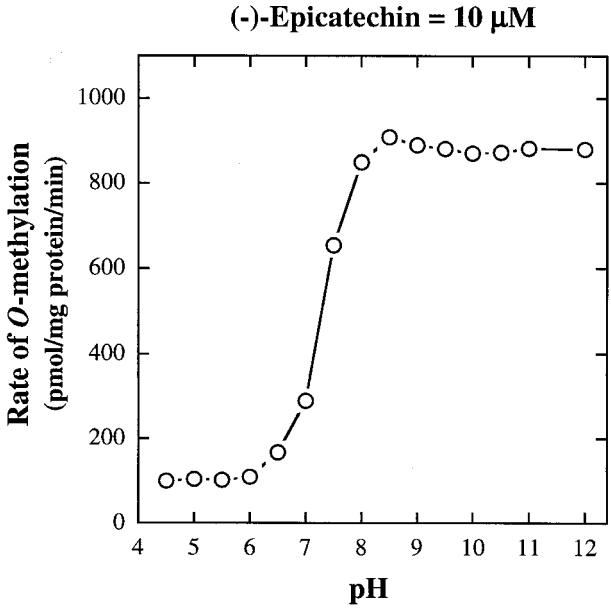


Figure 5. pH dependence of the *O*-methylation of (−)-epicatechin by rat liver cytosol. The indicated pH was the original pH of the 20 mM Tris-HCl stock buffer solution. The incubation conditions and HPLC analyses were as described in the Materials and methods. Each point is the mean of duplicate determinations.

Table 1. Rates of *O*-methylation of several catechol-containing tea polyphenols by rat liver cytosolic COMT at pH 7.4 and 10.0.

Tea polyphenol substrate	Concentration (μM)	Rate of *O*-methylation (pmol mg protein^{-1} min^{-1}) pH 7.4	pH 10.0
(−)-Epicatechin	10	713 ± 19	870 ± 14
	100	1530 ± 29	1230 ± 6
(+)-Epicatechin	10	401 ± 13	777 ± 8
	100	1261 ± 3	1226 ± 10
(−)-Epigallocatechin	10	793 ± 18	580 ± 9
	100	1296 ± 5	927 ± 7
(−)-Epicatechin gallate	10	246 ± 9	145 ± 1
	100	243 ± 11	170 ± 6
(−)-Epigallocatechin gallate	10	262 ± 3	160 ± 2
	100	285 ± 8	160 ± 3

Incubation mixture consisted of 10 or 100 μM of a tea polyphenol substrate, 50 μM [^{3}H-methyl]*S*-adenosyl-L-methionine (containing 0.2 μCi), 0.5 mg ml^{-1} rat liver cytosolic protein, 1 mM dithiothreitol and 1.2 mM $MgCl_2$ in a final volume of 1.0 ml Tris-HCl buffer (10 mM, pH 7.4 or 10.0). Incubations were carried out at 37°C for 20 min. Each point is the mean ± SD of triplicate determinations. Measurements for most of the tea polyphenols substrates were repeated three times and similar results were observed.

and 2882 pmol mg protein^{-1} min^{-1}, respectively, at pH 7.4, and 17 μM and 2093 pmol mg protein^{-1} min^{-1}, respectively, at pH 10.0. Similarly, the rate of metabolic *O*-methylation of (−)-epigallocatechin by rat liver cytosol also followed typical Michaelis–Menten kinetics (data not shown). The apparent K_m for the

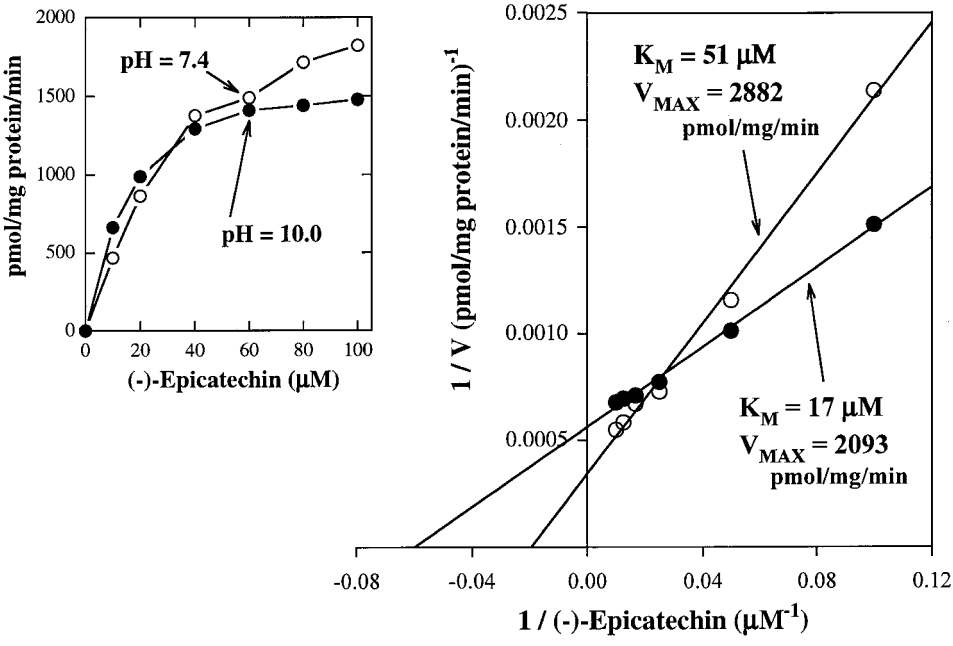


Figure 6. Double-reciprocal plots of the *O*-methylation of (−)-epicatechin by rat liver cytosol. The left inset depicts the relationship between the increasing concentrations of (−)-epicatechin and the rate of its enzymatic *O*-methylation *in vitro*. Open circles represent the data obtained at pH 7.4; solid circles at pH 10.0. The incubation conditions and HPLC analyses were as described in the Materials and methods. Each point is the mean of duplicate determinations.

O-methylation of (−)-epigallocatechin at pH 7.4 was 64 μM, and the V_{max} was 1832 pmol mg protein^{-1} min^{-1}.

Inhibition of rat liver O*-methylation of tea polyphenols by* S*-adenosyl-*L*-homocysteine*

When 5 μM (−)-epicatechin or (−)-epigallocatechin was used as substrate, their *O*-methylation by rat liver cytosol was inhibited by *S*-adenosyl-L-homocysteine (the demethylated product of *S*-adenosyl-L-methionine) in a concentration-dependent manner, with an IC_{50} ~10 μM. Kinetic analysis showed that *S*-adenosyl-L-homocysteine inhibited the COMT-catalysed *O*-methylation of (−)-epicatechin with a mixed (competitive plus non-competitive) mechanism (data not shown).

Discussion

It is known that polyphenolic dietary compounds (such as tea catechins and bioflavonoids) undergo extensive metabolism in the body to form more water-soluble conjugates, largely glucuronides and sulphates. Recently, we reported that the catechol-containing tea polyphenols could also be rapidly metabolized to monomethyl ethers by the cytosolic COMT present in human term placenta (Zhu *et al.* 2000). In the present study, it was found that several tea polyphenols

were rapidly *O*-methylated by rat liver cytosolic COMT. The results showed that (−)-epicatechin, (+)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate are all substrates for COMT-mediated *O*-methylation. Under the same conditions for *in vitro O*-methylation, (−)-epicatechin, (+)-epicatechin and (−)-epigallocatechin were rapidly *O*-methylated by rat liver cytosol, whereas (−)-epicatechin gallate and (−)-epigallocatechin gallate were *O*-methylated at significantly lower rates.

When (−)-epicatechin was used as a representative substrate, its *O*-methylation by rat liver cytosolic COMT showed characteristic dependence on the incubation pH, with maximal velocity observed at pH 8–12 (figure 5). Notably, when the same incubation conditions and the same substrate were used, human placental cytosolic COMT showed a narrow pH optimum at ~7.4 (Zhu *et al.* 2000), which is very different from the pH dependence observed in this study with rat liver cytosolic COMT. The reasons for the observed differences in pH dependence are not clear at present.

The results showed that the apparent K_m of *S*-adenosyl-L-methionine (the methyl donor) for the *in vitro O*-methylation of (−)-epicatechin is ~13 μM (figure 2). Since this *S*-adenosyl-L-methionine concentration is within the range of its tissue levels as previously reported for animals (Ueland 1982, Wagner *et al.* 1984, Zhu and Liehr 1996), it suggests that the COMT-catalysed rapid *O*-methylation of tea catechins is a biochemically feasible reaction *in vivo*.

During the metabolic *O*-methylation of catechol substrates (such as tea catechins), *S*-adenosyl-L-methionine is converted to *S*-adenosyl-L-homocysteine in equimolar amounts with the monomethyl products formed. It is known that *S*-adenosyl-L-homocysteine is an important feedback inhibitor for COMT-catalysed *O*-methylation of a variety of catechol substrates (Ueland 1982). The present results also showed that the rapid metabolic *O*-methylation of tea polyphenols is subject to the inhibitory regulation by *S*-adenosyl-L-homocysteine. This potent inhibition of the *O*-methylation of tea catechols by *S*-adenosyl-L-homocysteine may effectively prevent the endogenous *S*-adenosyl-L-methionine pool from being readily depleted, which would be important for maintaining the homeostasis of the universal methyl donor *S*-adenosyl-L-methionine during metabolic *O*-methylation of excess amounts of endogenous and/or exogenous catechols.

Regarding the possible structures of the methylated tea polyphenols, earlier studies with norepinephrine and quercetin (a catechol-containing flavonoid) showed that COMT at pH 7.4 predominantly methylates the *meta*-hydroxyl group of these substrates (Creveling 1970, Creveling *et al.* 1972, Zhu *et al.* 1994). Our HPLC analysis of the methylated products of (−)-epicatechin showed two less polar peaks with ~5:1 ratio (figure 2). Further LC/MS analysis confirmed that the quantitatively major metabolite of (−)-epicatechin was a monomethylated derivative. It is thus suspected that the major monomethylated metabolite of (−)-epicatechin likely is the 3′-*O*-methyl isomer, with the 4′-*O*-methyl isomer a minor metabolite (see figure 1 for the 3′ and 4′ positions). Similarly, it is suspected that the major methylated metabolite of (−)-epigallocatechin would be the 3′-*O*-methyl isomer. This suggestion is consistent with our chromatographic observation showing that one major monomethylated metabolite (likely the 3′-*O*-methyl metabolite) was detected on HPLC (figure 2). The largely undetectable formation of 4′-*O*-methyl metabolite from (−)-epigallocatechin as substrate might be, in part, due to the following two reasons: (1) (−)-epigallocatechin has two identical

meta-hydroxyl groups (i.e. two 3′-hydroxyl groups) that could be *O*-methylated at pH 7.4, and this would quantitatively favour the formation of 3′-*O*-methyl product relative to the 4′-*O*-methyl product when compared to (−)-epicatechin as substrate; and (2) the 4′-hydroxyl group of (−)-epigallocatechin might have lower accessibility by the enzyme than that of (−)-epicatechin.

Several recent studies showed that many common tea polyphenols such as (−)-epigallocatechin and (−)-epicatechin could be readily absorbed in human subjects after drinking tea, and some of them can reach submicromolar concentrations (0.1–0.4 μM) in the plasma (Lee *et al.* 1995, Unno *et al.* 1996, Yang *et al.* 1998, van het Hof *et al.* 1998, Li *et al.* 2000). A recent study using ^{3}H-labelled (−)-epigallocatechin gallate also showed that this tea catechin is widely distributed in the mouse tissues (Suganuma *et al.* 1998). It is thus expected that a significant fraction of the ingested catechol-containing tea polyphenols would be *O*-methylated in the body (Hackett and Griffiths 1981, 1982, Kuhnle *et al.* 2000). It will be of considerable interest to determine whether there are any important biological actions that are associated with methylated tea polyphenols. Noteworthily, Sano *et al.* (1999) reported that two quantitatively minor methylated catechins isolated from oolong tea showed a stronger anti-allergic activity than an unmethylated catechin. Likewise, it will also be of interest to determine whether tea polyphenols can effectively inhibit the metabolic *O*-methylation of endogenous catecholamines and catechol oestrogens, and to determine whether some of tea's effects are related to inhibition of the methylation metabolism of these endogenous catechols. Effective inhibition of the *O*-methylation of endogenous catecholaimes by tea components would be expected to alter their physiological functions as neurotransmitters/neurohormones, and inhibition of COMT-catalysed methylation metabolism of levodopa (the most effective agent for treatment of Parkinson's disease) would markedly increase its bioavailability in the central nervous system and reduce its peripheral side-effects. In the case of catechol oestrogens, inhibition of COMT-catalysed *O*-methylation of endogenous 2- and 4-hydroxylated oestrogens by tea polyphenols may lead to elevated tissue levels of the procarcinogenic 4-hydroxyoestradiol (Liehr 2000) plus decreased tissue levels of the anticarcinogenic 2-methoxyoestradiol (Zhu and Conney 1998). Although our earlier studies showed that chronic administration of dietary quercetin (a strong inhibitor of catechol oestrogen *O*-methylation *in vivo*) enhanced oestradiol-induced kidney tumour formation in male Syrian hamsters (Zhu *et al.* 1994, Zhu and Liehr 1994), the effects of tea polyphenols on hormonal carcinogenesis remain to be determined.

In summary, several catechol-containing tea polyphenols are very rapidly *O*-methylated by rat liver cytosolic COMT. This metabolic *O*-methylation of tea polyphenols is subject to inhibition by *S*-adenosyl-L-homocysteine. The results of the present study call for further research to determine whether some of the important biological actions of tea polyphenols may be caused by their methylated products. Studies are also needed to determine the potential inhibitory effects of tea polyphenols on the COMT-catalysed *O*-methylation of endogenous catecholamines and catechol oestrogens.

Acknowledgements

The authors thank Mao-Jung Lee and Chung S. Yang for advice on the HPLC separation of tea catechins, and Chuan Li, Bozena Winnik and Brian Buckley for

help with the liquid chromatography-mass spectrometry analysis of the *O*-methylated catechins.

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