Biological Activity of the Folacin Oxidation Products 10-Formylfolic Acid and 5-Methyl-5,6-dihydrofolic Acid

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The biological activity of 10-formylfolic acid (10-formyl-FA) and 5-methyl-5,6-dihydrofolic acid (5-methyl-DHF) was examined to determine the potential contribution of these oxidized folates to the folacin activity of foods. Both compounds exhibited high folacin activity for *Lactobacillus casei* in microbiological assays under standard conditions in the presence of ascorbate. Chick bioassays with plasma folacin as the indicator of biological activity revealed approximately 100% activity for 10-formyl-FA and 80% activity for the natural *l* isomer of 5-methyl-DHF, respectively, relative to folic acid. Tritiated forms of these compounds were synthesized and compared with tritiated folic acid for their incorporation into the liver folacin pools of rats after oral dosing. Fractionation of conjugase-treated liver extracts by HPLC indicated that 10-formyl-FA and 5-methyl-DHF were absorbed and metabolized in a manner that was qualitatively and quantitatively similar to that of tritiated folic acid. These results suggest that the formation of 10-formyl-FA and 5-methyl-DHF in foods by oxidation of reduced folates would not yield important losses of folacin activity.

The nutritional quality of foods with respect to folacin is of great importance in view of the marginal deficiency that is common in certain population groups (Bailey et al., 1979, 1980). Complete evaluation of the nutritional quality of foods requires information concerning the concentration, biological activity, and bioavailability of the various forms of folacin present. Complete assessment of the effects of storage, processing, and preparation of foods cannot be performed accurately without information concerning the biological properties of the oxidation products of folacin vitamers.

Although the chemical mechanism of the loss of folacin in complex foods has not yet been elucidated fully, a great deal of research concerning the chemistry of folates suggests that oxidation reactions largely would be responsible for the loss of folacin in foods. The principal naturally occurring vitamers of folacin in animal tissues are polyglutamyl forms of tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid (5-methyl-THF), and 10-formyltetrahydrofolic acid (10-formyl-THF), while the folacin in many plant tissues is comprised mainly of polyglutamyl forms of 5-methyl-THF (Gregory et al., 1984). The polyglutamyl forms of folacin exhibit the same general stability as their monoglutamyl forms. THF, which is more labile than the other folates, is susceptible to oxidative cleavage of the C9–N10 bond to yield biologically inactive pterins and (*p*-aminobenzoyl)glutamates (Blair and Pearson, 1974; Reed and Archer, 1980). The presence of the methyl group at the N5 position of 5-methyl-THF increases the resistance of this folate to oxidative cleavage (Blair et al., 1975). Oxidation of 5-methyl-THF at or near neutral pH yields 5-methyl-5,6-dihydrofolic acid (5-methyl-DHF; Larrabee et al., 1961, 1963; Donaldson and Keresztesy, 1962; Scrimgeour and Vitols, 1966; Gupta and Huennekens, 1967; Blair et al., 1975). Although 5-methyl-DHF can be reduced back to nutritionally active 5-methyl-THF by agents such as borohydride, thiols, and ascorbate (Donaldson and Keresztesy, 1962; Larrabee et al., 1963; Gupta and Huennekens, 1967), the inherent folacin activity of 5-methyl-DHF is unclear on the basis of studies published to date (Ratanasthien et al., 1977; Kennelly et al., 1982). A secondary oxidation product of 5-methyl-THF has been identified and previously described as 4a-hydroxy-5-methyl-THF (Gapski et al., 1971; Blair et al., 1975). Later studies have indicated that this compound is actually a pyrazino-*s*-triazine derivative formed through rearrangement of the pteridine ring (Jongejan et al., 1979). Pyrazino-*s*-triazine compounds have been shown to be biologically inactive (Gapski et al., 1971; Ratanasthien et al., 1977; Kennelly et al., 1982).

In a manner similar to that of 5-methyl-THF, 10-formyl-THF is resistant to cleavage of the C9–N10 bond but is converted to 10-formylfolic acid (10-formyl-FA) under oxidative conditions (Maruyama et al., 1978; Lewis and Rowe, 1979). 10-Formyl-FA has been shown to be a potent inhibitor of dihydrofolate reductase, which is a key enzyme in folate metabolism (Bertino et al., 1965; d'Urso-Scott et al., 1974), although the in vivo folacin activity is unclear in view of conflicting published data (Beavon and Blair, 1975; Connor and Blair, 1979; Pheasant et al., 1981; Saleh et al., 1982).

The factors responsible for the variable results in studies of the biological activity of 5-methyl-DHF and 10-formyl-FA are unclear. Variation in the route of administration, dosage levels, and purity of the compounds may have contributed to the uncertainty. In view of the probable occurrence of these compounds in foods via oxidation of naturally occurring reduced folates, further characterization of their biological properties is essential. In addition, the results of common methods of biological assay used in research concerning folacin would be ambiguous unless the response to the oxidized folates is understood. The objective of this research was to evaluate the folacin activity of unlabeled forms of 5-methyl-DHF and 10-formyl-FA in chick bioassays and in microbiological assays employing *Lactobacillus casei* and also to evaluate the comparative metabolism of radiolabeled forms of these compounds and folic acid after oral administration to rats.

MATERIALS AND METHODS

Folacin Compounds. Unlabeled folic acid, *dl*-THF, *dl*-5-methyl-THF (barium salt) and *dl*-5-formyl-THF (calcium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). [The designations *d* and *l* refer to the stereochemical orientation at the C6 asymmetric center of the reduced folates. The *l* form is the naturally occurring isomer. All folates discussed in this paper are of the L form, which refers to the orientation at the α-carbon of the glutamyl side chain.] 10-Formyl-FA was prepared from

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folic acid by incubation in 98% formic acid (K & K Laboratories Division, ICN Pharmaceuticals, Plainview, NY) as described by Blakley (1959). *dl*-5-Methyl-DHF was prepared from *dl*-5-methyl-THF by treatment at pH 9 with potassium permanganate and hydrogen peroxide (Maruyama et al., 1978). The purity of all folates was assessed by using high-performance liquid chromatography (HPLC) and by examination of UV absorption spectra. The concentration of folate solutions was determined spectrophotometrically (Blakley, 1969) during the preparation of standards for all analyses.

Tritiated folic acid, which was labeled in the 3′, 5′, 7′, and 9′positions (35–45 Ci/mmol), was purchased from Amersham Corp. (Arlington Heights, IL). This was used for the preparation of [^{3}H]-10-formyl-FA as described above. The natural *l* isomer of [^{3}H]-5-methyl-THF was prepared for use in the synthesis of [^{3}H]-5-methyl-DHF by using the method of Horne et al. (1977). Dihydrofolate reductase (from *L. casei*) employed in this synthesis was obtained from The Enzyme Center Inc. (Malden, MA). The preparations of [^{3}H]-5-methyl-THF, [^{3}H]-10-formyl-FA, and [^{3}H]folic acid were purified by preparative HPLC by the ion-pair method of Horne et al. (1981) using a Partisil M9/25 ODS-3 column (9.4 mm i.d. × 25 cm, octadecylsilica; Whatman, Inc.; Clifton, NJ). The purified [^{3}H]-5-methyl-DHF was desalted by using a 1.5 cm i.d. × 71 cm column of Sephadex G-10 (Pharmacia Fine Chemicals; Piscataway, NJ) that was eluted with 0.01 M sodium phosphate buffer, pH 7.0 (degassed by flushing with nitrogen gas), while [^{3}H]-10-formyl-FA, and [^{3}H]folic acid were chromatographed by using the same buffer except with 0.5% (w/v) sodium ascorbate. Residual amounts of tetrabutylammonium phosphate, which is intensely bitter, were removed by passage of the compounds through a column (2.5 cm i.d. × 42 cm) packed with CM-Sephadex A-25 (Pharmacia Fine Chemicals) and eluted isocratically with the pH 7.0 buffers described for use with the Sephadex G-10 column. The purity of the tritiated folates was evaluated by the HPLC method of Horne et al. (1981) using a Whatman Partisil 10 ODS-3 analytical column (4.6 mm i.d. × 25 cm; octadecylsilica). The ability of 5-methyl-DHF to be reduced to 5-methyl-THF by 2-mercaptoethanol (Donaldson and Keresztesy, 1962), as monitored by HPLC, also was used as a criterion of purity and identity.

Radioactivity of collected chromatographic fractions was quantified with a Beckman LS-9000scintillation counter by using a commercial scintillation fluid (Aqualyte; J. T. Baker Chemical Co.; Phillipsburg, NJ). Disintegrations per minute (dpm) were determined by using an external standard-channels ratio procedure.

HPLC Methods. All HPLC analyses were performed using an Altex Scientific (Berkeley, CA) Model 312 HPLC system that included two Model 110A solvent metering pumps, a Model 410 gradient programmer, a Model 905-42 injection valve, and a Model 153 ultraviolet absorption monitor (280-nm filter). A Perkin-Elmer (Norwalk, CT) LS-5 spectrophotofluorometer equipped with a 20-μL flow cell was employed as the detector (290 nm for excitation, 357 nm for emission) in the analysis of reduced folates in liver.

Preparative and analytical separations involving 5-methyl-DHF and 10-formyl-FA were performed, as described above, using the basic method of Horne et al. (1981), which employs ion pairing at neutral pH. Analytical separations involving 10-formyl-FA also were performed using reverse-phase HPLC (Day and Gregory, 1981; Gregory et al., 1984). Because of the lability of 5-methyl-DHF in acidic media, reverse-phase methods based on an acidic mobile phase could not be employed for separations involving this vitamer. The extraction, enzymatic treatment, purification, and HPLC separation of radiolabeled folates in the livers of rats were performed by the method of Gregory et al. (1984). This procedure involved extraction using a pH 4.9 buffer at 100 °C for 60 min, which yielded quantitative conversion of 5,10-methenyl-THF and 10-formyl-THF to the 5-formyl-THF isomer, followed by deconjugation to the monoglutamyl form with partially purified hog kidney conjugase. After purification of the extracts using DEAE-Sephadex A-25 (Pharmacia Fine Chemicals), HPLC separations were performed by gradient elution using an acidic phosphate-acetonitrile mobile phase and a μBondapak phenyl column (3.9 mm i.d. × 30 cm; Waters Associates, Milford, MA). Tritiated folates were quantified by scintillation counting of collected 1-mL fractions of the HPLC effluent.

Chick Bioassay. The chick bioassay was conducted using the casein–glucose basal diet of Scott et al. (1969), as described in our previous publication (Ristow et al., 1982). Fortification of the diets with the various folates was performed by the addition of 50 mL of appropriate aqueous solutions (50 mL/12 kg of diet) of folic acid (0.0, 1.13, 2.27, and 3.40 nmol/g of diet), *dl*-5-methyl-DHF (0.837 nmol/g, 0.418 nmol/g with respect to the *l* isomer), and 10-formyl-FA (1.60 nmol/g). The diets were stored at 4 °C and removed in small aliquots as needed for feeding. Microbiological analysis indicated negligible loss of these folates during storage.

Day-old male color-sexed chicks (Cobb × Cobb) were randomly divided into groups of 15 each. Each chick was weighed and wing banded at the beginning of the study. The chicks were housed five per pen, with three pens per treatment in stainless steel heated batteries (Petersime Incubator Co., Gettysburg, OH), and fed ad libitum for 21 days. Consumption of feed was determined for each pen. The pens were randomly assigned to the batteries. At the end of the 21-day feeding period, each chick was weighed, and a blood sample was taken by anterior heart puncture into a heparinized syringe. After centrifugation of the blood, the plasma samples were transferred to polyethylene vials, frozen on dry ice, and then stored at −20 °C for no more than 3 weeks until analyzed microbiologically.

Microbiological Assay. The microbiological determination of total folacin was based on the method of Herbert (1966) using *L. casei* (ATCC 7469). Duplicate assay tubes were run for all samples of plasma, diet extracts, and folacin compounds at each level analyzed. Bacterial growth during the 18-h incubation period was evaluated by measuring turbidity (absorbance at 650 nm vs. an inoculated blank. Absorbance values of duplicates typically exhibited agreement within at least 5–8%. Folacin activity of plasma and diet samples was calculated relative to a folic acid standard curve. Duplicate preparations of the oxidized folates, 5-methyl-DHF and 10-formyl-FA, were synthesized and assayed in two independent trials over a range of concentrations to permit a multilevel assessment of their biological activity relative to that of folic acid. A commercial assay medium was employed in all analyses (Folic Acid Casei Medium; Difco Laboratories, Detroit, MI) with 0.1% (w/v) sodium ascorbate (5.05 mM). The pH of the medium was approximately 6.7 prior to incubation. Samples of diets were extracted and treated with crude hog kidney conjugase prior to analysis, as previously described (Ristow et al., 1982).

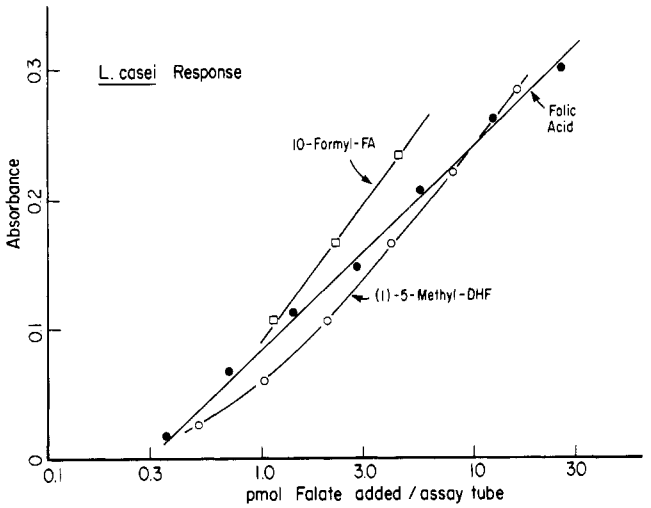

Figure 1. Dose–response curves for microbiological assay of folacin activity of folic acid, 10-formylfolic acid, and *dl*-5-methyl-5,6-dihydrofolic acid (concentration expressed as the natural *l* isomer). Data points are means of two tubes (10-mL volume).

Hepatic Metabolism of Radiolabeled Folates. Male rats (500–600 g; Crl:CD(SD)BR Sprague Dawley; Charles River Breeding Laboratories, Wilmington, MA) were housed individually in stainless steel cages and fed ad libitum a commercially pelleted diet (Purina Rodent Chow No. 5001, Ralston Purina Co., St. Louis, MO). For several days prior to the administration of the radiolabeled folates, the rats were presented daily with 12 mL of a 25% (w/v) solution of sucrose (in 0.02 M sodium phosphate, pH 7.0) in a small bottle with an attached drinking tube. When the rats were accustomed to receiving the sucrose solution, they typically consumed the entire 12-mL volume within approximately 15 min. The following oral doses of the tritiated folates were administered: [^{3}H]folic acid, 1.81 μCi; [^{3}H]-*l*-5-methyl-DHF, 0.64 μCi; [^{3}H]-10-formyl-FA, 2.03 μCi. Dosage levels corresponded to approximately 82, 29, and 92 pmol/kg of body weight, respectively. These compounds were given to three rats each. The rats were killed by decapitation 24 h after dosing. Livers were rapidly excised and packed in crushed ice. Homogenization and extraction for HPLC analysis were begun within 20 min.

Statistical Analysis. Dose–response curves from microbiological assays were evaluated by using an iterative nonlinear regression method that fit the curves to the Michaelis–Menten equation (Duggleby, 1981). The results of the hepatic metabolism study were evaluated by using analysis of variance and the Tukey procedure for multiple comparisons (Neter and Wasserman, 1974).

RESULTS

The oxidized folates used in this study were evaluated to determine their purity prior to evaluation of their biological properties. Unlabeled and tritiated forms of 10-formyl-FA were found to coelute in both ion-pair (Horne et al., 1981) and conventional reverse-phase HPLC systems (Day and Gregory, 1981). The UV absorption spectrum (pH 7.0) of unlabeled 10-formyl-FA yielded maxima and absorptivity values that were in agreement with those reported by Blakley (1969). UV absorption spectra of unlabeled 5-methyl-DHF at pH 13, 7 and 1 also were in agreement with those previously reported (Donaldson and Keresztesy, 1962; Larrabee et al., 1961, 1963; Maruyama et al., 1978). Reduction of both radiolabeled and unlabeled 5-methyl-DHF preparations using 2-mercaptoethanol or ascorbate to yield 5-methyl-THF, as previously reported (Donaldson and Keresztesy, 1962), provided additional evidence of authenticity.

Table I. Results of Chick Bioassay of Folacin Activity of 5-Methyldihydrofolic Acid and 10-Formylfolic Acid

diet fortification[a]	concn added, nmol/g	wt gain, g	feed consumption, g chick^{-1} day	plasma folacin, ng/mL
folic acid	0.00	180 ± 5	11.3 ● 1.3	3.2 ± 0.6
folic acid	1.13	518 ± 13	32.7 ± 2.3	9.6 ± 1.6
folic acid	2.27	583 ± 13	35.4 ± 1.2	19.9 ± 2.7
folic acid	3.40	538 ± 21	36.7 ± 8.0	31.0 ± 2.7
10-formyl-FA	1.60	523 ● 18	32.9 ± 1.2	14.7 ± 2.8
dl-5-methyl-DHF[b]	0.84	392 ± 23	27.6 ± 4.8	6.3 ± 1.9

[a] Values are means and SEM. [b] 5-Methyl-DHF fortification was 0.42 nmol/g with respect to the biologically active *l* isomer.

Table II. Folacin Activity of 10-Formylfolic Acid and *l*-5-Methyl-5,6-dihydrofolic Acid As Determined by Chick Bioassay

compound[a]	concentration, nmol/g of diet: added to diet	concentration, nmol/g of diet: bioassay response	rel act., %[b]
10-formyl-FA	1.60	1.56 ± 0.34	97.5 ± 21.3
l-5-methyl-DHF	0.42	0.33 ± 0.06	79.5 ± 13.1

[a] Values are means and SEM. Bioassay response calculated from the dietary folic acid–plasma folacin dose–response curve. [b] Folacin activity relative to that of folic acid.

Microbiological Assay. The results of microbiological assays comparing the folacin activity of folic acid, 5-methyl-DHF, and 10-formyl-FA are presented in Figure 1. Similar dose–response curves were obtained in a second independent trial. The activity of the *l* isomer of 5-methyl-DHF, relative to that of folic acid, was dose dependent and approached 100% activity at the upper region of the curve (25–30 pmol/tube.) The folacin activity of *l*-5-methyl-DHF is attributed to its essentially complete reduction to *l*-5-methyl-THF by the ascorbate in the assay medium. *l*-5-Methyl-DHF exhibited a molar response that was approximately 5–25% lower than that of folic acid over the lower range of the dose–response curve (0.5–10 pmol/tube). 10-Formyl-FA exhibited greater molar folacin activity (5–20%) than that of folic acid under these conditions.

Chick Bioassay. The raw data from the chick bioassay are presented in Table I. This study was performed simultaneously with an experiment involving the bioavailability of folacin; thus, the data for the folic acid standards have been reported previously (Ristow et al., 1982). The plasma folacin concentration was a linear function of dietary folic acid (r = 0.801), whereas growth was not affected by the concentration of dietary folic above approximately 1 nmol/g. The results concerning plasma folacin indicate that 10-formyl-FA and 5-methyl-DHF exhibited folacin activity in the bioassay. This conclusion also is supported by the observed growth of chicks in these dietary groups. On the basis of the linear regression equation calculated for the dose–response curve of the concentration of plasma folacin as a function of dietary folic acid dosage, the relative biological activity of 10-formyl-FA and 5-methyl-DHF (Table II) was found to be 97.5 ± 21.3% and 79.5 ± 13.1% (mean and SEM), respectively.

Hepatic Metabolism of Radiolabeled Folates. By comparison of the hepatic content and distribution of radiolabeled folates following oral dosing, the relative extent of intestinal absorption and hepatic metabolism and re-

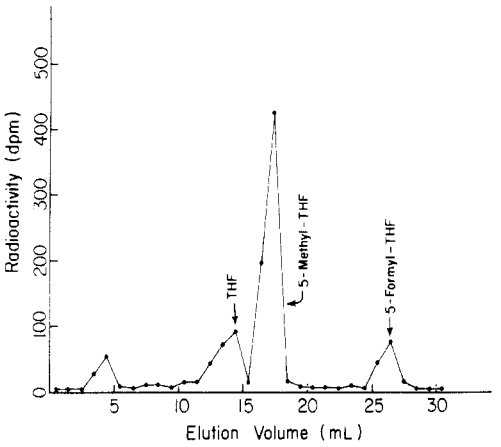

Figure 2. Representative chromatogram from HPLC analysis of tritiated hepatic folates. This liver sample was from a rat dosed orally with 2.03 μCi of [^{3}H]-10-formylfolic acid 24 h prior to sacrifice.

Table III. Hepatic Retention and Distribution of Tritiated Folates 24 h after Oral Dose of [^{3}H]Folic Acid, [^{3}H]-10-Formylfolic Acid, and [^{3}H]-*l*-5-Methyl-5,6-dihydrofolic Acid

folate[a]	oral dose, μCi	hepatic 5-CH_3-THF,[b] % of oral dose	rel folate concn, % THF	5-CH_3-THF	5-CHO-THF
folic acid	1.81	5.9 ± 0.6a	27 ± 2	58 ± 3	16 ± 2
10-CHO-FA	2.03	10.3 ± 1.0b	25 ± 2	63 ± 2	12 ± 2
l-5-CH_3-DHF[c]	0.64	4.5 ± 1.0a	20–30	55–65	10–20

[a] Values are means and SEM; n = 3 per group. [b] In the hepatic 5-CH_3-THF column, values followed by the same letter are not significantly different at the 5% level. [c] Because of the low counts observed for THF and 5-formyl-THF in livers of rats given *l*-5-CH_3-DHF due to the low dose, the absolute concentration of these vitamers and the relative distribution of liver folates could only be approximated. Therefore, the relative distribution of hepatic folates is expressed only as approximate ranges rather absolute percentages. The radioactivity of hepatic 5-CH_3-THF was sufficient for accurate quantitation.

tention of tritiated forms of folic acid, *l*-5-methyl-DHF, and 10-formyl-FA could be determined. As previously observed with this HPLC method (Gregory et al., 1984), THF, 5-methyl-THF, and 5-formyl-THF were the sole hepatic folates detected (Figure 2). The quantitative results of this study are presented in Table III. Because of the low yield obtained in the synthesis of [^{3}H]-5-methyl-DHF, the dose of this compound administered was less than optimal. The resulting low radioactivity of the hepatic folates of rats given [^{3}H]-5-methyl-DHF made quantitation of the minor radiolabeled folacin vitamers (i.e., [^{3}H]-THF and [^{3}H]-5-formyl-THF) difficult, although the radioactivity of hepatic [^{3}H]-5-methyl-THF *was* sufficient for accurate quantitation. For the same reason, *total* radiolabeled hepatic folacin and the percentage of folate vitamers could only be approximated in rats fed [^{3}H]-5-methyl-DHF. The relative hepatic retention of the three tritiated folate compounds (as a percentage of oral dose) and the relative distribution of radiolabeled folacin vitamers were similar for each compound. Statistical analysis indicated that the net hepatic retention of radiolabeled folates in rats fed [^{3}H]-10-formyl-FA was significantly greater than that observed for the [^{3}H]-folic acid reference. Overall, these results indicate that the uptake and metabolic utilization of both 5-methyl-DHF and 10-formyl-FA in the rat are quantitatively and qualitatively similar to that of folic acid.

DISCUSSION

The major objective of these studies was to evaluate the biological activity of *l*-5-methyl-DHF and 10-formyl-FA because of their likely presence in many foods through the oxidation of naturally occurring reduced folates. Another primary objective was to determine the response of these compounds in biological assay procedures that are used commonly in studies of the content, stability, and bioavailability of folacin in foods.

In studies of the total content or bioavailability of folacin in foods, the chemical diversity of the folacin group imposes many potential analytical problems. The results of biological analyses, whether using microbiological, mammalian (rat), or avian (chick) bioassay techniques, would be ambiguous if the folates present exhibited widely differing molar responses. The results of this study indicate that if food samples were to contain 5-methyl-DHF and/or 10-formyl-FA as components of their folacin pool, these compounds would yield a response essentially equivalent to that of folic acid. It is important to note that, in commonly used microbiological assays for folacin, *L. Casei* exhibits a slightly greater growth response to folic acid than to *l*-5-methyl-THF when performed with media initially at pH 6.7 (Phillips and Wright, 1982). The observed disparity in molar response between *l*-5-methyl-DHF and folic acid over the range of about 0.5 to 10 pmol/tube (Figure 1) is comparable to the difference in response between *l*-5-methyl-THF and folic acid reported by Phillips and Wright (1982) for this assay medium at pH 6.7. These authors reported that *l*-5-methyl-THF and folic acid exhibit an equivalent response when the initial pH is 6.1–6.3, as has been recently confirmed in our laboratory. Presumably, *l*-5-methyl-DHF would behave similarly. This effect and the slightly greater response observed for 10-formyl-FA (Figure 1) would not yield important analytical errors in most microbiological assays because the differences in response are nearly within the limits of precision of the analytical methods.

Although the oxidation of 5-methyl-THF to form 5-methyl-5,6-DHF has been known for many years (Larrabee et al., 1961, 1963; Donaldson and Keresztesy, 1962; Scrimgeour and Vitols, 1966; Blair et al., 1975), the extent of the formation of 5-methyl-DHF in foods has not yet been determined. Day and Gregory (1983) employed liquid model systems fortified with 5-methyl-THF to simulate milk products in studies of the thermal degradation of folacin in retort processing. In these relatively anaerobic systems (typically ≤5 ppm of oxygen) the formation of 5-methyl-DHF could not be detected by thin-layer chromatography, although the extent of formation of the biologically inactive pyrazino-*s*-triazine was not investigated. Further research is needed to determine the extent of formation of 5-methyl-DHF and the triazine product, as well as to determine the stability characteristics of 5-methyl-DHF in foods. Because of the rapid reduction of 5-methyl-DHF to 5-methyl-THF by ascorbate and mercaptoethanol, which are common antioxidants in folacin analysis, the specific presence of 5-methyl-DHF would not be detected in most chromatographic methods of folacin analysis.

The presence of 10-formyl-FA in foods has been suggested by Butterworth et al. (1963) and Santini et al. (1964) on the basis of chromatographic fractionation and microbiological analysis of food extracts; however, conclusive identification was not performed. Although no other studies have detected 10-formyl-FA in foods, the known

susceptibility to oxidation of 10-formyl-THF, which is a major naturally occurring folate, suggests that 10-formyl-FA would be present as a significant proportion of the total folacin in certain foods. The adaptation of recent chromatographic methods to the determination of 10-formyl-FA in foods is, therefore, warranted.

Numerous contradictions exist in the literature concerning the biological activity of these oxidized folates. 10-Formyl-FA has been shown to be a potent inhibitor in vitro of dihydrofolate reductase from rat liver and Ehrlich ascites carcinoma cells (Bertino et al., 1965; d'Urso-Scott et al., 1974). In vivo metabolic studies by Beavon and Blair (1975) suggested that orally administered 10-formyl-FA was poorly utilized and largely ineffective in the folacin nutrition of the rat. Later work in the same laboratory yielded contrasting data that indicated that the hepatic uptake and metabolism of 10-formyl-FA were similar to those of folic acid in the rat (Connor and Blair, 1979; Pheasant et al., 1981). In view of the reported inhibitory effects and lack of substrate activity of 10-formyl-FA for dihydrofolate reductase, the mechanism of its entry into the hepatic reduced-folate metabolism of the rat is unclear. The slightly greater hepatic retention of tritiated folates observed after oral administration of tritiated 10-formyl-FA (Table III) is suggestive of preferred cellular uptake or intracellular metabolic trapping of this folate. The metabolic and nutritional significance of this phenomenon is uncertain at present. Large doses of 10-formyl-FA (5 mg) in humans have been reported to be excreted intact and not metabolized, as well as to retard the metabolism of accompanying small doses (60 μg) of folic acid (Saleh et al., 1982). The results of the present (Tables II and III) and previous studies (Connor and Blair, 1979; Pheasant et al., 1981), which indicate that low doses of 10-formyl-FA are effectively utilized in mammalian (rat) and avian species, suggest the need for further evaluation of the role of 10-formyl-FA in human nutrition.

Previous research concerning 5-methyl-DHF also has yielded apparent contradictions. In vitro studies have indicated that 5-methyl-DHF is not a substrate for avian or mammalian dihydrofolate reductase (Donaldson and Keresztesy, 1962; Bertino et al., 1965). Since hepatic nonprotein thiols are present at millimolar concentrations (Sedlak and Lindsay, 1968), which would be sufficient for conversion of 5-methyl-DHF to 5-methyl-THF, the nonenzymatic reduction of 5-methyl-DHF by compounds such as reduced glutathione may provide an explanation the observed folacin activity in rats and chicks (Tables II and III). A key variable in the observed in vivo activity of 5-methyl-DHF is its susceptibility to cleavage (Maruyama et al., 1978; Lewis and Rowe, 1979) or rearrangement (Larrabee et al., 1961, 1963; Jongejan et al., 1979) in acidic media to yield biologically inactive products. The rates of these reactions in the acidic environment of the stomach would influence the net gastric stability of the compound and, thus, influence the observed folacin activity in experiments using oral routes of dosing. In the present study, the dose of 5-methyl-DHF was given to rats orally in a comparatively large volume (12 mL) of dilute phosphate buffer. Kennelly et al. (1982) observed that oral doses of 5-methyl-DHF in phosphate buffer underwent decomposition in the gastrointestinal tract of the rat, while intraperitoneal doses were effectively utilized. Similar work with human subjects indicated that 5-methyl-DHF administered with sodium bicarbonate was well absorbed and utilized (Ratanasthien et al., 1977). These data, along with the results of the present study (Tables II and III) suggest that 5-methyl-DHF would not be extensively degraded in the gastrointestinal tract under many dietary conditions.

In summary, this research has provided further information indicating that the oxidized folates 5-methyl-DHF and 10-formyl-FA exhibit essentially full folacin activity. The measurement of the folacin activity of foods using biological methods of analysis (i.e., bioassays using rats, chicks, or *L. casei*) would yield an essentially full molar response to these compounds. Although further studies are needed to characterize the properties of these compounds and their folacin activity in humans, the observed activity in nonhuman species suggests that 5-methyl-DHF and 10-formyl-FA in foods would contribute significantly to the folacin nutrition of humans.

Registry No. 10-Formyl-FA, 134-05-4; 5-methyl-DHF, 28581-42-2; folacin, 59-30-3.

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Studies on the Enzymic Reduction of 1-Octen-3-one in Mushroom (*Agaricus bisporus*)

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The enzymic reduction of 1-octen-3-one into 1-octen-3-ol and 3-octanone in mushroom (*Agaricus bisporus*) was investigated by using synthetic 1-octen-3-one blended with mushrooms and the pH adjusted from 5.0 to 9.0. 1-Octen-3-ol can be converted from 1-octen-3-one, but the formation does not seem to be affected by the pH value while the formation of 3-octanone was favored at pH 8.0–9.0. It is possible there exists two enzymes that are responsible for the formation of 1-octen-3-ol and 3-octanone. However, the presence of a reduction enzyme explains only in part about the origin of 1-octen-3-ol in mushroom (*A. bisporus*).

1-Octen-3-ol, an unsaturated alcohol, occurs in many mushroom species (Cronin and Ward, 1971; Picardi and Issenberg, 1973; Pyysalo, 1976; Maga, 1981). It contributes significantly to the flavor of edible mushrooms such as *Agaricus campestris* (Tressl et al., 1980, 1982) and *Agaricus bisporus* (Wurzenberger and Grosch, 1983; Chen and Wu, 1983). Tressl et al. (1980, 1982) demonstrated that 1-octen-3-ol was formed enzymically from linoleic acid, which was shown by Holtz and Schisler (1971) to be the major fatty acid from *A. bisporus*. Enzymes involved in the pathway of formation of 1-octen-3-ol as proposed by Tressl et al. (1980) included lipoxygenase, hydroperoxide cleavage enzyme, and alcohol oxidoreductase. The activity of lipoxygenase was found in mushrooms by de Lumen et al. (1978). The activities of hydroperoxide cleavage enzyme were found in cucumbers and tomatoes (Galliard and Matthew, 1977; Wardale et al., 1978) and tea (Hatanaka et al., 1977), in which the cleavage of linoleic acid occurs via the 13- or 9-hydroperoxide. In mushroom *A. campestris*, the presence of a hydroperoxide cleavage enzyme was also proposed by Tressl et al. (1980) for the conversion of 13-hydroperoxide to 1-octen-3-ol and two 10-carbon compounds. On the contrary, Wurzenberger and Grosch (1983) proposed that a 10-peroxide might be the intermediate for the formation of 1-octen-3-ol, since they found that incubation of 13-hydroperoxide with mushroom homogenate only reduced the hydroperoxide into the corresponding 13-hydroxy fatty acid. The above two different proposals lead to two different possibilities about the formation of 1-octen-3-ol; that is, 1-octen-3-one is reduced to 1-octen-3-ol by an alcohol oxidoreductase if the proposal of Tressl et al. (1980) is followed; on the contrary, 1-octen-3-ol may be formed directly from the cleavage of 10-hydroperoxide if the proposal of Wurzenberger and Grosch (1982) is followed.

The purpose of this study was to clarify the pathway by which 1-octen-3-ol is formed. This paper presents the results of experiments in which mushrooms were blended with added 1-octen-3-one at different pH values followed by the determination of the product formed.

EXPERIMENTAL SECTION

Sample Preparation. 1-Octen-3-one was synthesized from the chromic acid oxidation of 1-octen-3-ol (97%, Fluka AG) as shown by Brown and Garg (1961). 3-Octanone (97%) was obtained from Fluka AG. Fresh mushrooms of approximately the same size (ca. 16 g) were picked daily from the local cultivation houses near Hsinchu, Taiwan. Mushrooms (250 g) were blended under room temperature for 5 min with 750 mL of distilled water, while the pH of the whole mixture was maintained at a constant value by adding 0.1 N HCl or 0.1 N NaOH solution. The pH value of the mixtures ranged from 5.0 to 9.0 with a 1.0-unit interval. The same mixtures were prepared and 160 mg of synthetic 1-octen-3-one was added during each preparation. Volatile components in each mixture were extracted for 1 h in a Likens-Nickerson apparatus (Römer and Renner, 1974). Glass-distilled pentane and ether (1:1) were used as extracting solvents. 1-Nonanol (Haarman and Reimer GmbH) was added as an internal standard. The extracted volatiles were injected directly into the gas chromatograph without further concentration.

Gas Chromatography. Gas chromatography was carried out on a Shimadzu GC-8APF equipped with dual flame ionization detectors and dual glass columns (2 m × 2.6 mm i. d.). The column packing was 10% Carbowax-20M (Varian aerograph) coated on Chromosorb W A/W DMCS (80–100 mesh, Supelco, Inc.). The oven temperature was programmed from 60 to 200 °C/min at 2 °C/min. The injector and detector temperatures were 250 °C. The

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