Inhibition of Catechol O-Methyltransferase-catalyzed O-Methylation of 2- and 4-Hydroxyestradiol by Quercetin

POSSIBLE ROLE IN ESTRADIOL-INDUCED TUMORIGENESIS*

Bao Ting Zhu‡ and Joachim G. Liehr§
From the Department of Pharmacology and Toxicology, The University of Texas Medical Branch, Galveston, Texas 77555-1031

Catecholestrogens have been postulated to mediate the induction of kidney tumors by estradiol in male Syrian hamsters. In this study, we examined the mechanism of inhibition by quercetin of the catechol O-methyltransferase-catalyzed O-methylation of catecholestrogens as a basis for the previously reported enhancement of estradiol-induced tumorigenesis by this flavonoid. In hamsters treated with 50 μg of [6,7-3H]estradiol, quercetin increased concentrations of 2- and 4-hydroxyestradiol in kidney by 80 and 59%, respectively. In animals treated with two 10-mg estradiol implants, quercetin also decreased by 62% the urinary excretion of 2- and 4-hydroxyestradiol monomethyl ethers. Taken together, these results demonstrate the in vivo inhibition of the O-methylation of catecholestrogens by quercetin. S-Adenosyl-L-homocysteine, produced by the methylation of catecholestrogens, noncompetitively inhibited the O-methylation of 2- and 4-hydroxyestradiol by hamster kidney cytosolic catechol O-methyltransferase (IC_{50} approximately 10–14 μM). Due to the rapid O-methylation of quercetin itself, quercetin decreased renal concentrations of S-adenosyl-L-methionine by approximately 25% in control or estradiol-treated hamsters and increased concentrations of S-adenosyl-L-homocysteine by 5–15 nmol of wet tissue, which was estimated to cause a 30–70% inhibition of the enzymatic O-methylation of catecholestrogens. Quercetin or fisetin (a structural analog) inhibited the O-methylation of 2- and 4-hydroxyestradiol by a competitive plus noncompetitive mechanism (IC_{50} approximately 2–5 μM). These results suggest that the in vivo O-methylation of catecholestrogens is inhibited more by S-adenosyl-L-homocysteine than by quercetin. The accumulation of 2- and 4-hydroxyestradiol during co-administration of estradiol and quercetin may enhance metabolic redox cycling of catecholestrogens and thus estradiol-induced kidney tumorigenesis.

The chronic administration of natural or synthetic estrogens such as estradiol (E_2) or diethylstilbestrol to male Syrian hamsters induces kidney tumors with an incidence approaching 100% (1). Estrogens are complete carcinogens, i.e. tumor initiators and promoters in this animal model, which is thus useful for studying the multiple roles of estrogens in the development of hormone-associated cancers. The metabolic redox cycling of CE metabolites or of diethylstilbestrol between their hydroquinone and quinone forms has been established as a mechanism of metabolic activation (2), because this process generates potentially mutagenic free radicals in addition to chemically reactive estrogen semiquinone/quinone intermediates (3, 4, 5). These reactive chemical species mediate damage to DNA and other cellular components in the target organ of carcinogenesis in analogy to some classical chemical carcinogens (4–8) and thus may participate in the initiation of tumors. This mechanistic hypothesis has been probed by the co-administration of quercetin to hamsters treated chronically with E_2 (9), because this flavonoid is a good inhibitor of the in vitro O-methylation of CE by purified porcine liver COMT (9). Quercetin itself is also an excellent substrate for COMT (10). The inhibition of the enzymatic conversion of CE to methoxyestrogens in hamster kidney by quercetin was expected to result in an accumulation of CE, the substrates for metabolic redox cycling, and thus to enhance the induction of renal tumors by E_2. Consistent with this hypothesis, a diet supplemented with quercetin significantly enhanced the severity of E_2-induced tumorigenesis (9), but it did not enhance (rather, slightly decreased) the induction of tumors by diethylstilbestrol, which may directly undergo metabolic redox cycling without initial conversion to catechol metabolites (2). The enhancement of E_2-induced kidney tumorigenesis by quercetin also contrasts sharply with the inhibition by this flavonoid of tumors in several other animal models. For instance, this flavonoid decreases the incidence of 7,12-dimethylbenz(a)anthracene- and N-nitrosomethylurea-induced mammary tumors in rats (11) and azoxymethanol-induced colonic neoplasms in mice (12). Taken together, these observations suggest that the selective enhancement by quercetin of E_2 (but not diethylstilbestrol)-induced kidney tumorigenesis in hamsters may be due to a specific potentiating effect rather than to a nonspecific co-carcinogenesis by this flavonoid. The inhibition of renal O-methylation of 2- and 4-OH-E_2, which may accumulate in kidneys of hamsters treated with E_2 and quercetin, may increase the concentrations of substrates for metabolic redox cycling of CE (2), increase the production of potentially mutagenic free radicals (3–5), and thereby potentiate E_2-induced renal tumorigenesis.

To evaluate the in vivo inhibition of the O-methylation of CE metabolites by quercetin, we first determined the concentrations of unmetabolized CE in kidney and of methoxyestrogens...
Inhibition of Catecholestrogen O-Methylation by Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone), hesperidin (3,3′,4′,5-tetrahydroxyflavone), and naringenin (3,3′,4′-trihydroxyflavone) in DMN-induced hepatic tumors also previously served a protective role against urinary bladder carcinomas (15, 16) and lung tumors (17). In addition, a significant decrease in urinary excretion of mutagenic tests in both hamsters (18) and rats (19) treated with 3% quercetin in the diet or a diet supplemented with 3% quercetin has been consistently observed by others (10, 13, 14). The reaction mixture was then treated with two subcutaneous implants of 10 mg of E2. Portions (100 μl) of the first 48-h urine samples following E2 implantation were collected in metabolic cages (caged by Lab Products Inc., Maywood, NJ) and were extracted 3 times with 7 ml of diethyl ether for the determination of concentrations of unconjugated methoxyestrogens. For the determination of total methoxyestrogen pools (unconjugated methoxyestrogens, conjugated metabolites), 1000 μl aliquots of urine were first hydrolyzed at 37 °C for 24 h by β-glucuronidase (10,000 units) and sulfatase (2000 units) in a final volume of 0.5 ml of sodium acetate buffer (0.5 M, pH 6.0). The enzymatic hydrolyses were arrested by the addition of 1 ml of 1 M citric acid and 0.1 ml of 50 n HCl. The mixtures were then extracted 3 times with 7 ml of diethyl ether. The combined ether extracts were evaporated to dryness under a stream of nitrogen gas and then assayed by gas chromatography as described previously (15, 16).

Rats—To avoid variations caused by ingestions of differing amounts of quercetin by individual hamsters, 30 mg of quercetin (in 2 ml of syrup) was administered to each hamster (8 weeks old) by intragastric intubation. Control animals received 2 ml of syrup (vehicle). After a 4-h period, each hamster was decapitated and the kidneys and livers were immediately removed and washed 2–3 times in ice-cold normal saline solution. Two kidneys or 0.4–0.6 g liver tissues were weighed and homogenized for 1–2 min in 3 ml of 0.2 n HCl containing 50 mM ascorbic acid. The homogenates were dialyzed 3 times with 10 ml of ethyl acetate saturated with 0.2 n HCl. The pooled extracts were treated with 1 g of anhydrous sodium sulfate for 30 min to remove any water component, transferred to another container, and dried under a stream of nitrogen gas. After the dried extracts were redissolved in 200 μl of methanol, 800 μl of 100 mM Tris base buffer (pH 8.3) and 25 mg of neutral alumina were added. The neutral alumina (which adsorbs quercetin) was precipitated by a brief centrifugation, and the supernatant cytosolic fractions were filtered (0.45-m pore size). The filtrates were then redissolved in 1 ml of water and analyzed by HPLC as described previously (20).

MATERIALS AND METHODS

Chemicals—The chemicals used in this study were obtained from the following sources. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), hesperidin (3,3′,4′,5-tetrahydroxyflavone), and naringenin (3,3′,4′-trihydroxyflavone) were purchased from Sigma, SAH, dithiobisretol, porcine liver COMT (1720 units/ml of protein, purified by affinity column procedure), β-glucuronidase (Type IA), and sulfatase (Type H-1) from Sigma. Fisetin (3,3′,4′,7-tetrahydroxyflavone) was from Aldrich, and [methyl-3H]SAM (specific activity, 11.2–13.5 Ci/mmol) and [6,7-3H]E2 (specific activity, 53.5 Ci/mmol) were from DuPont NEN. A special diet supplemented with 3% quercetin (by weight) was prepared by Dyets (Houston, TX). To determine the contribution of increased tissue levels of SAH to the noncompetitive component of inhibition of the O-methylation of CE by quercetin as observed previously (9), mice were allowed to acclimatize for at least one week prior to any experiment.

Preparation of Hamster Kidney Cytosolic Fractions—All procedures were carried out at 0–4 °C. Kidneys from 2-month-old male Syrian hamsters were homogenized in 1.4% potassium chloride solution containing 10 mM EDTA, pH 7.4. Tissue homogenates were centrifuged at 9,000 × g for 10 min, and supernatants were pooled and filtered through two layers of cheesecloth to remove lipid clots. The filtrates were then recentrifuged at 105,000 × g (4 °C) for 60 min, and the supernatant cytosolic fractions were filtered (0.45-μm pore size). The proteins in the filtrate were precipitated by slowly adding ice-cold ethanol to a final concentration of 80%. The protein precipitates were collected by centrifugation at 9,000 × g for 10 min, and then resuspended in 10 ml of Tris-HCl (pH 7.4) to a protein concentration of 2 mg/ml. Aliquots of these cytosolic preparations were stored at −80 °C.

Inhibition of COMT-catalyzed O-Methylation of CE by Quercetin and SAH—The reaction mixture consisted of 250–500 μl of cytosolic protein from hamster kidney, 1.2 mM MgCl₂, 200 μM SAM iodide (containing 0.5 μCi of [methyl-3H]SAM), 1 mM dithiobisretol, and varying concentrations of CE in a final volume of 1.0 ml of Tris-HCl buffer (50 mM, pH 7.4). The reaction was started by addition of cytosolic protein of hamster kidney, and carried out at 37 °C for 30 min. The reaction was arrested by rapidly cooling to ice temperatures. The reaction mixture was then immediately extracted with 7 ml of ice-cold n-heptane. After centrifugation at 1000 × g for 10 min, 3-ml aliquots of the organic extracts were analyzed for radioactivity content by liquid scintillation counting (Packard Instruments, model LS 5000TD).

Tissue Concentrations of 2-OH-E2 and 4-OH-E2—To avoid variations caused by ingestions of differing amounts of quercetin by individual hamsters, 30 mg of quercetin (in 2 ml of syrup) was administered to each hamster (8 weeks old) by intragastric intubation. Control animals received 2 ml of syrup (vehicle). After a 4-h period, each hamster was decapitated and the kidneys and livers were immediately removed and washed 2–3 times in ice-cold normal saline solution. Two kidneys or 0.4–0.6 g liver tissues were weighed and homogenized for 1–2 min in 3 ml of 0.2 n HCl containing 50 mM ascorbic acid. The homogenates were dialyzed 3 times with 10 ml of ethyl acetate saturated with 0.2 n HCl. The pooled extracts were treated with 1 g of anhydrous sodium sulfate for 30 min to remove any water component, transferred to another container, and dried under a stream of nitrogen gas. After the dried extracts were redissolved in 200 μl of methanol, 800 μl of 100 mM Tris base buffer (pH 8.3) and 25 mg of neutral alumina were added. The neutral alumina (which adsorbs quercetin) was precipitated by a brief centrifugation, and the supernatant cytosolic fractions were filtered (0.45-m pore size). The filtrates were then redissolved in 1 ml of water and analyzed by HPLC as described previously (20).

Tissue Concentrations of Quercetin—4-week-old male hamsters received a diet supplemented with 3% quercetin for 2 weeks or 6.5 months. Blood samples (1–3 ml), obtained by cardiac puncture of animals anesthetized with CO₂, were centrifuged at approximately 3000 × g for 10 min. Aliquots (300 μl) of supernatant plasma were transferred to tubes containing 1 ml of 0.2 n Tris base buffer (pH 8.2), 5 mM ascorbic acid, and 100 mg of neutral alumina. After the cardiac puncture, the animals were immediately decapitated and kidneys were removed, weighed, and homogenized in 4 volumes (v:w) of 30% aqueous methanol. Tris base buffer (0.5 ml, pH 8.2) containing 5 mM ascorbic acid and 100 mg of neutral alumina were added. The neutral alumina (which adsorbs quercetin) was precipitated by a brief centrifugation, and the supernatant was removed with a Pasteur pipette. The neutral alumina precipitates were washed 3 times with 5 ml of 20 mM Tris base solution containing 0.2% EDTA. Quercetin was eluted from the neutral alumina with 300 μl of 0.25 M HCl-50% methanol solution and analyzed by HPLC using a reversed phase C₁₈ column (150 × 4.6 mm, particle size 5 μm; Rainin Instrument Co., Torrance, CA) with UV detection at 340 nm. The HPLC system consisted of a Waters model 510 pump, a model 501 solvent delivery system, a model 490 multi-wavelength detector, and a model 740 data module. The mobile phase was 50%
aqueous methanol containing 10 mM KH$_2$PO$_4$, adjusted to pH 2.40 with H$_3$PO$_4$.

**RESULTS**

**Effect of Quercetin on Estrogen Metabolite Concentrations in Tissue and Urine**

Tissue Concentrations of 2-OH-E$_2$ and 4-OH-E$_2$—1 h after an intraperitoneal injection of 50 µg of [6,7-$^3$H]E$_2$ to male Syrian hamsters, concentrations of 2-OH-E$_2$ and 4-OH-E$_2$ in kidney were 2.7 ± 0.4 and 1.3 ± 0.3 ng/g of wet tissue, respectively, and corresponding concentrations in liver were 2.1 ± 0.3 and 0.6 ± 0.2 ng/g of wet tissue, respectively (Fig. 1). When hamsters were treated with 30 mg of quercetin by intragastric intubation 15 min prior to the intraperitoneal injection of 50 µg of E$_2$ (in 50 µl of corn oil, containing 100 µCi of [6,7-$^3$H]E$_2$), the concentrations of 2-OH-E$_2$ and 4-OH-E$_2$ were increased by 80% (*p < 0.01) and 59% (**p < 0.01), respectively (Fig. 1).

**Urinary Excretion of Methoxyestrogen Metabolites—**The urinary excretion of 2- and 4-methoxyestradiol in the first 48 h after implantation of two 10-mg E$_2$ pellets to male Syrian hamsters was 9.2 ± 4.1 and 1.4 ± 0.9 µg/24 h, respectively (Fig. 2). Pretreatment of hamsters with a diet supplemented with 3% quercetin for 2 weeks decreased the urinary excretion of 2- and 4-methoxyestradiol in the first 48 h by 65% (p < 0.05) and 53%, respectively (Fig. 2).

In summary, concentrations of CE metabolites in kidney of male hamsters injected with 50 µg of [6,7-$^3$H]E$_2$ were comparable to those in liver. Treatment of hamsters with quercetin significantly increased CE concentrations in kidney and concomitantly decreased urinary concentrations of methoxysterogens. Taken together, these results demonstrate an in vivo inhibition by quercetin of the O-methylation of CE metabolites during co-treatment of hamsters with E$_2$.
Inhibition of Catecholestrogen O-Methylation by Quercetin

In Vitro Inhibition of the O-Methylation of CE by SAH—The in vitro O-methylation of 2-OH-E2 or 4-OH-E2 (at 10 and 40 μM concentrations) by hamster kidney cytosolic COMT was inhibited by the addition of SAH in a concentration-dependent manner. SAH inhibited the O-methylation of two different concentrations (10 and 40 μM) of 2-OH-E2 or 4-OH-E2 with very similar inhibition potencies (IC50 values of approximately 10–14 μM; Fig. 3). The rates of O-methylation of 2.5–50 μM 2-OH-E2 or 4-OH-E2 in the absence of inhibitors were of typical hyperbolic patterns and reached plateau rates at about 30–50 μM substrate concentrations (Fig. 4, inset). The Km values for 2-OH-E2 and 4-OH-E2 were 4.6–4.8 and 9.5–11.3 μM, respectively, and corresponding Vmax values were 82.3–84.6 and 66.7–68.8 pmol/mg of protein/min, respectively (Fig. 4 and Tables I and II). In the presence of varying concentrations (5, 10, 20, and 40 μM) of SAH, the maximal velocities (Vmax) for the O-methylation of 2-OH-E2 and 4-OH-E2 were inhibited in a concentration-dependent manner, whereas the corresponding Km values for 2- and 4-OH-E2 substrates were not altered (Fig. 4 and Table II), indicating a pure noncompetitive mechanism of enzyme inhibition with respect to CE substrates.

To determine the mechanism of the noncompetitive inhibition of CE O-methylation by SAH, we examined the possibility of decreased interaction of the methyl donor SAM with COMT in the presence of SAH. The effect of varying concentrations of SAH on the rates of O-methylation of 50 μM 2-OH-E2 by kidney cytosolic COMT exhibited a hyperbolic curve pattern, with the Km value for SAM approximately 7 μM (Fig. 5). In the presence of 5, 10, and 20 μM SAH, the apparent Km values for SAM in the O-methylation of 50 μM 2-OH-E2 were increased proportionally to the SAH concentrations present, whereas the Vmax values were not altered (Fig. 5), thus indicating that SAH competitively inhibited the interaction of SAM with COMT.

| Treatment (No. of animals) | SAM | SAH | Ratio of SAH/SAM |
|----------------------------|-----|-----|------------------|
| Control (n = 4)            | 42.2 ± 5.1 | 16.6 ± 4.2 | 0.38              |
| E2 (n = 3)                 | 40.6 ± 4.8 | 18.9 ± 3.1 | 0.47              |
| 3% quercetin (n = 5)       | 31.4 ± 6.0 | 22.7 ± 3.6 | 0.72              |
| 3% quercetin + E2 (n = 4)  | 32.1 ± 3.2 | 31.3 ± 4.3 | 0.98              |

*p < 0.05 (one-way analysis of variance) compared with the control.

Inhibition of the O-Methylation of CE Metabolites by Quercetin

The in vitro O-methylation of 2-OH-E2 or 4-OH-E2 by hamster kidney cytosolic COMT was inhibited by the addition of quercetin or its structural analog, fisetin, in a concentration-dependent manner (Fig. 6). Quercetin or fisetin displayed similar inhibition potencies for the O-methylation of 10 μM 2-OH-E2 (both IC50 values approximately 8 μM; Fig. 6, upper panel) or 4-OH-E2 (both IC50 values approximately 2 μM; Fig. 6, lower panel). In contrast, hesperetin, a monomethylated flavonoid, showed little or no inhibitory effect. In the presence of varying
concentrations of quercetin or fisetin, the V\textsubscript{max} values of this enzymatic O-methylation of 2-OH-E\textsubscript{2} and 4-OH-E\textsubscript{2} were markedly inhibited in a concentration-dependent manner (Fig. 7 and Table II). The marked decreases in V\textsubscript{max} values in the presence of flavonoids indicated a substantial contribution by a noncompetitive mechanism of enzyme inhibition. In addition to the observed decreases in V\textsubscript{max} values, the K\textsubscript{m} values were simultaneously increased in the presence of quercetin or fisetin (Fig. 7 and Table II), thus indicating a mixed (competitive plus noncompetitive) mechanism of enzyme inhibition as reported previously with a purified porcine liver COMT preparation (4).

Tissue Levels of Quercetin—The concentrations of unmetabolized quercetin in plasma, kidney, and liver of hamsters treated with a dietary supplement of 3% quercetin for 2 weeks were 0.22 ± 0.19 μM, 0.43 ± 0.37, and 0.37 ± 0.22 nmol/g of wet tissue, respectively (Table III). Similar concentrations of unmetabolized quercetin were obtained in plasma, liver, and kidney of hamsters treated with this 3% quercetin diet for 6.5 months (Table III). In contrast, concentrations in tissues from animals on a control diet were below the detection limit (<0.05 μM quercetin in plasma or <0.05 nmol of quercetin/g of wet tissue; Table III).

In summary, quercetin inhibited the COMT-catalyzed O-
Inhibition of Catecholestrogen O-Methylation by Quercetin

The CE concentrations in the kidney of male hamsters injected with 50 μg of [6,7-3H]E2 were comparable with those in liver. In contrast, the enzyme activities in kidney catalyzing the 2- and 4-hydroxylation of E2 are at least 1 order of magnitude lower than those in liver (21). These relatively high concentrations of CE metabolites in kidney may be explained in part by the lower detoxifying enzyme activities in this organ compared with those in liver (22). The larger increase in renal CE concentrations compared with hepatic concentrations in male hamsters co-treated with quercetin and E2 also suggests that detoxification of CE in liver may remain intact, whereas that in kidney may be compromised. Second, the concentrations of endogenous catecholamines (substrates and competitive inhibitors of COMT-catalyzed O-methylation) in hamster kidney are more than 40-fold higher than those in liver (14), which inhibits the O-methylation of CE (14) and thereby may contribute to the high levels of CE in kidney. Finally, in addition to direct aromatic hydroxylation of parent estrogen, CE may be formed by metabolic deconjugation of estrogen conjugates such as estrogen glucuronides and methyl ethers (16).

This metabolic deconjugation has been shown to be an important source of CE production in hamster kidney but is less important in liver compared with hepatic CE production by direct hydroxylation of parent estrogens (16).

Our study clearly demonstrates that the COMT-catalyzed O-methylation of CE is inhibited by quercetin via two different mechanisms, i.e. the direct inhibition of COMT by quercetin and the indirect inhibition by elevated tissue concentrations of SAH. Quercetin itself is a substrate for COMT (10) and thus competitively inhibits the O-methylation of CE substrates by competing for the methylating enzyme. Although SAH inhibited COMT in a noncompetitive fashion with respect to CE substrates, the kinetic analysis revealed that SAH competitively inhibited the association of the methyl donor SAH with the methylating enzyme. Thus, SAH may decrease concentrations of the COMT-SAM complex and increase those of the COMT-SAH complex. A decrease in the concentration of the COMT-SAM complex is consistent with a decrease in the Vmax value and unchanged Km value (a noncompetitive inhibition). This noncompetitive inhibition by SAH also explains the noncompetitive component of enzyme inhibition by quercetin or fisetin in vitro, because the O-methylation of either flavonoid will increase the concentrations of SAH. In addition to these two mechanisms, an approximately 25% decrease in renal pools of SAM (the methyl donor) during quercetin administration may also be a contributing factor for the decreased metabolism of CE by O-methylation in vivo.

In hamster kidney, the inhibition of CE O-methylation by SAH likely dominates over the direct inhibition by quercetin for the following reasons. (i) Despite the chronic administration of a high dose of quercetin to animals (3% in the diet), plasma or tissue concentrations of unmetabolized quercetin do not exceed 0.5 nmol/ml or g of wet tissue, respectively. Quercetin has previously been shown to undergo rapid O-methylation and/or other conjugation reactions (10). The low concentrations of unmetabolized quercetin in blood and in tissues observed in this study are in close agreement with previous studies (23). (ii) The marked increase in renal concentrations of SAH and in SAH/SAM ratios during treatment with quercetin makes it likely that inhibition by SAH is the dominant form of inhibition in quercetin-treated animals. The increase in tissue levels of

| Treatment (No. of animals) | Plasma | Liver | Kidney |
|---------------------------|--------|-------|--------|
| 3% Quercetin diet         |        |       |        |
| 2 weeks (n = 4)           | 0.22 ± 0.19 | 0.42 ± 0.37 | 0.37 ± 0.22 |
| 6.5 months (n = 4)        | 0.28 ± 0.12 | 0.58 ± 0.34 | 0.50 ± 0.13 |
| Control diet              |        |       |        |
| 2 weeks (n = 1)           | <0.1   | <0.1  | <0.1  |
| 6.5 months (n = 2)        | <0.1   | <0.1  | <0.1  |

**DISCUSSION**

Our data show that administration of quercetin to male Syrian hamsters treated with E2 increased concentrations of CE metabolites in kidney (the target organ of tumorigenesis) and concomitantly decreased excretion of methoxyestrogens in the urine. These results demonstrate an in vivo inhibition of the O-methylation of CE metabolites during co-treatment of hamsters with quercetin and E2. Our results also show that treatment of hamsters with quercetin decreased concentrations of SAM (the cofactor for COMT-catalyzed O-methylation reactions) and concomitantly increased concentrations of SAH (the demethylated product of SAM), and thereby markedly increased the SAH/SAM ratios in hamster kidney.

The CE concentrations in the kidney of male Syrian hamsters treated with E2 increased concentrations of 3% Quercetin diet and concomitantly decreased excretion of methoxyestrogens in the urine. These results demonstrate an in vivo inhibition of the O-methylation of CE metabolites during co-treatment of hamsters with quercetin and E2. Our results also show that methylation of 2-OH-E2 and 4-OH-E2 in vitro by a competitive plus noncompetitive mechanism. The concentrations of quercetin in circulation and in tissues were low relative to the concentrations required for inhibiting the enzymatic O-methylation of CE in vitro. A comparison of the in vitro inhibition of CE O-methylation by SAH and quercetin with their available tissue concentrations suggests that the direct inhibition of COMT by quercetin may be less pronounced in vivo, whereas the inhibition by SAH may be a dominant mechanism.

**FIG. 7.** Double-reciprocal plots of the O-methylation of 2.5-40 μM 2-OH-E2 and 4-OH-E2 catalyzed by hamster kidney cytosolic COMT in the absence or presence of two different concentrations of quercetin or fisetin. The incubation conditions are described under "Experimental Procedures." Each value is the mean of replicate determinations. Intra-assay variations were within 7%.

*TABLE III*

Plasma and tissue levels of quercetin in male Syrian hamsters given a diet supplemented with 3% quercetin for 2 weeks or 6.5 months.

The quercetin content of this diet was confirmed by an HPLC analysis to be 97 ± 2% of the indicated concentration, whereas the content in normal rodent chow was below the detection limit of the assay. Blood samples, obtained by cardiac puncture, and freshly exercised liver and kidney samples were analyzed for quercetin concentrations by an HPLC method described under "Materials and Methods."

| Treatment (No. of animals) | Plasma | Liver | Kidney |
|---------------------------|--------|-------|--------|
| 3% Quercetin diet         |        |       |        |
| 2 weeks (n = 4)           | 0.22 ± 0.19 | 0.42 ± 0.37 | 0.37 ± 0.22 |
| 6.5 months (n = 4)        | 0.28 ± 0.12 | 0.58 ± 0.34 | 0.50 ± 0.13 |
| Control diet              |        |       |        |
| 2 weeks (n = 1)           | <0.1   | <0.1  | <0.1  |
| 6.5 months (n = 2)        | <0.1   | <0.1  | <0.1  |
SAH during 3% quercetin treatment results from rapid and extensive O-methylation of this flavonoid as demonstrated previously (10). Based on our enzyme kinetic studies, the magnitude of increase in renal SAH concentrations (approximately 10 μM) is estimated to significantly inhibit the metabolic O-methylation of CE in vivo. Thus, it is suggested that a markedly increased demand on the circulating one-carbon pool due to the O-methylation of quercetin or other catechols may result in an increase in tissue pools of SAH and a concomitant inhibition of the O-methylation in vivo of CE metabolites. This metabolic change may be the basis of the previously observed increase in the severity of kidney tumorigenesis in hamsters treated with E₂ and quercetin (9) and supports the postulated role of CE, in particular 4-OH-E₂, in the induction of estrogen-associated tumors (4, 5). CE have previously been shown to undergo metabolic redox cycling, a process to generate potentially mutagenic free radicals in addition to other chemically reactive species such as estrogen semiquinones and quinones (3, 6–8). Details of this mechanism of DNA damage induced by redox cycling of CE are discussed in a recent review (5).

In summary, the administration of quercetin to male Syrian hamsters treated with E₂ inhibits the O-methylation of CE metabolites and thereby increases their concentrations in tissues and decreases urinary excretion of methoxyestrogen conjugates. The increase in CE concentrations together with the previously reported increase in the severity of E₂-induced kidney tumorigenesis in hamsters is taken as evidence in support of a critical role of redox cycling of CE metabolites and free radical generation in the induction of hormone-associated cancers.

REFERENCES
1. Kirkman, H. (1959) Natl. Cancer Inst. Monogr. 1, 1–57
2. Liehr, J. G., Ulubelen, A. A., and Strobel, H. W. (1986) J. Biol. Chem. 261, 16865–16870
3. Roy, D., and Liehr, J. G. (1988) J. Biol. Chem. 263, 3646–3651
4. Liehr, J. G., and Roy, D. (1990) Free Radical Biol. Med. 8, 415–423
5. Liehr, J. G. (1994) Polycyclic Aromatic Compounds 6, 229–239
6. Han, X., and Liehr, J. G. (1994) Cancer Res 54, 5515–5517
7. Han, X., and Liehr, J. G. (1994) Carcinogenesis 15, 997–1000
8. Han, X., and Liehr, J. G. (1995) Carcinogenesis 16, 2571–2574
9. Zhu, B. T., and Liehr, J. G. (1994) Toxicol. Appl. Pharmacol. 125, 149–158
10. Zhu, B. T., Ezell, E. L., and Liehr, J. G. (1994) J. Biol. Chem. 269, 232–239
11. Verma, A. K., Johnson, J. A., Gould, M. N., and Tainer, M. A. (1988) Cancer Res. 48, 5754–5758
12. Deschner, E. E., Ruperto, J., Wong, G., and Newmark, H. L. (1991) Carcinogenesis 12, 1193–1196
13. Hoffman, A. R., Paul, S. M., and Axelrod, J. (1979) Cancer Res. 39, 4584–4587
14. Zhu, B. T., and Liehr, J. G. (1993) Arch. Biochem. Biophys. 304, 248–256
15. Roy, D., Hachey, D. L., and Liehr, J. G. (1991) J. Chromatogr. Biomed. Appl. 567, 309–318
16. Zhu, B. T., Enongene, E. N., Antoniak, S. K., Sarabia, S. F., Ricci, M. J., and Liehr, J. G. (1996) Toxicol. Appl. Pharmacol., in press
17. Hersey, R. M., Williams, K. I. H., and Weisz, J. (1981) Endocrinology 109, 1912–1920
18. Mondschein, J. S., Hersey, R. M., Walega, M. A., and Weisz, J. (1985) Endocrinology 117, 2339–2346
19. Bui, Q. D., and Welsz, J. (1989) Endocrinology 124, 1085–1087
20. Chun, B.-G., Park, W. K., and Kim, S. (1983) J. Chromatogr. 264, 321–328
21. Welsz, J., Bui, Q. D., Roy, D., and Liehr, J. G. (1992) Endocrinology 131, 655–661
22. Roy, D., and Liehr, J. G. (1989) Cancer Res. 49, 1475–1480
23. Gugler, R., Leschik, M., and Dengler, H. J. (1975) Eur. J. Clin. Pharmacol. 9, 229–234