COOH), 135 (M^+ – OAc), 133 (m/z 151 – H_2O), 105 (m/z 151 – COOH), 77, 43. This mass spectrum is consistent with the reference standard of *o*-(acetoxymethyl)benzoic acid (**10**).

A trimethylsilyl derivative of component B-4-1 was also analyzed by mass spectroscopy. Characteristic ions were seen at m/z 266 (M^+), 251 ($M^+ - CH_3$), 223 ($M^+ - CH_3C{=}O$), 207 (M^+ – OAc), 133 (m/z 223 – Me_3SiOH), 117 ($COOMe_3Si$), 105, 90, 89, 75, and 73. This spectrum was consistent with the trimethylsilyl derivative of **10**. The retention times of the metabolite and standard were also nearly identical. Component B-4-1 is confirmed as *o*-(acetoxymethyl)benzoic acid.

Component B-4-2 was methylated prior to GC/MS analysis. This radioactive component eluted at approximately 250 °C. An expanded chromatogram showed that it actually consisted of two closely related components. The mass spectra of these two components were identical and showed the presence of a molecular ion (M^+) at m/z 376 and other fragment ions at m/z 344, 318, 227, 167, 149, 133, 119, and 43. These mass spectra are consistent with the mass spectrum of the methyl ester of 9-(acetoxymethyl)-α-carboxycinmethylin (**11**). Component B-4-2 was also analyzed by GC/MS as the trimethylsilyl derivative. Both the retention time and the mass spectrum of the metabolite matched those of the standard.

It is evident that 9-(acetoxymethyl)-α-carboxycinmethylin (**11**) was generated as the *O*-acetyl conjugate of 9-hydroxy-α-carboxycinmethylin (**4**) (also isolated as two diastereomers). **10** and **11** were recovered as unique metabolites of cinmethylin (**1**) since they were not detected during the isolation and identification of their corresponding precursors, *o*-(hydroxymethyl)benzoic acid (**6**) and 9-hydroxy-α-carboxycinmethylin (**4**), respectively, under identical conditions. Similar results were observed with methanol as the extraction solvent of the silica gel.

CONCLUSION

Conjugations are biosynthetic reactions in which xenobiotics or their metabolites react with readily available, endogenous substances such as glucuronic acid, sulfate, acetate, amino acid, etc. These endogenous substrates are transferred from the coenzymes participating in intermediary metabolism to usual conjugation reaction sites such as the hydroxyl, carboxyl, epoxide, halogen, thiono, and amino functional groups. The best understood acylation reaction to date is limited to the transfer of acetate from acetyl coenzyme A to an amino group [see examples provided by Iwan (1976)]. Several examples of the N-acylation reaction of pesticide molecules are known and include metobromuron (Tweedy et al., 1970) and 4,6-dinitro-*o*-cresol (Smith et al., 1953). O-Acylation of xenobiotics has not yet been reported. In the rat metabolism study of cinmethylin, O-acylation products of *o*-(hydroxymethyl)benzoic acid and 9-hydroxy-α-carboxycinmethylin, principal metabolites of cinmethylin, were isolated. The formation of these novel conjugates is of biochemical significance since these conjugates have a higher lipid solubility than the free molecule.

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Assessment of Folate Bioavailability in the Rat Using Extrinsic Dietary Enrichment with Radiolabeled Folates

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The relative bioavailability of tritiated folates was examined in the rat by a single-dose protocol. Hepatic [^{3}H]folate retention 24 h postdose and 24-h urinary tritium excretion were used as primary response criteria. The in vivo retention of the tracer dose of [^{3}H]folic acid (0.11 nmol) was not significantly affected by the level of total dietary folate over the range of 0–10 nmol of added folic acid but was reduced by the presence of 100 nmol of folic acid. Monoglutamyl [^{3}H]tetrahydrofolates (mainly 5-formyl) from bacterial synthesis exhibited greater in vivo retention than either their polyglutamyl analogues or [^{3}H]folic acid monoglutamate. In additional studies, the presence of cabbage and orange juice in test meals significantly retarded the bioavailability of [^{3}H]folic acid and bacterial polyglutamyl [^{3}H]folates, while pectin, wheat bran, and kidney beans exhibited trends toward reduced bioavailability of the labeled folates.

The folate nutriture of humans and animals depends on the content and bioavailability of the dietary folates relative to the nutritional requirement for the vitamin. A great deal of uncertainty exists concerning the bioavailability of folate. In the context of this study, we define bioavailability as the overall utilization of the vitamin including intestinal absorption and function in folate

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metabolism. The bioavailability of dietary folate could be affected by dietary factors or physiological conditions influencing the rate or extent of intestinal deconjugation of polyglutamyl folates, intestinal absorption, entry into folate metabolism and enterohepatic circulation, or the rate of urinary excretion.

Numerous experimental approaches have been used in the study of folate bioavailability including bioassays with rats, chicks, or human subjects and intestinal perfusions (Babu and Srikantia, 1976; Graham et al., 1980; Keagy and Oace, 1979, 1984; Ristow et al., 1982a,b; Tamura and Stokstad, 1973; Tamura et al., 1976; Anderson and Talbot, 1981; Rodriguez, 1978; Retief, 1969; Nelson et al., 1975). The results of these studies have often been variable and conflicting. Studies by Babu and Srikantia (1976) and Tamura and Stokstad (1973) using human subjects suggested that pronounced differences in folate bioavailability exist among common foods. Similar studies of fortified grain products indicated that the food matrix may affect the rate or extent of folic acid utilization (Colman et al., 1975a,b; Margo et al., 1975).

The extent of inhibition of intestinal pteroylpolyglutamate hydrolases (conjugases) by dietary components has not been determined. Apparent conjugase inhibitors, possibly nucleic acids, have been reported (Mims et al., 1947; Swendseid et al., 1947), yet their influence on folate bioavailability is unclear (Grossowicz et al., 1975). Butterworth et al. (1974) have reported that various legumes contain a component that inhibits the conjugases of human plasma, chick pancreas, and rat liver in vitro.

Bioassay procedures have been developed and applied to a limited extent. Previous studies in this laboratory using chick bioassays indicated that the bioavailability of folic acid monoglutamate was not affected by various forms of dietary fiber (Ristow et al., 1982b). Similar conclusions were reached by Keagy and Oace (1984) using a rat bioassay procedure that they developed (1982). Recent studies of the efficacy of rat bioassays have indicated the potential for overestimation of biologically available folate in certain foods (Abad and Gregory, 1987).

The major objectives of this study using radiolabeled folates were (a) to develop an experimental protocol for the evaluation of factors affecting folate bioavailability in the rat, (b) to compare the utilization of several mono- and polyglutamyl folates, and (c) to determine the effects of several foods on the bioavailability of folic acid and polyglutamyl reduced folates.

MATERIALS AND METHODS

Folate Compounds and Test Foods. Unlabeled folic acid and other monoglutamyl folates were obtained from Sigma Chemical Co. (St. Louis, MO). Tritiated folic acid (3′,5′,7,9-^{3}H; 35–45 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). The concentration of unlabeled folates used for analytical standards was determined spectrophotometrically using published molar absorptivities (Blakley, 1969). Folic acid triglutamate and pentaglutamate were synthesized in this laboratory (Krumdieck and Baugh, 1980). Folic acid heptaglutamate was purchased from Dr. Charles Baugh, University of South Alabama, Mobile, AL.

[^{3}H]Polyglutamyl folates were prepared from [^{3}H]folic acid by biological synthesis using *Lactobacillus casei* essentially as described by Buehring et al. (1974). *L. casei* was grown in Folic Acid Casei Medium (Difco Laboratories, Detroit, MI) to which [^{3}H]folic acid had been added at 10 ng/mL (0.9 μCi/mL) for 18 h at 37 °C. The cells were harvested by centrifugation at 2000*g*, washed with the unsupplemented growth medium, and suspended in 0.05 M sodium acetate, pH 4.9, containing 57 mM ascorbic acid in a volume equivalent to the original growth medium. The cells were lysed by incubation in a boiling water bath for 15 min, and the cell debris was sedimented by centrifugation (10000*g*) for 20 min at 4 °C. Gel filtration chromatography using Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) was employed for the isolation of long-chain polyglutamyl folates using the acetate–ascorbate buffer described above as the eluent (Shin et al., 1972). The elution characteristics of the column were determined of synthetic pteroylpolyglutamates of varying chain length. The composition of the bacterial folates was determined following enzymatic deconjugation by the high-performance liquid chromatographic (HPLC) procedures of Gregory et al. (1984b) and Horne et al. (1982).

For the study of the effects of diet composition on folate utilization, cabbage and kidney beans were evaluated in gelled test diets at a level of 10% (w/w, wet-weight basis). Orange juice solids were added at 10% (w/w). Locally purchased fresh cabbage was finely chopped and then blanched under flowing steam at 1 atm for 10 min, followed by homogenization in 1.5 volumes of distilled water. This treatment was sufficient to inactivate the endogenous conjugase activity as determined by chromatographic assay (Day and Gregory, 1985). Orange juice solids were obtained in vacuum-dried form (Crystals International, Inc., Plant City, FL). The kidney beans used were commercially canned red kidney beans. Hard red wheat bran (American Association of Cereal Chemists, St. Paul, MN) and citrus pectin (Sunkist Growers, Inc., Ontario, CA) were used at 1% w/w (wet-weight basis) in the diets.

The concentration of total folate in test materials assayed by the procedure of Phillips and Wright (1983) was as follows: kidney beans, 0.33 nmol/g, wet weight; orange juice solids, 7.1 nmol/g, dry weight; blanched cabbage, 2.2 nmol/g, wet weight. No folate was detected in the pectin preparation. The folate concentration reported for wheat bran was 5.8 nmol/g dry weight (Perloff and Butrum, 1977).

Experimental Protocols. In each experiment, male Sprague–Dawley rats (Crl:CD(SD)BR: Charles River Breeding Laboratories, Wilmington, MA) ranging in weight from 180 to 220 g were individually housed in stainless-steel metabolism cages with wire mesh floors. The rats were maintained by feeding a commercially pelletted nonpurified diet (5001; Ralston Purina, St. Louis, MO). Water was provided ad libitum. Over a period of 1 week prior to each experiment, the rats were trained to consume a gelled test meal by daily presentation of 10 g of gelatin gel (1.9% w/w USP gelatin; Knox Gelatin Inc., Englewood Cliffs, NJ), which contained 17 g of sucrose/100 g of gel, between 9:00 and 11:00 a.m. daily. After approximately 4 days of conditioning in this way, the rats consumed the entire 10-g portion within the 2-h time period. During the conditioning period, the rats were presented with four pellets of the commercial diet (approximately 16–18 g) each day at 11:00 a.m. The pellets were consumed by the following morning.

The objective of experiment 1 was to determine the effect of total folic acid consumption on the utilization of a tracer dose of [^{3}H]folic acid. Twenty-four rats were divided into four groups of six each. After the 7-day conditioning period, the rats were fed 6 g of the gelatin–sucrose gel containing 4.5 μCi of [^{3}H]folic acid (0.11 nmol) and either 0.0, 0.68, 10, or 100 nmol of unlabeled folic acid. Aliquots of each gel were retained in this and all other experiments for HPLC analysis (Gregory et al., 1984) to

determine the precise amount of the labeled folate consumed. All of the gels were consumed within 2 h, at which time the commercial nonpurified diet was reintroduced. Urine was collected for 24 h in foil-wrapped flasks containing 5 mL of 0.1 M sodium acetate buffer (pH 4.9) containing 114 mM ascorbic acid. The rats were killed by decapitation 24 h after the test meal. Blood was collected from the cervical vessels into heparinized tubes, plasma was separated by centrifugation, and the livers were rapidly excised. All samples were stored at −20 °C until analysis within 2 weeks.

Experiment 2 was performed as described for experiment 1, except that the objective was to determine whether the form of [^{3}H]folate presented influenced the net bioavailability in this protocol. Eighteen rats were divided into three groups of six rats each. Each group was fed a gel containing 4.5 μCi of either [^{3}H]folic acid, [^{3}H]polyglutamyl folates from *L. casei*, or the bacterial [^{3}H]folates that had been deconjugated to the monoglutamyl form by treatment with conjugase from hog kidneys (as described later). In this manner, the effects of oxidation state and one-carbon substitution, as well as chain length, could be evaluated. Preliminary conditioning, gel administration, and collection of urine, plasma, and liver were performed as in experiment 1.

Experiment 3 was conducted by the same procedure to determine the bioavailability of [^{3}H]folic acid and [^{3}H]-polyglutamyl folate as influenced by the presence of various materials added to the test gels. This was accomplished by administering an oral dose of 4.5 μCi of [^{3}H]folic acid or [^{3}H]polyglutamyl folate to the rats in the gelatin gel as in previous experiments or in gelatin gels, which had been formulated with either cabbage or homogenized kidney beans (each at 10% wet weight basis), orange juice solids (10% w/w), or wheat bran or citrus pectin (each at 1% w/w). Four separate trials were performed in order to test all of these substances. The gels employed in the conditioning period, which contained no radioactive folates, were unsupplemented in the case of the controls or contained the appropriate supplement (cabbage, etc.). This experiment was conducted in two phases of two trials each with the following design: phase 1, control (unsupplemented), 10% cabbage, and 10% orange juice solids: phase 2, control, 10% kidney beans, 1% wheat bran, and 1% citrus pectin. In each trial, three rats were fed the appropriate gelled meal, with the remainder of the protocol conducted as in the other experiments.

Analytical Procedures. The distribution of radiolabeled folates in the gelled diets, liver samples, and bacterial folate preparations was determined by using preparative procedures and HPLC methods developed in this laboratory (Gregory et al., 1984). Portions of liver and diet were homogenized in four volumes of 0.05 M sodium acetate buffer, pH 4.9, containing 57 mM ascorbic acid. The homogenates were flushed with nitrogen gas, incubated in a boiling water bath for 1 h, and then centrifuged. This treatment provided extraction of folates as well as complete conversion of 10-formyltetrahydrofolates to the 5-formyl isomers. An aliquot of each supernatant was treated with hog kidney conjugase at 37 °C for 1 h, which yielded complete conversion of polyglutamyl folates to the monoglutamyl forms. These extracts were analyzed by HPLC without further purification. The retention time of the folate standards [tetrahydrofolic acid (H_4folic acid), 5-methyltetrahydrofolic acid (5-Me-H_4folic acid), and 5-formyltetrahydrofolic acid (5-CHO-H_4folic acid)] was 19 determined by monitoring their native fluorescence. During HPLC separation of the sample extracts, fractions of the effluent (0.5 mL) were collected directly into scintillation vials.

The measurement of radioactivity in HPLC fractions, urine, liver, and diet extracts, and unfractionated plasma was performed on a Beckman LS-2800 liquid scintilltion counter (Beckman Instruments, Irvine, CA) with a commercial scintillation cocktail (Aqualyte; J. T. Baker Chemical Co., Jackson, TN). The conversion of counts per minute (cpm) to disintegrations per minute (dpm) was performed with use of the efficiency determined by a channels ratio procedure and appropriate quench curves. The counting efficiency was typically 45%.

The concentration of each radiolabeled folate compound in gelled diets and livers was calculated by multiplying the total radioactivity by the proportion of each compound determined by HPLC. Values reported for hepatic retention of tritiated folate refer specifically to tritiated folates, rather than total radioactivity.

Statistical Analysis of Data. Statistical evaluation of the data was performed by analysis of variance. When significant main effects were observed in the analysis of variance, differences between treatments were determined by the Tukey procedure for multiple comparisons or Dunnett's test for comparisons with the control mean. All statistical procedures were performed as described by Steel and Torrie (1980).

Table I. Concentration (Percent of Oral Dose) of Tritiated Hepatic Folate and Total Urinary and Plasma Tritium 24 h after Oral Dose of Tritiated Folic Acid (0.11 nmol) in the Presence of Various Levels of Unlabeled Folic Acid (Experiment 1)[a,b]

added folic acid, nmol per rat	hepatic [^{3}H]folate	urinary tritium	plasma tritium
0.00	11.4 (0.8) a	37.0 (3.0) ab	0.352 (0.016) a
0.68	13.0 (1.2) a	30.3 (1.2) a	0.397 (0.012) a
10.0	10.1 (0.3) ab	37.6 (4.6) ab	0.346 (0.012) a
100.0	7.38 (0.55) b	43.6 (2.7) b	0.371 (0.020) a

[a] Values are means and SEM (in parentheses), six rats per group. [b] Values within a column followed by the same letter were not significantly different as determined by the Tukey procedure ($P < 0.05$).

RESULTS

Experiment 1. The results of experiment 1, which was designed to investigate the relationship between the quantity of folic acid ingested and the bioavailability of a simultaneously administered [^{3}H]folic acid tracer dose, are shown in Table I. Only the group fed 100 nmol of unlabeled folic acid exhibited hepatic retention of [^{3}H]-folates that was significantly less than the control ($P < 0.05$). Additional intermediate dosage levels would be required to determine the exact nature of the dose–response relationship. No significant difference in urinary tritium was detected between the control and the various folate-supplemented groups.

The hepatic distribution of tritiated folates was only slightly affected by the level of dietary folic acid (Table II). 5-Me-H_4folic acid was the major tritiated hepatic folate compound in all cases. Although the relative concentration of the various folates was similar among dietary groups, the following differences were observed. In rats fed 10 and 100 nmol of folic acid, the proportions of tritiated hepatic H_4folic acid and 5-Me-H_4folic acid were each significantly greater than the control. The proportion of tritiated 5-CHO-H_4folic acid of the 100-nmol group was significantly lower than that of the control ($P < 0.05$). The metabolic implications of these observations are not fully clear, although the overall magnitude of these changes in hepatic folate distribution was small.

Table II. Percentage Distribution of Tritiated Folates in Livers of Rats 24 h after Administration of an Oral Tracer Dose (0.11 nmol) of [^{3}H]Folic Acid in the Presence of Varying Amounts of Unlabeled Folic Acid (Experiment 1)[a,b]

added folic acid, nmol per rat	H_4folate	5-Me-H_4folate	5-CHO-H_4-folate
0.00	12.1 (0.8) a	68.7 (1.8) a	19.2 (1.9) a
0.68	14.0 (0.8) ab	67.5 (1.1) a	18.5 (1.0) a
10.0	17.3 (1.1) b	67.5 (0.6) ab	15.2 (1.0) ab
100.0	15.2 (0.9) ab	72.9 (0.8) b	11.8 (0.5) b

[a] Values are means and SEM (in parentheses), six rats per group. [b] Values within a column followed by the same letter were not significantly different as determined by the Tukey procedure ($P < 0.05$).

Table III. Concentration (Percent of Oral Dose) of Tritiated Hepatic Folate and Total Urinary and Plasma Tritium 24 h after Oral Dose of Tritiated Folic Acid, Bacterial Reduced Folylpolyglutamates, or Bacterial Reduced Folylmonoglutamates (0.11 nmol Each; Experiment 2)[a,b]

tritiated folate	hepatic [^{3}H]folate	urinary tritium	plasma tritium
folic acid	12.8 (0.4) a	31.6 (0.9) ab	0.368 (0.024) a
bacterial polyglutamates	13.7 (0.6) a	27.7 (1.9) a	0.280 (0.014) b
bacterial monoglutamates	16.9 (1.1) b	33.6 (1.6) b	0.328 (0.016) ab

[a] Values are means and SEM (in parentheses), 12 rats per group. [b] Values within a column followed by the same letter were not significantly different as determined by the Tukey procedure ($P < 0.05$).

Experiment 2. This experiment was conducted to examine potential differences in the utilization of [^{3}H]folic acid and tritiated mono- and polyglutamyl folates produced by biological synthesis. The bacterial folyl polyglutamates eluted near the void volume of the Sephadex G-25 column at a point equivalent to the pteroylheptaglutamate marker. In view of the limited resolution of this column for long-chain polyglutamyl folates, it was concluded that the isolated tritiated polyglutamates were hexaglutamates or longer. After enzymatic deconjugation, analysis of the tritiated bacterial folates indicated 89% 5-CHO-H_4folic acid and 11% 5-Me-H_4folic acid, with no unreduced folic acid. This pattern also was observed in the preparations of tritiated polyglutamyl folates employed in experiment 3.

The hepatic retention of tritiated folates and tritium content of plasma and urine are presented in Table III. The tritiated bacterial folates yielded a significantly higher hepatic retention when fed in monoglutamyl rather than polyglutamyl form and also differed significantly from rats fed [^{3}H]folic acid ($P < 0.05$). A similar relationship was observed for urinary tritium excretion. With respect to plasma tritium retention, the group fed [^{3}H]folic acid was highest, followed by monoglutamyl and polyglutamyl bacterial reduced folates, respectively ($P < 0.05$).

Observed distributions of labeled hepatic folates (Table IV) were similar to those of experiment 1. No significant differences in percentages of tritiated hepatic folates were observed between dietary groups.

Experiment 3. The third experiment was conducted to determine the bioavailability of [^{3}H]folic acid and tritiated bacterial polyglutamyl folates in the presence of representative foods and food components including cabbage, orange juice, kidney beans, wheat bran, and pectin. The data regarding hepatic retention of tritiated folates and the urinary tritium excretion in phases 1 and 2 of this experiment are presented in Table V.

Table IV. Percentage Distribution of Tritiated Folates in Livers of Rats 24 h after Administration of an Oral Tracer Dose (0.11 nmol) of [^{3}H]Folic Acid, Tritiated Bacterial Polyglutamyl Folates, or Tritiated Bacterial Monoglutamyl Folates (Experiment 2)[a,b]

tritiated folates	H_4folate	5-Me-H_4-folate	5-CHO-H_4-folate
folic acid	17.7 (1.1) a	69.1 (1.5) a	14.1 (0.6) a
bacterial polyglutamates	18.6 (0.8) a	69.3 (1.0) a	12.0 (0.7) a
bacterial monoglutamates	17.2 (1.4) a	70.9 (1.0) a	11.8 (0.6) a

[a] Values are means and SEM (in parentheses), six rats per group. [b] Values within a column followed by the same letter were not significantly different as determined by the Tukey procedure ($P < 0.05$).

For the study of diets containing cabbage and orange juice (phase 1), the analysis of variance indicated no overall difference between forms of dietary [^{3}H]folate with respect to the hepatic retention of tritiated folates ($P > 0.05$). The overall effect of dietary composition on hepatic retention was significant at $P < 0.05$. The mean values for tritiated hepatic folate retention of groups fed diets containing cabbage and orange juice were significantly lower than the mean value obtained for the control diet. Using the hepatic retention of tritiated folates as an index of bioavailability, the mean values for diets containing orange juice and cabbage were 75% and 69%, respectively, relative to the bioavailability of folates in the control diets. No differences were noted in urinary tritium among the control, orange juice, and cabbage diets. In addition, analysis of variance indicated no overall significant differences in urinary tritium excretion of the groups fed [^{3}H]folic acid and those fed tritiated bacterial polyglutamyl folates.

In phase 2 of experiment 3 (Table V), analysis of variance indicated no significant main effects of tritiated dietary folate or diet composition ($P > 0.05$). Treatment means for the tritiated hepatic folate retention of groups fed kidney beans, wheat bran, and citrus pectin were consistently lower than the mean value observed for the control diet, although the difference was not statistically significant ($P > 0.05$). On the basis of tritiated hepatic folate retention, the mean bioavailability of tritiated folates in diets containing kidney beans, wheat bran, and pectin would be 82%, 84%, and 83%, respectively, relative to the bioavailability of folates in the controls, although these values were not significantly less than 100% ($P > 0.05$). No significant main effects on urinary tritium excretion were detected for tritiated dietary folate or diet composition ($P > 0.05$).

The distribution of tritiated hepatic folates was highly consistent among the treatments in both phases of experiment 3 (Table VI), with several weak but statistically significant effects noted. For both the cabbage and orange juice diets, there was less tritiated hepatic H_4folic acid and 5-CHO-H_4folic acid, with proportional increases in 5-Me-H_4folic acid, relative to the control diets. Also, the concentration of 5-Me-H_4folic acid was slightly higher in groups fed diets containing tritiated reduced polyglutamyl folates compared to those with [^{3}H]folic acid. The concentration of 5-Me-H_4folic acid also was higher in groups fed tritiated reduced folates relative to those fed [^{3}H]folic acid, while the converse was true for hepatic 5-CHO-H_4folic acid. In phase 2, rats fed diets containing bran or pectin exhibited lower proportions of tritiated H_4folic acid and 5-CHO-H_4folic acid relative to controls. The presence of kidney beans in the diet had little effect on the distribution of labeled hepatic folates.

DISCUSSION

The purpose of this research was to evaluate and apply

Table V. Concentration (Percent of Oral Dose) of Tritiated Hepatic Folate and Total Urinary Tritium 24 h after Oral Dose of Tritiated Folic Acid or Bacterial Polyglutamyl Folates (0.11 nmol) in the Presence of the Following Materials (Experiment 3): Phase 1, Control, Orange Juice Solids (10% w/w) or Blanched Cabbage (10% Wet-Weight Basis); Phase 2, Control, Kidney Beans (10% Wet-Weight Basis), Wheat Bran or Pectin (1% w/w)[a,b]

diet	dietary [^{3}H]folate	hepatic [^{3}H]folate[c]		urinary tritium[d]	
		group mean	diet mean	group mean	diet mean
		Phase 1			
control	FA	11.0 (0.1)	10.7 (1.0)	37.5 (2.0)	40.9 (2.2)
	PG	10.6 (1.6)		44.4 (3.5)	
orange juice	FA	8.92 (0.74)	8.17 (0.48)**	42.0 (2.3)	44.8 (1.5)
	PG	7.43 (0.49)		47.7 (1.4)	
cabbage	FA	6.47 (0.42)	7.96 (0.76)**	42.0 (3.2)	40.9 (2.3)
	PG	9.46 (1.22)		39.8 (3.6)	
		Phase 2			
control	FA	8.06 (0.61)	7.66 (0.67)	40.6 (2.9)	45.9 (2.5)
	PG	7.26 (1.24)		51.2 (2.6)	
kidney beans	FA	7.19 (0.64)	6.28 (0.44)	47.3 (1.1)	46.8 (2.1)
	PG	5.37 (0.35)		38.3 (4.3)*	
wheat bran	FA	6.95 (0.24)	6.52 (0.34)	46.2 (3.8)	46.8 (2.1)
	PG	6.10 (0.61)		47.1 (2.4)	
pectin	FA	6.52 (0.45)	6.38 (0.60)	36.3 (5.6)	41.2 (3.1)
	PG	6.24 (1.16)		46.0 (1.8)	

[a] Values are means and SEM (in parentheses), six rats per group. Abbreviations: FA, [^{3}H]folic acid; PG, tritiated polyglutamatyl folates. [b] Within data sets for each phase of the experiment, values followed by an asterix (*) were significantly different from their respective control ($P < 0.05$) as determined by Dunnett's test. Values followed by a double asterix (**) were significantly different at $P < 0.01$. [c] In phase 1, effect of diet composition was significant at $P < 0.05$, while folate effects and the diet × folate interaction were not significant ($P > 0.05$). No significant factor effects or interactions were noted in phase 2 ($P > 0.05$). [d] In phase 2, a significant diet × folate statistical interaction was detected ($P < 0.05$). Dunnett's test was not performed on diet means.

Table VI. Percentage Distribution of Tritiated Folates in Livers of Rats 24 h after Administration of an Oral Tracer Dose (0.11 nmol) of [^{3}H]Folic Acid in the Presence of Selected Food Components Added to the Gel Diet (Experiment 3)[a-c]

diet	dietary [^{3}H]folate	H_4folate	5-Me-H_4folate	5-CHO-H_4folate
		Phase 1		
control	FA	19.6 (1.2) a	61.5 (2.4) a	19.0 (2.6) a
	PG	18.8 (1.6) ab	64.0 (3.6) a	17.0 (1.0) ab
cabbage	FA	16.8 (0.7) ab	65.9 (4.4) ab	16.6 (1.7) ab
	PG	12.4 (2.8) bc	73.6 (2.2) ab	14.6 (2.2) ab
orange juice	FA	16.9 (0.9) ab	73.9 (2.6) ab	11.6 (1.1) ab
	PG	9.3 (1.2) c	80.1 (3.1) b	10.6 (0.6) b
		Phase 2		
control	FA	16.2 (2.3) ab	69.0 (4.0) a	14.8 (2.9) a
	PG	18.0 (2.0) a	75.3 (2.4) a	6.7 (3.1) a
kidney beans	FA	16.8 (1.2) ab	73.5 (2.9) a	9.8 (0.9) a
	PG	15.5 (1.3) ab	76.9 (3.1) a	8.1 (1.2) a
wheat bran	FA	13.4 (1.4) ab	77.3 (3.4) a	9.2 (1.6) a
	PG	11.6 (0.7) ab	79.1 (2.4) a	9.3 (1.1) a
pectin	FA	11.5 (1.6) ab	78.0 (4.4) a	10.5 (1.1) a
	PG	11.1 (1.1) b	80.0 (3.9) a	8.8 (2.1) a

[a] Values are means and SEM (in parentheses), six rats per group. Abbreviations: FA, [^{3}H]folic acid; PG, tritiated polyglutamyl folates. [b] For each phase of the experiment, values within a column followed by the same letter were not significantly different as determined by the Tukey procedure ($P < 0.05$). [c] Significant effects in two-way analysis of variance for each phase: phase 1, H_4folate, dietary composition ($P < 0.01$), diet folate ($P < 0.01$); phase 1, 5-Me-H_4folate, diet composition ($P < 0.01$); phase 1, 5-CHO-H_4folate, diet composition ($P < 0.01$); phase 2, H_4folate, diet ($P < 0.01$).

a single-dose procedure for the determination of folate bioavailability in the rat. The major indicators of bioavailability were the retention of tritiated hepatic folates and urinary tritium excretion. While urinary tritium excretion is a good indicator of intestinal absorption, it is not necessarily reflective of in vivo utilization of absorbed folates. Consequently, the hepatic retention of tritiated folates may be more accurate as the primary criterion of overall absorption and metabolic utilization of the vitamin. The hepatic retention of tritiated folates provided a direct indication of the proportion of dietary dose of tritiated folates incorporated into the folate metabolism of the animals. It was noted that all of the labeled hepatic folates were fully reduced. No residual [^{3}H]folic acid was detected in any of the livers or representative urine samples.

Experiment 1 was conducted to determine the range of dietary folate ingestion that could be tolerated in this protocol without reducing the bioavailability of the tracer dose of [^{3}H]folic acid (0.11 nmol). Quantities of unlabeled folic acid up to 10 nmol did not reduce the utilization of the tritiated folic acid, as indicated by the retention of tritiated hepatic folates. These conclusions were supported, although less clearly, by the trend toward increased urinary tritium excretion with increasing dietary folate concentration. These results are indicative or impaired folate retention at the 100-nmol dosage level, which may be due to saturation of folylpolyglutamate synthetase. Because this enzyme is involved in intracellular metabolic trapping of folates in polyglutamyl form (Steinberg, 1984; Foo and Shane, 1982; Cichowicz et al., 1981), saturation

of the enzyme by high levels of ingested folate would presumably be associated with reduced retention of the tritiated oral dose by an isotopic dilution effect. The total folate dose in the test diets of experiment 3 was less than 10 nmol/rat; thus, dose dependence of the bioavailability of the [^{3}H]folate tracers would be negligible in that experiment.

The comparative bioavailability of [^{3}H]folic acid and tritiated bacterial mono- and polyglutamyl reduced folates was investigated to determine the influence of form of the vitamin presented to the rats in this experimental protocol. The monoglutamyl preparation of tritiated bacterial folates, which existed mainly as tritiated 5-CHO-H_4folic acid, exhibited significantly higher bioavailability on the basis of hepatic retention of tritiated folates. This difference between [^{3}H]folic acid and a formyl-substituted folate is in agreement with the previous observation that oral doses of tritiated 10-formylfolic acid in a similar protocol were incorporated more extensively than [^{3}H]folic acid into the hepatic folate pool (Gregory et al., 1984b). Enhanced hepatic retention of orally administered formylfolates, relative to folic acid, may by hypothesized as being due to differences in the extent of metabolic trapping and disposition of the various folates being presented to the liver (Steinberg, 1984; Steinberg et al., 1979; McGuire et al., 1980). The differences in retention of tritiated plasma and liver folates between groups in experiment 2 were consistent with the hypothesis of greater hepatic metabolic trapping of formyl folates. Rats fed the monoglutamyl bacterial folates tended to exhibit lower plasma radioactivity than rats fed [^{3}H]folic acid.

The results of experiment 2 also indicated that the bioavailability of polyglutamyl bacterial folates was 81% of that exhibited by monoglutamyl bacterial folates on the basis of the hepatic retention of tritiated hepatic folates. This conclusion was supported by the observed lower urinary tritium excretion by rats fed polyglutamyl vs monoglutamyl bacterial folates (Table III). These results suggest that the incomplete bioavailability of polyglutamyl folates in this protocol was due to reduced intestinal absorption. While evidence suggests that the intralumenal hydrolysis of dietary polyglutamyl folates in humans occurs at least partially at the brush-border membrane (Halsted et al., 1975; Reisenauer et al., 1977), the site of hydrolysis in the rat is unclear. Because of the lack of significant conjugase activity in the intestinal brush border of the rat (Day and Gregory, 1984; Wang et al., 1985), the conjugase activity of bile or pancreatic fluid (Horne et al., 1981; Kesevan and Norhona, 1983) may be major factors in the deconjugation of dietary folates in this species. Irrespective of the source of conjugase activity, polyglutamyl bacterial folates were utilized less effectively than their monoglutamyl analogues under the conditions of this experiment. These results are in contrast to the full bioavailability of unlabeled folic acid pentaglutamate, which was observed recently using a longer term (14-day) rat bioassay (Abad and Gregory, 1987).

The bacterial polyglutamyl folates used in these studies were found to be long-chain species on the basis of their behavior in gel filtration chromatographic analysis. This is in agreement with the report by Cichowicz et al. (1981) that *L. casei*, when cultured in the presence of [^{3}H]folic acid under conditions similar to those employed in this study, produces intracellular folates of mainly octa- and nonaglutamate chain length. This bacterial procedure for the preparation of tritiated polyglutamyl folates is a convenient and less costly alternative to the synthesis of tritiated folic acid polyglutamates, although the latter would eliminate oxidation state and one-carbon substitution as variables.

As applied in experiment 3, this single-dose protocol was suitable for the detection of dietary factors that may alter the bioavailability of dietary folates. In this experiment, only single levels of incorporation of the tested materials were employed. The formulation of gels containing cabbage, kidney beans, pectin, and bran was selected to yield 0.1 g of solids/g of gel, thus permitting direct comparisons among the test substances at a single dosage. In constrast, the 10% level of orange juice solids was selected to be similar to that of single-strength juice. This protocol will permit an evaluation of dose dependence of inhibitory effects in future studies. A major advantage of this protocol, as compared to conventional bioassay procedures, is that the diets are fed in fully hydrated form. Thus, potential artifacts that could arise in studies involving diets containing dry hydrophilic materials (Struthers, 1986) are avoided.

Using the retention of tritiated hepatic folates as the primary response indicator, all of the materials examined, including cabbage, orange juice, kidney beans, pectin, and bran, tended to reduce the utilization of the labeled dietary folates, with only cabbage and orange juice inducing significant impairment utilization at $P < 0.05$. Comparisions of the various treatment groups with their respective controls indicated consistent, but not always statistically significant, reductions in utilization of both [^{3}H]folic acid and tritiated polyglutamyl bacterial folates by the dietary components (Table V). This suggests that the impairment of bioavailability occurred primarily at the level of the intestinal absorption of the monoglutamyl folates, rather than by selective inhibition of the intestinal deconjugation of polyglutamyl folates. It should be noted that the magnitude of these effects was sufficiently small that little or no difference in urinary tritium excretion was generally detected.

The results based on extrinsic dietary enrichment as employed here may not be reflective of the bioavailability of endogenous polyglutamyl folates in the foods tested. In the case of the diets containing cabbage, the incomplete bioavailability of the exogenous labeled folates is in general agreement with apparent bioavailability values for endogenous folate in cabbage of 68% based on a rat bioassay (Abad and Gregory, 1987) and 40% using a chick bioassay (Ristow et al., 1982a). Tamura and Stokstad (1973) reported an apparent bioavailability of folate in cabbage of 47% as determined in human subjects. The incomplete bioavailability of tritiated folates in diets containing orange juice solids is consistent with the earlier observations (Tamura and Stokstad, 1973; Tamura et al., 1976). Tamura et al. (1976) reported that a reduction in pH of the intestinal contents may have been responsible for the incomplete bioavailability of polyglutamyl folate in their study. Whether a similar effect of intestinal pH would occur at the proportionally lower dose used in the present study has not been determined. Rhode et al. (1983) reported that long-term ingestion of orange juice as a folate supplement was as effective as oral doses of folic acid. A weak antagonistic effect of kidney beans on the utilization of polyglutamyl folates was noted in this study, which may be related to a possible inhibition of intestinal conjugases by a component of legumes (Butterworth et al., 1974). The nutritional significance of mildly antagonistic effects of dietary fiber on the bioavailability of various folates also is unclear at this time (Keagy and Oace, 1979, 1984; Ristow et al., 1982b; Keagy, 1985). The results indicate that wheat bran and pectin weakly retard the utilization of dietary

monoglutamyl and polyglutamyl folates under the conditions of this study.

Significant differences in physical characteristics and mode of action have been reported beween intestinal conjugases of the chick, rat, and human (Rosenberg and Neumann, 1974; Elsenhans et al., 1984; Day and Gregory, 1984; Wang et al., 1985). The consequences of these differences regarding folate bioavailability have not been determined. The rat and human have been shown to exhibit highly similar carrier-mediated folate absorption characteristics (Said et al., 1987). While the suitability of the rat is uncertain as a model in studies of polylgutamyl folate deconjugation, the rat would appear to be an ideal model for the study of dietary factors that retard folate absorption as observed in this study. It may be concluded from this study that food components widely differing in chemical composition influence the bioavailability of dietary folate in the rat. Further study is needed to elucidate more fully the effects of dietary components on the bioavailability of the various forms of folate. Several of the dietary components did induce small but statistically significant alterations in the relative proportions of the hepatic folates. The metabolic significance of these effects is unclear.

In summary, a protocol has been devised and investigated in which the short-term bioavailability of various radiolabeled dietary folates, as influenced by diet composition, may be determined. This protocol would be suitable for systematic evaluation of the effects of diet composition on folate bioavailability using extrinsic dietary enrichment as performed in this study. These results also indicate that the protocol would be suitable for investigations of the bioavailability of folate in foods intrinsically enriched with radiolabeled forms of the vitamin.

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Registry No. Folic acid, 59-30-3; 5-methyltetrahydrofolate, 134-35-0; 5-formyltetrahydrofolate, 58-05-9.

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Residues of Macrolide Antibiotic Sedecamycin and Its Major Metabolites in Swine Blood and Tissues

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Pigs were fed a diet containing 50–500 ppm of the macrolide antibiotic sedecamycin for 14 or 28 days starting at 6 or 10 weeks of age. Tissues were collected to determine sedecamycin and its three biologically active metabolites at designated times after the drug was withdrawn. Of the edible tissues, liver retained the highest metabolite concentrations at 2 h after withdrawal. Blood, muscle, and fat showed no drug residue at any time even at the 500 ppm level of medication. The small intestine showed higher concentrations of the unchanged drug and metabolites than the liver. All compounds disappeared rapidly from all the tissues and were not detected in any tissue at 1 day after withdrawal and thereafter.

Sedecamycin (I, Figure 1), one of the lankacidin group of antibiotics produced by *Streptomyces rochei* var. volubilis (Harada et al., 1969), possesses preeminent activity against *Treponema hyodysenteriae*, the pathogenic organism for swine dysentery (Narukawa et al., 1984; Yamazaki et al., 1986), in addition to antibacterial activities against gram-positive organisms (Tsuchiya et al., 1971). In Japan, I has been used at a concentration of 25–75 ppm in feed to treat swine dysentery since it was approved in 1985. Before approval was granted, it was obligatory to examine the residue of I in swine tissues to determine the withdrawal period before slaughter.

When [^{14}C]I was administered orally to swine, 61% of the radioactivity was excreted as approximately 20 lipophilic and some polar metabolites in the bile, urine, and feces within 48 h (Okada et al., 1984). Among the lipophilic metabolites, lankacidin C (II), lankacidinol A (III), and lankacidinol (IV) exhibited antibacterial activity against *T. hyodysenteriae* (Narukawa et al., 1984). As most lipophilic metabolites showed polarities between those of I and IV, these four compounds were considered to represent the tissue affinity of the other metabolites. For these reasons, I–IV were monitored in swine tissues to know the residual profile of I after it was administered continuously in feed.

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MATERIALS AND METHODS

Chemicals. Sedecamycin used in these residue studies was technical grade of 97.8% purity; it was produced in the Applied Microbiology Laboratories of Takeda Chemical Industries. Analytical standard compounds were prepared in the laboratories and their purities were as follows: I, 99.2%; II, 99.7%; III, 94.2%; IV, 93.1%.

Hexane and 2-propanol were HPLC grade from Wako Pure Chemical Industries Ltd., Osaka. Silica gel 60 (70–230 mesh, Merck) and Florisil (100–200 mesh, Floridin Co.) were dried 6 h at 130 °C before use. The other reagents were reagent grade from Wako Pure Chemical Industries.

Animal Treatment. Two experiments were carried out. In experiment 1, six Landrace pigs (five castrated and one female) aged 6 weeks and nine Landrace pigs (five castrated and four female) aged 10 weeks produced at the Fukuchiyama Experimental Farm, located in Kyoto Prefecture, of Takeda Chemical Industries, were used. The 15 pigs were divided randomly into two groups: a control

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