Improving the response of the rat liver folate bioassay

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The effect of 21-day depletion and extending the repletion period from 7 to 14 days within the constructs of a 35-day rat liver bioassay was investigated. Male weanling Sprague-Dawley rats were depleted on a low folate AIN-76A formulated basal diet for 21 days. In repletion diets, folic acid or brewer's yeast were added to provide 0.57, 1.13, and 2.27 μM/kg. The 14-day slopes calculated for folic acid and brewer's yeast were compared to 7-day slopes from four previous studies. The present slopes were significantly different in two studies for folic acid and in one study for brewer's yeast. However, the longer repletion period improved liver folate resolution in response to dietary folate levels and the efficiency of the 35-day assay protocol.

Keywords: rat; liver folate; repletion; bioassay

Introduction

New developments, advantages, and limitations of major methods to assess vitamin bioavailability using men and animals have been reviewed recently by Gregory.[1] Earlier folate bioavailability studies depended on non-specific responses such as growth or hematological changes in depleted animals. In bioassay with rats, sulfa drugs were often included to prevent the synthesis of folate by intestinal bacteria.[2] With improved analytical methods, it was shown that tissue concentrations of folate were highest in the liver and could be affected by the level in the diet.[3-5] The potential for a more specific and quantitative rat liver bioassay using a semipurified diet without sulfa drugs was first reported by Keagy and Oace.[6] Subsequent studies,[7] using an AIN-76A diet without folic acid, (PGA, pteroylmonoglutamic acid) confirmed the dose response relationship. Since, others have reported results of rat bioassays for folate with modified protocols.[8] Recently, the modifications in protocol and their effects on the bioassay as well as the factors that affect folate bioavailability have been evaluated.[8,9]

Previously, after 21 and 28 days' depletion, a 7-day repletion rat liver bioassay was used to investigate the bioavailability of folate and the effect of drugs on deconjugation and absorption.[10-12] This study focused to improve the overall efficiency of the 35-day rat liver folate bioassay by limiting the depletion period to 21 days and increasing the repletion period to 14 days.

Materials and methods

Male weanling CRL:CD (SD) BR Sprague-Dawley rats (43–61 g) were purchased from Charles River, St. Constant, P.Q., Canada. The lightest and heaviest animals were sacrificed on the day received to determine initial liver folate concentration. During the 21 days of depletion, the rats were fed a low folate AIN-76A basal diet that contained 0.11 μM/kg of residual folate from the vitamin-free casein that had 0.62 μM/kg. To follow the changes in liver folate concentrations and promote similar body size, on days 7, 14, and 21 the lightest and heaviest (n = 2) animals were sacrificed. On day 21, the remaining depleted rats were randomized according to body weight and allocated to 7 test diets (8/diet group) for a 14-day repletion period. The composition of the test diets and the levels of the folate sources are shown in *Table 1*. Although the folate sources are added to the basal diet to provide the nominal standard concentrations, the test diets are sampled and analyzed for actual folate content before use to account for variations in weighing and mixing that may occur during preparation. The actual folate contents of the test diets are shown in *Table 2*. All test diets were adjusted to be isocaloric and isonitroge-

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Table 1 Composition of basal and test diets

Diet no.	Folate added mg/Kg	Test source	% Casein vitamin-free	Corn starch	Sucrose	Fiber
1	—	Basal[a]	20.0	40.0	25.0	5.0
		PGA[b]				
2	0.25	+	20.0	40.0	25.0	5.0
3	0.50	+	20.0	40.0	25.0	5.0
4	1.00	+	20.0	40.0	25.0	5.0
		B. yeast[c]				
5	0.25	2.0	19.5	39.0	25.0	4.5
6	0.50	4.0	18.5	39.0	25.0	3.5
7	1.00	8.0	17.0	38.5	24.0	2.5

[a] Based on the AIN-76 recommended levels for rats[14,15] including AIN-76A vitamin mix, folic acid omitted, 1%; AIN-76 mineral mix, 3.5%; DL-methionine, 0.3%; and choline bitartrate, 0.2%; U.S. Biochemical Corp., Cleveland, OH, USA; corn oil, 5%. Fiber is non-nutritive cellulose.
[b] U.S. Biochemical Corp.
[c] Fisher Scientific Co.; freeze-dried, moisture 2%. Protein 38.8 g; fiber 1.7 g; energy 283 kcal per 100 g dry-matter (USDA Handbook No. 8, 1963). *L. casei* folate; total 28.32, free 3.28 μM/kg.

Table 2 Fourteen-day repletion data for food consumed, liver weight, and liver folate

Source	Folate in test diet μM/kg	Food consumed g/14 day	Liver weight g	Liver folate nM/g
Basal	0.11	315 ± 11[a]	18.4 ± 2.0	5.26 ± 0.93
PGA	0.87	305 ± 16	18.7 ± 2.3	9.43 ± 2.41
	1.39	315 ± 18	18.6 ± 2.6	13.32 ± 2.48
	2.37	313 ± 17	17.5 ± 1.6	18.24 ± 2.34
Brewer's yeast	0.70	310 ± 19	18.2 ± 2.2	9.78 ± 1.96
	1.24	299 ± 14	17.7 ± 1.9	14.17 ± 2.43
	2.34	296 ± 15	17.8 ± 1.7	17.94 ± 1.44

[a] Mean ± SD; $n = 8$.

nous. Individual animals were housed in mesh-bottom stainless steel cages with free access to food and water. Body weights and food consumption were recorded weekly for the depletion and repletion periods. After repletion (day 35), the animals were killed by CO_2 inhalation and the livers were removed. Analysis of the liver and diet samples for total folate was carried out in duplicate on different days by microbiological assay with *Lactobacillus casei* as described previously.[7] Data are reported as mean ± SD.

The slopes calculated for PGA and brewer's yeast for 14-day repletion were compared with those obtained from previous 7-day repletion studies [10, 11 (expts. I, II), 12 (expt. I)] as summarized in *Table 3* as experiments I, II, III, and IV, respectively. The extra sum of squares principle was used[13] to test the hypothesis that a common slope existed for all diets in each of the PGA and brewer's yeast group. The differences between the regression sum of squares for a model that allowed each diet in the two groups to have a unique intercept and slope and a model that forced all lines to have a common slope was calculated. These values, divided by the difference in the degrees of freedom for the regression sum of squares for the two models, were compared to the variance estimate for the first model using an F-test with a significance level of 0.05. If there was evidence against the hypothesis of a common slope, then contrasts were used to determine which diets had slopes that were significantly different from the slope calculated for the PGA and brewer's yeast diet in the present 14-day repletion study. Food intake and liver weight were analyzed by ANOVA.[13]

Results

Initial liver folate levels (10.54 ± 1.13, $n = 8$) of the rats fed the low folate basal diet declined to 5.26 nM/g. These baseline liver folate levels are similar to those obtained previously after 21 days of repletion. As shown in *Table 2*, food consumption and liver weight were not affected by diet ($P > 0.05$).

There was evidence against the hypothesis of a common slope for each of the PGA and brewer's yeast group from previous experiments I, II, III, and IV, *Table 3* [($P = 0.0025$) for the PGA group and ($P = 0.028$) for the brewer's yeast group] with a 7-day repletion protocol. Multiple comparisons showed that

Table 3 Seven-day repletion data for folate in test diets and liver folate from previous experiments[a]

Source	Basal		PGA		Brewer's yeast	
	Dietary folate μM/kg	Liver folate nM/g	Dietary folate μM/kg	Liver folate nM/g	Dietary folate μM/kg	Liver folate nM/g
Expt. I	0.18	4.40 ± 0.88[b]	0.64	5.64 ± 1.22	0.81	6.28 ± 0.86
			1.41	9.00 ± 1.06	1.36	7.82 ± 1.29
			2.45	10.79 ± 1.61	2.46	13.30 ± 1.50
Expt. II	0.30	5.73 ± 1.70	0.96	7.70 ± 1.68	0.71	7.95 ± 1.27
			1.42	10.91 ± 1.50	1.18	8.72 ± 1.52
			2.31	12.51 ± 1.77	2.14	12.15 ± 1.65
Expt. III	0.29	5.44 ± 1.09	0.72	7.21 ± 1.83	0.73	7.39 ± 1.45
			1.28	8.88 ± 1.59	1.27	9.06 ± 1.68
			1.77	11.28 ± 1.29	2.39	14.39 ± 3.85
Expt. IV	0.28	7.50 ± 2.56	0.86	8.43 ± 1.16	0.82	8.70 ± 1.77
			1.38	9.97 ± 1.20	1.75	10.54 ± 1.68
			2.32	12.24 ± 1.77	3.08	14.98 ± 2.22

[a] Experimental data for Expt. I are cited in reference 10; Expt. II and III in reference 11; Expt. IV in reference 12.
[b] Mean ± SD; $n = 8$.

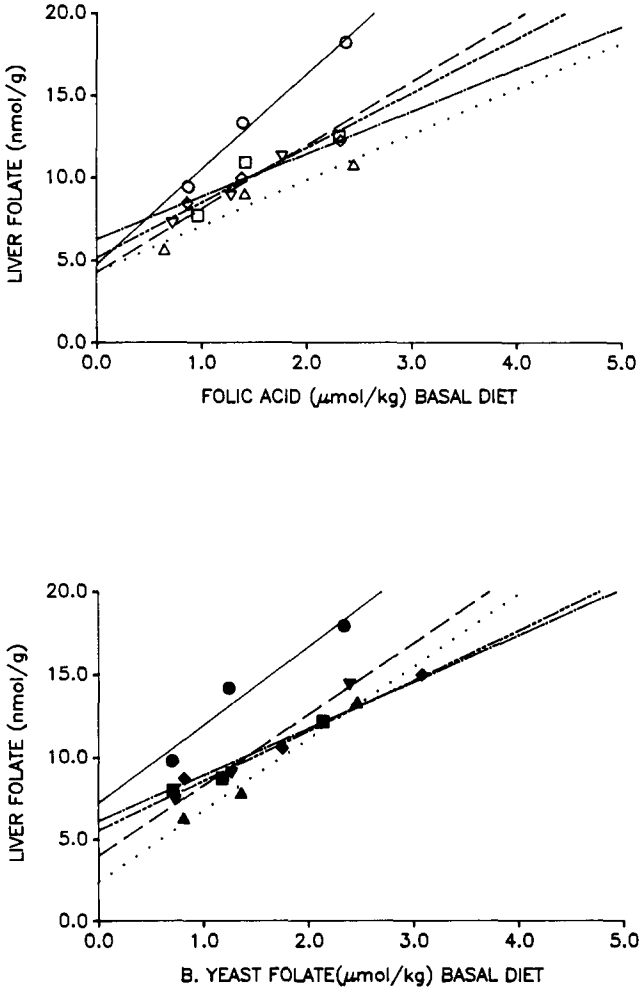

Figure 1 Liver folate as a function of folate in the diet. Upper, open symbols: PGA. Lower, closed symbols: Brewer's yeast. 14-day repletion = circles + _____ line. 7-day repletion from Expt. I = triangles + ... line; Expt. II = squares + _._._. line; Expt. II = inverse triangles + _ _ _ line; Expt. IV = diamonds + _._._._ line.

among the PGA diets only those from experiments I and II, and for the brewer's yeast diets from experiment IV, had slopes that were significantly different from the slopes calculated for the PGA and brewer's yeast diet in the present 14-day repletion protocol. *Figure 1* compares the individual regression lines of the PGA and brewer's yeast groups from previous 7-day repletion data with the present 14-day repletion data. The ratios of the slopes of 14 day/7 day repletion ranged from 1.5 to 2.8 for PGA, and from 1.1 to 1.7 for brewer's yeast, indicating that 14-day repletion provides a steeper slope with more precision and improved sensitivity of the assay to low concentrations of dietary folate.

Discussion

From depletion and repletion data, liver folate was shown to follow saturation kinetics as characterized by the Henri-Michaelis-Menten equation and its integrated form. The rate constant for folate leaving the liver estimated from the experimental data gave an 8-day half-life for liver folate.[16] This value compares favorably with the 4–8-day half-life observed in tracer studies in rats and does not depend on the folate status of the animal.[17,18] Correspondingly, a 24–30-day period was found experimentally to be adequate for liver folate depletion.[6,7] In recent studies, it was observed that these baseline liver folate levels usually are reached after 21 days of depletion.

Within the constructs of a 35-day rat liver folate bioassay, extending the repletion period to 14 days offered an improvement of the assay protocol. The repletion period defines the range of folate intake and sensitivity of the standard curve with liver folate as the response parameter. A steep slope is desirable for maximum sensitivity to low concentrations of dietary folate and changes in liver folate when the effects of drugs are investigated. Too short a repletion period

may result in a low slope with less precision.[6,7] Recently, the coefficients of variation for standards of bioassays have been reported to range from 6 to 12%. For liver folate bioassays, 28-day repletion gave lower coefficients while those with larger variations were from 7-day repletion protocols.[8] Excessively long depletion and repletion periods are also not desirable. It appears that rapid growth rates are associated with the lowest liver folate values.[6,19] Hence, as the growth rate in maturing animals decreases more dietary folate becomes available for liver storage without any increase in dietary folate concentrations. In the present 35 day assay protocol, this is minimized by being restricted to the first 8 weeks of the growth curve for the rats.[14]

Changing the protocol to 21-day depletion and extending the repletion period to 14 days resulted in an overall improvement in efficiency within the confines of the 35-day rat liver bioassay. This should be reflected in future studies on folate bioavailability and the effects of drugs on intestinal deconjugation and absorption of ingested food folate.

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