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THE EFFECTS OF pH AND RAT INTESTINAL CONTENTS ON THE LIBERATION OF ELLAGIC ACID FROM PURIFIED AND CRUDE ELLAGITANNINS

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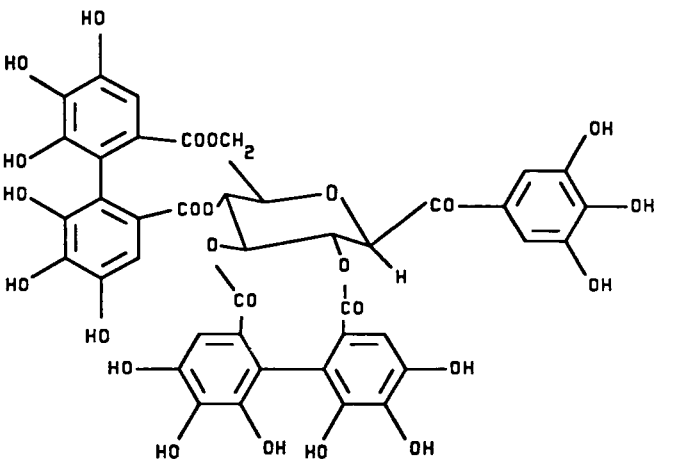
ABSTRACT.—This study was undertaken to measure the liberation in vitro of ellagic acid [**2**], a naturally occurring inhibitor of carcinogenesis, from precursor ellagitannins under conditions found in the gut tract. Enzymes, namely β-glucosidase, esterases, and α-amylase, were incubated with raspberry extract. In addition, raspberry extract and casuarictin [**1**] were treated at different pH's and with the contents of small intestine and cecum from rats fed AIN-76A diet. The esterase activity of the enzyme samples was measured spectrophotometrically using *p*-nitrophenol acetate as the substrate, and the amount of ellagic acid [**2**] released from all samples was analyzed by hplc. The hydrolysis of the ellagitannins was not catalyzed by any of the purified enzymes tested, and components of the raspberry extract were found to inhibit the purified esterases noncompetitively. Casuarictin [**1**] was hydrolyzed to yield high quantities of ellagic acid [**2**] when placed in buffer at pH 7 and 8, or when incubated with cecal contents for two hours. The release of ellagic acid [**2**] from the raspberry extract was optimal at pH 8, and maximal release in cecal contents occurred with 1 h. Small intestinal contents had no significant effect on ellagic acid liberation from either casuarictin [**1**] or raspberry extract.

Epidemiologic studies have shown that populations who consume a diet rich in fruits and vegetables tend to have a decreased risk of developing cancer (1). Numerous constituents of fruits and vegetables such as phenols, indoles, flavones, isothiocyanates, and vitamins, to name a few, have demonstrated anticarcinogenic activity (2). Over the past decade, ellagic acid [**2**], a phenolic compound present in blackberries, raspberries, strawberries, cranberries, walnuts, and pecans, has shown promise as a dietary inhibitor of carcinogenesis (3,4).

In vitro studies have shown that ellagic acid [**2**] inhibits DNA adduct formation by benzo[*a*]pyrene and benzo[*a*]pyrene-7,8-dihydrodiol in mouse lung explants (5), *N*-



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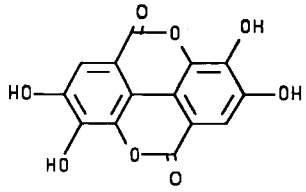

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nitrosobenzylmethylamine in rat esophageal tissues (6), and aflatoxin B_1 in rat and human tracheobronchial tissues (7). When given in the diet, ellagic acid [**2**] has been shown to reduce *N*-nitrosobenzylmethylamine-induced esophageal tumors in the rat (8), benzo[*a*]pyrene-induced lung tumors in strain A mice (9), and 2-acetylaminofluorene-induced liver tumors in rats (10). Topically applied ellagic acid [**2**] protects against skin carcinogenesis in mice induced by benzo[*a*]pyrene-7,8-diol-9,10-epoxide-2 (11), 7,12-dimethylbenz[*a*]anthracene (9), and 3-methylcholanthrene (12). Therefore, both in vitro and in vivo, ellagic acid [**2**] has demonstrated inhibition against different classes of chemical carcinogens and is effective in several tissues.

Ellagic acid [**2**] is present in plants as ellagitannins, which consist of a central core of glucose esterified with hexahydroxydiphenic acid. These precursor molecules may undergo hydrolysis with acid or base to yield ellagic acid [**2**]. In a recent report, we hydrolyzed the ellagitannins in various fruits and nuts by heating with a strong acid to quantitate the total amount of ellagic acid [**2**] present. It is not known to what extent ellagic acid is liberated from ingested ellagitannins. In the present study, the liberation of ellagic acid [**2**] from raspberry extract and casuarictin [**1**], a purified ellagitannin, was measured under different conditions of acidity and after treatment with extracts of rat intestinal contents. In addition, the effects of the raspberry extract on purified esterases were examined.

EXPERIMENTAL

CHEMICALS.—Reagent grade extraction solvents and hplc grade MeOh were obtained from Curtin Matheson Scientific (Houston, TX). Na_3PO_4, *p*-nitrophenol, Ac_2O, and ellagic acid [**2**] dihydrate (>97% purity as determined by hplc) were obtained from Aldrich Chemical (Milwaukee, WI). General esterases, β-glucosidase, and α-amylase were purchased from Sigma Chemical Co. (St. Louis, MO).

p-Nitrophenol acetate was prepared by reacting *p*-nitrophenol with an excess of Ac_2O for 30 min at 100°. The purity of the *p*-nitrophenol acetate was confirmed to be >97% by tlc, determination of melting point, and uv spectroscopy.

The raspberry extract was made from 1 g of freeze-dried raspberries (*Rubus ideaus*) that were extracted with MeCOMe-H_2O (70:30) as previously described (3).

ISOLATION OF CASUARICTIN [**1**].—Frozen strawberries (*Fragaria ananassa*) of the Kent variety (450 g obtained from a local grower) were homogenized in MeCOMe-H_2O (7:3) (4 × 1 liter), and the resulting extract was filtered. After removal of the MeCOMe, the aqueous solution was extracted successively with Et_2O (5 × 500 ml), MeCOMe (20 × 1 liter), and *n*-BuOH (10 × 1 liter). The MeCOMe extract was chromatographed over a Sephadex LH-20 column and eluted with EtOH and then with a mixture of EtOH and increasing amounts of H_2O-MeCOMe (7:3). Eight fractions were collected, and the sixth fraction was re-chromatographed over a Sephadex LH-20 column using EtOH-H_2O-MeCOMe (90:7:3) as the mobile phase. Casuarictin [**1**] was isolated from the seventh fraction and its structure was confirmed by uv, ir, nmr (^1H and ^{13}C) spectra together with hplc.

Casuarictin [**1**].—^1H nmr (400 MHz, D_2O) δ 7.06 (2H, s, galloyl), 6.73, 6.69, 6.64, 6.47 (1H each, s, 2 × hexahydroxydiphenoyl), 6.09 (1H, d, J = 8, H-1), 5.48 (1H, t, J = 9.2, H-3), 5.26 (1H, dd, J = 8, 9.2, H-2), 5.25 (1H, d, J = 13.6, H-6), 5.07 (1H, t, J = 9.6, H-4), 4.14 (1H, t, J = 9.6, H-5), 3.90 (1H, d, J = 13.6, H-6); ^{13}C nmr (100 MHz, D_2O) 170.12, 169.73, 169.30, 169.16, 165.66 (ester C=O), 118.82, 110.61, 144.92, 139.42 (galloyl carbons), 114.96, 114.88, 113.75, 113.50, 125.07,

124.93, 124.77, 124.56, 108.26, 107.81, 107.24, 107.14, 144.81, 143.91, 136.19, 135.92 (hexahydroxydiphenoyl carbons), 91.47 (C-1), 75.25 (C-2), 76.47 (C-3), 68.90 (C-4), 72.43 (C-5), 63.26 (C-6) (glucose carbons).

HARVEST OF INTESTINAL CONTENTS.—Four male F-344 rats (wt 300–400 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals were housed two per cage in rooms with 12-h light-dark cycles. The rats were acclimated to the facility for at least 1 week and observed to be free of disease. At least one full week prior to sacrifice, the rats were given AIN-76A diet (Teklad, Winfield, IA) and H_2O ad libitum.

Rats were killed by CO_2 asphyxiation, and the small intestine and cecum were excised. The contents of the small intestine and cecum of both rats were removed, pooled, and weighed. Both the small intestine and cecal fractions were diluted with 25 ml of ice-cold 10 mM Na_3PO_4, pH 7. The contents were vortexed thoroughly and centrifuged at 4000 *g* for 10 min. The supernatant was removed and stored at 4°.

MEASUREMENT OF ESTERASE ACTIVITY.—Intestinal extract (0.4 ml) was incubated with 50, 100, 250, and 500 μM *p*-nitrophenol acetate in a total volume of 2.0 ml. Duplicate samples of the reaction mixtures were kept at 37° for 10 min, and the amount of *p*-nitrophenol released was measured spectrophotometrically at 403 nm in a Beckman DU-7 spectrophotometer (Irvine, CA).

INHIBITION OF ESTERASES BY RASPBERRY EXTRACT.—Esterases (500 μl), raspberry extract (325 μl), and *p*-nitrophenol acetate were added to 10 mM Na_3PO_4, pH 7 to give 50, 100, 250, and 500 μM concentrations of *p*-nitrophenol acetate (total volume 2.0 ml). Duplicate samples were vortexed, and incubated at 37°, and the amount of *p*-nitrophenol at 7, 12, and 30 min was measured spectrophotometrically at 403 nm.

HYDROLYSIS OF CASUARICTIN [**1**] AND RASPBERRY EXTRACT.—In pH hydrolysis experiments, 100 μl of casuarictin [**1**] (1.0 mg/ml) or raspberry extract (200 μl) was added to 10 mM Na_3PO_4, pH 2, pH 7, and pH 8 (total volume 2 ml). Four replicate samples for each time point and pH were incubated at 37° for 0, 60, and 120 min in a shaking H_2O bath. The samples were stored at −20° prior to analysis.

Intestinal extract (400 μl), casuarictin [**1**] (200 μl), or raspberry extract (400 μl) was added to 10 mM Na_3PO_4, pH 7 (total volume 2 ml). Four replicate samples from each variable were incubated at 37° for 0, 30, 60, and 120 min in a shaking H_2O bath. The samples were stored at −20° prior to analysis.

Stock solutions of β-glucosidase (6.9 units/ml), esterases (5 units/ml), and α-amylase (410 units/ml) were prepared. β-Glucosidase (500 μl), esterases (500 μl), α-amylase (20 μl), and raspberry extract (150 and 325 μl) were added to 10 mM Na_3PO_4, pH 7 (total volume 2 ml). Four replicate samples for each enzyme and concentration of raspberry extract were incubated at 37° for 0, 15, and 30 min. The samples were stored at −20° prior to analysis.

ANALYSIS OF HYDROLYZED SAMPLES.—Thawed samples containing intestinal extract or purified enzymes were diluted with 3.5 ml of MeOH and centrifuged at 12,000*g* for 10 min. Buffer samples were thawed and analyzed without further treatment. All samples (200 μl) were analyzed for ellagic acid content using a Waters hplc system equipped with two model 510 pumps, a 710 WISP injection system and a model 484 Multiwavelength Detector (Waters, Milford Corp., Milford, MA). Separations were done on a 4.6 × 250 mm C-18 reversed-phase column (Altech Associates, Deerfield, IL) as described previously (3). Results obtained with casuarictin [**1**] samples were expressed as ng ellagic acid /μg of casuarictin [**1**] (± SD) and those obtained with the raspberry extract samples as μg ellagic acid /g of raspberry (dry wt) (± SD).

STATISTICAL ANALYSIS.—Results obtained from the hydrolysis experiments were analyzed for statistical significance ($p<0.05$) by one-way analysis of variance (ANOVA) and the Tukey post hoc comparison test (13).

RESULTS AND DISCUSSION

Casuarictin [**1**] was characterized by ^1H and ^{13}C nmr as well as by hplc. The ^1H- and ^{13}C-nmr spectra agree with the reported data (14–16). When chromatographed as previously (3), casuarictin [**1**] has a retention time of 22.0–22.5 min, and was 85% pure with ellagic acid [**2**] present as 5% of the impurity. The other 10% impurity was an undetermined early eluting fraction.

The activity of the commercially purified esterases and the intestinal enzymes was measured spectrophotometrically using *p*-nitrophenol acetate as the substrate. The Km and Vmax data from the enzymes are given in Table 1. Purified esterases demonstrated

TABLE 1. Determination of Vmax and Km of Esterase Enzymes.[a]

	Vmax (mmol/min)	Km (mM)
Small Intestinal Contents[b]	0.10	0.82
Cecal Contents[b]	0.10	0.12
Esterases (purified)	0.42	4.21
Esterases + Rsp.[c] 7 min	0.28	4.08
Esterases + Rsp. 12 min	0.17	4.21
Esterases + Rsp. 30 min	0.06	3.67

[a]Average measurement of the ability of the enzymes to cleave actate groups.
[b]Absorbance measured after 10 min.
[c]Raspberry extract.

more than four times the activity of those enzymes present in the small intestine that liberate acetate groups. Measured in this system, esterase activity in the cecum was minimal.

The ability of purified esterases to liberate *p*-nitrophenol was inhibited over time by the components of raspberry extract. The Km values of the esterases did not change significantly and therefore indicate a noncompetitive mode of enzyme inhibition (Table 1). Numerous studies have reported the inhibition of enzymes by the constituents of plant materials. In vitro, condensed tannins have demonstrated noncompetitive inhibition against trypsin, amylases, lipases, cellulases, and pectinases (17–19). Because raspberries have been shown to contain significant amounts of condensed tannins (20), the inhibiton of esterase activity by raspberry extract is not surprising.

Table 2 demonstrates the hydrolysis data of raspberry extract treated with purified β-glucosidase, esterases, and α-amylase. The hydrolysis of the ellagitannins in the extract was not catalyzed by any of the enzymes tested; i.e., the ellagic acid [**2**] released under the experimental conditions was not significantly different ($p<0.05$) from the control extract samples incubated in pH 7 buffer.

TABLE 2. Hydrolysis of Raspberry Extract Using Purified Enzymes.[a]

Enzyme	Time in Minutes		
	0	15	30
Control[b]	479 ± 53	N.D.[c]	574 ± 55
β-Glucosidase	479 ± 53	545 ± 47	537 ± 66
Esterases	479 ± 53	550 ± 71	560 ± 104
α-Amylase	479 ± 53	582 ± 80	578 ± 69

[a]Data expressed at μg ellagic acid/g dried raspberry ± SD.
[b]Raspberry extract control.
[c]N.D. = not determined.

Table 3 illustrates the hydrolysis data of casuarictin [**1**]. The casuarictin [**1**] was stable at pH 2 for 120 min at 37°. However, after 60 min at pH 7 and 8, the compound hydrolyzed to yield significant amounts of ellagic acid [**2**]. After incubation with contents from the small intestine, the ellagic acid [**2**] present at the various time points was not significantly different from the amount found in the control samples at pH 7. In contrast, casuarictin [**1**] was hydrolyzed to the greatest extent in cecal contents at 30, 60, and 120 min.

TABLE 3. Hydrolysis of Casuarictin Using pH and Gut Contents.[a]

	Time in Minutes			
	0	30	60	120
pH 2	20.1 ± 0.3	N.D.[b]	20.0 ± 0.4	20.0 ± 0.2
pH 7	20.1 ± 0.3	20.1 ± 0.3	47.1 ± 1.6[c]	61.0 ± 3.4[c]
pH 8	20.1 ± 0.3	N.D.	61.1 ± 3.1[c]	69.1 ± 7.3[c]
Small Intestine	20.1 ± 0.3	38.8 ± 1.2	42.4 ± 1.1[c]	55.0 ± 0.7[c]
Cecum	20.1 ± 0.3	53.9 ± 1.1[c,d]	57.3 ± 0.9[c,d]	76.3 ± 1.9[c,e]

[a]Data expressed as ng ellagic acid/ μg casuarictin ± SD.
[b]N.D. = not determined.
[c]Significantly different from pH 2 ($p<0.05$).
[d]Significantly different from pH 7 and small intestine ($p<0.05$).
[e]Significantly different from pH 7, pH 8, and small intestine ($p<0.05$).

The liberation of ellagic acid [**2**] from raspberry extract under different conditions is shown in Table 4. The extract is stable at pH 2 over all the time points, but the ellagic acid [**2**] concentration increased significantly over time at pH 7 and pH 8. In all extract samples incubated with contents from the small intestine, the ellagic acid [**2**] liberated was not significantly different from the amount present in the pH 7 controls. However, in the samples treated with cecal contents, the ellagic acid [**2**] measured at 30 and 60 min was greater than either the small intestine or the pH 7 control samples. After 120 min, the amount of ellagic acid liberated in the small intestine, cecal or pH 7 control samples was not significantly different.

TABLE 4. Hydrolysis of Raspberry Extract Using pH and Gut Contents.[a]

	Time in Minutes			
	0	30	60	120
pH 2	400 ± 20	378 ± 10	385 ± 7	N.D.[b]
pH 7	481 ± 40	611 ± 46[c]	687 ± 45[c]	775 ± 52[c]
pH 8	400 ± 20	620 ± 59[c]	853 ± 34[c,d]	N.D.
Small Intestine	481 ± 40	621 ± 70[c]	648 ± 59[c]	765 ± 43[c]
Cecum	481 ± 40	729 ± 33[c,e]	853 ± 20[c,e]	791 ± 54[c]

[a]Data expressed as mg ellagic acid /g dried raspberry ± SD.
[b]N.D. = not determined.
[c]Significantly different from pH 2 ($p<0.05$).
[d]Significantly different from pH 7 ($p<0.05$).
[e]Significantly different from pH 7 and small intestine ($p<0.05$).

The results of the hydrolysis experiments involving raspberry extract and casuarictin [**1**] indicate that ellagitannins can be hydrolyzed to yield ellagic acid [**2**] under physiological conditions. The effects of pH on the hydrolysis of hydrolyzable tannins has been noted by other investigators (3,21). Klocke *et al.* (21) reported the partial hydrolysis of geraniin, a complex ellagitannin, in a pH 8 buffer. Although various enzymes have been suggested to hydrolyze ellagitannins (19,22,23), none of the three specific enzymes that were used in our study were shown to have any effect. In addition, in samples from the small intestine that contained considerably more esterase activity than the colon, the hydrolysis of both casuarictin [**1**] and the ellagitannins present in raspberry extract was not significantly different from the pH 7 controls (Tables 3 and

4). Because ellagitannins are comprised mostly of ester linkages, it is surprising that esterases do not liberate ellagic acid [**2**] under the experimental conditions. Possibly, the bulky structures of the ellagitannin molecules prohibit enzyme catalysis by causing steric hinderance of the enzyme-substrate complex. Another explanation for the absence of esterase hydrolysis could be the inhibition of the enzymes by the protein-binding ability of the condensed tannins in the extract. However, given the strong activity of the purified esterases and the concentration of raspberry extract used, it is unlikely that the enzymes would have been completely inhibited (Table 1).

With both casuarictin [**1**] and raspberry extract, there was an increased liberation of ellagic acid [**2**] in samples incubated with cecal contents (Tables 3 and 4). In the case of casuarictin [**1**], the hydrolysis was greatest in the cecal contents at 2 h (Table 3). With raspberry extract, the release of ellagic acid [**2**] was optimal at 1 h in the cecal samples. After 2 h the ellagic acid [**2**] content was not significantly different from the pH 7 control (Table 4). The presence of condensed tannins and other constituents in the raspberry extract may have limited the hydrolysis of the ellagitannins in the cecal contents at 2 h.

Collectively, these results indicate the presence of enzymes in the cecum that enhance the liberation of ellagic acid [**2**]. Because the pH of the cecal contents was 7.4, it is unlikely that the increased hydrolysis in the cecum was due to pH. Bacterial microflora have been shown to act on ellagic acid [**2**] and ellagitannin molecules resulting in changes in their structures (18,24). Because the bacterial content of the cecum is high, it is possible that the microflora increased the liberation of the ellagic acid [**2**] in the cecal samples.

The amount of ellagic acid [**2**] from dietary sources that could be available for possible antimutagenic or anticarcinogenic effects in vivo has not been determined to our knowledge. Such determinations would be difficult in view of the low amounts of ellagic acid [**2**] present in fruit extracts (3) and the difficulty of detecting the compound in blood (8). In vitro experiments to examine the hydrolysis of various compounds in intestinal contents have been reported in the literature (25,26), and in this study, those procedures were modified and used to measure the hydrolysis of crude ellagitannins and casuarictin [**1**]. It is apparent from our results that ellagic acid [**2**] can be liberated from dietary sources at pH's found in the small intestine and in the cecum but not in the stomach. In addition, the microflora of the cecum may participate in the hydrolysis of ellagitannins. The chemopreventive value of dietary ellagitannins would likely depend on the absorption of the ellagic acid [**2**] released. More information about the absorption of ellagic acid [**2**] from the gastrointestinal tract is needed to assess the chemopreventive potential of dietary sources of ellagic acid [**2**].

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